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

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Bacterial strain PETP02T 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 PETP02T 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 PETP02T (=LMG 22712T = CECT 7015T). 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 PETP02T, 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 PETP02T belongs to a novel species of Phyllobacterium.

Strain PETP02T 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 PETP02T 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 PETP02T were Gram-negative, rod-shaped, non-sporulating, motile by means of a polar flagellum and commonly observed as single cells.

Strain PETP02T 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...
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 MEKA2 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 PETP02T showed 98.0% similarity to that of *P. myrsinacearum*, suggesting that it belongs to a different species.

Strain PETP02T 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).

Results from the plasmid profile analysis and the Southern hybridization are shown in Fig. 2. The technique used revealed three plasmids in strain PETP02T (Fig. 2, lane 2). The specific probe detected a *nodD* gene in the three plasmids of strain PETP02T (Fig. 2, lane 4).

![Fig. 2. (a) Plasmid profile in horizontal 0-7% agarose gel: *Sinorhizobium meliloti* GR4 (lane 1) and strain PETP02T (lane 2). (b) Results of hybridization (marked by arrowheads) using the *nodD* gene probe: strain GR4 (lane 3) and strain PETP02T (lane 2).](image-url)
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 PETP02T 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 PETP02T gave DNA–DNA hybridization levels of 12.0% with two strains of P. myrsinacearum, LMG 1t1 and LMG 2t2T.

Phenotypic characterization of strain PETP02T was based on growth with different carbon sources (Bergersen, 1961) as described previously (Velázquez et al., 2001). P. myrsinacearum LMG 2t2T 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 PETP02T and strains LMG 2t2T and LMG 1t1 were also characterized by using API 20NE tests according to the manufacturer’s instructions (bioMérieux).

**Fig. 3.** Comparative sequence analysis of the nodD gene from Phyllobacterium trifolii PETP02T 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.

**Fig. 4.** Nodules induced in Trifolium repens by strain PETP02T (a) and by Rhizobium leguminosarum bv. trifolii ATCC 14480 (b). Bars, 2 cm. Nodules induced in Lupinus albus roots by strain PETP02T (c) and by Bradyrhizobium sp. ISLU35 (d). Bars, 0.2 cm.
The results indicated that strain PETP02<sub>T</sub> 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<sub>T</sub>. Additional phenotypic characteristics of strain PETP02<sub>T</sub> are given in the species description below.

We also compared the fatty acid composition of strain PETP02<sub>T</sub> with those of *P. myrsinacearum* strains LMG 1t1 and LMG 2t2<sup>T</sup>. 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 18:1<sup>o</sup>7c as the predominant fatty acid. It differs from *P. myrsinacearum* in that it contains more than 10% 16:0, more than 15% 18:1<sup>o</sup>7c 11Me, small amounts of 17:0 and 20:2 (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<sub>T</sub> 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.

### 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 rhamnose and arabinose. Acid production from sucrose, arabinose and adonitol was positive in *P. myrsinacearum* but weak in strain PETP02<sub>T</sub>. Additional phenotypic characteristics of strain PETP02<sub>T</sub> are given in the species description below.

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<table>
<thead>
<tr>
<th>Characteristic</th>
<th><em>P. trifolii</em> PETP02&lt;sub&gt;T&lt;/sub&gt;</th>
<th><em>P. myrsinacearum</em></th>
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<tr>
<td>Acid from:</td>
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<tr>
<td>Sucrose</td>
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<td>Rhamnose</td>
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<td>Trehalose</td>
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<td>Raffinose</td>
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<tr>
<td>Citrate assimilation</td>
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<tr>
<td>Polymyxin B</td>
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<tr>
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<tr>
<td>Neomycin</td>
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</table>

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.

*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<sup>i</sup> and/or 14:0 3-OH; summed feature 3 consists of 16:1<sup>o</sup>7c and/or iso-15:0 2-OH.

The type strain, PETP02<sub>T</sub> (=LMG 22712<sub>T</sub> = CECT 7015<sup>T</sup>), was isolated from a *Trifolium pratense* root nodule.

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References


