**Pseudomonas rhizosphaerae** sp. nov., a novel species that actively solubilizes phosphate *in vitro*

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A bacterial strain (designated IH5\(^T\)), isolated from rhizospheric soil of grasses growing spontaneously in Spanish soil, actively solubilized phosphates *in vitro* when bicalcium phosphate was used as a phosphorus source. This strain was Gram-negative, strictly aerobic, rod-shaped and motile. The strain produced catalase, but not oxidase. Cellulose, casein, starch, gelatin, aesculin and urea were not hydrolysed. Growth was observed with many carbohydrates as the carbon source. The main non-polar fatty acids detected were hexadecenoic acid (C\(_{16:1}\)), hexadecanoic acid (C\(_{16:0}\)) and octadecenoic acid (C\(_{18:1}\)). The hydroxy fatty acids detected were 3-hydroxydecanoic acid (C\(_{10:0}\) 3-OH), 3-hydroxydodecanoic acid (C\(_{12:0}\) 3-OH) and 2-hydroxydodecanoic acid (C\(_{12:0}\) 2-OH). Phylogenetic analysis of 16S rRNA indicated that this bacterium belongs to the genus *Pseudomonas* in the γ-subclass of the Proteobacteria and that the closest related species is *Pseudomonas graminis*. The DNA G+C content was 61 mol%. DNA–DNA hybridization showed 23% relatedness between strain IH5\(^T\) and *P. graminis* DSM 11363\(^T\). Therefore, strain IH5\(^T\) belongs to a novel species from the genus *Pseudomonas*, for which the name *Pseudomonas rhizosphaerae* sp. nov. is proposed (type strain, IH5\(^T\) = LMG 21640\(^T\) = CECT 5726\(^T\)).

Phosphorus is an essential plant nutrient that is added to soil as soluble inorganic phosphates, a large proportion of which becomes insoluble and therefore unavailable to plants (Singh & Kapoor, 1994). Many species of bacteria are able to solubilize phosphates *in vitro* and most of them live in the plant rhizosphere. At present, bacilli, rhizobia and pseudomonads are the most studied phosphate-solubilizers (Rodríguez & Fraga, 1999). Nevertheless, only a small number of species that presently belong to the genus *Pseudomonas sensu stricto* are known to be phosphate-solubilizers and they all belong to rRNA group I (Palleroni, 1992). *Pseudomonas putida* (Viveganandan & Jauhi, 2000; Kumar & Singh, 2001; Manna et al., 2001; Villegas & Fortin, 2002), *Pseudomonas aeruginosa* (Musarrat et al., 2000), *Pseudomonas corrugata* (Pandey & Palni, 1998), *Pseudomonas stutzeri* (Vázquez et al., 2000) and *Pseudomonas fluorescens* (Di Simine et al., 1998; Deubel et al., 2000) are the most studied species. Nevertheless, many rhizospheric phosphate-solubilizing bacterial species remain unknown and more studies are needed to reveal the high biodiversity of these bacteria. Although the study of rhizospheric bacteria is difficult, due to the high number of bacteria present in soil, characterization and identification of these bacteria are necessary for wide ecological studies of the plant rhizosphere.

During a wide study of phosphate-solubilizing, rhizospheric bacteria in soils from northern Spain, we isolated a strain that produced a yellow pigment in media that contained glucose as the carbon source and formed a transparent ‘halo’ around its colonies in media that contained bicalcium phosphate as the phosphorus source. A sample of rhizospheric soil of grasses was collected under aseptic conditions from land in northern Spain; 10 g was suspended in 90 ml sterile water and stirred for 30 min. From this suspension, 100 μl was spread on YED-P medium (7 g glucose l\(^{-1}\), 3 g yeast extract l\(^{-1}\), 3 g bicalcium phosphate l\(^{-1}\) and 17 g agar l\(^{-1}\)) and incubated at 28 °C. A bacterial strain that actively solubilized phosphate was isolated. This strain, designated IH5\(^T\), was maintained as a glycerol suspension [25% (v/v)] at −80 °C.

Strain IH5\(^T\) was grown in nutrient agar medium for 48 h at 22 °C to check for motility by phase-contrast microscopy. Cells were also stained according to the classical method and Gram-stained. Electron micrographs of strain IH5\(^T\) and a full phylogenetic tree are available as supplementary material in IJSEM Online.
Gram procedure described by Doetsch (1981). For electron microscopy, cells were grown on nutrient agar for 2 days then suspended gently in sterile water, stained with 0.2% uranyl acetate and examined at 80 kV with a Zeiss EM 209 transmission electron microscope. Strain IH5T is a Gram-negative, rod-shaped organism (1.2–2.4 × 0.8–0.9 μm). Cells are motile with a polar flagellum (see Supplementary Fig. I, available in IJSEM Online) and grow as weakly translucent, yellow-coloured colonies on nutrient agar.

For 16S rDNA sequencing, DNA extraction was carried out as described previously (Rivas et al., 2001). Amplification and sequencing of 16S rDNA were performed as described previously (Rivas et al., 2003a). The sequence obtained was compared with those in GenBank by using the FASTA program (Pearson & Lipman, 1988). Sequences were aligned by using CLUSTAL W software (Thompson et al., 1997). Distances were calculated according to the methods of Jukes & Cantor (1969), Kimura (1980), Tajima & Nei (1984) and Tamura & Nei (1993). Phylogenetic trees were inferred by using the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Yang, 1997) and parsimony (Felsenstein, 1983) analysis methods. Bootstrap analysis was based on 1000 resamplings. The MEGA 2.1.0 package (Kumar et al., 2001) was used for all analyses. Trees were rooted by using Burkholderia graminis as the outgroup.

The complete 16S rDNA sequence for isolate IH5T (GenBank accession no. AY152673) was obtained (1531 nt). Comparison with all 16S rDNA sequences of Pseudomonas species with validly published names held in GenBank indicated that the organism is related phylogenetically to members of this genus. A phylogenetic tree obtained with Kimura’s two-parameter model and the neighbour-joining method that included representative species of the genus Pseudomonas sensu stricto according to Anzai et al. (2000) and the recently described species Pseudomonas grimontii (Baida et al., 2002), Pseudomonas kilonensis (Sikorski et al., 2001), Pseudomonas lini (Delorme et al., 2002), Pseudomonas tremae, Pseudomonas cannabina (Gardan et al., 1999), Pseudomonas cremoricolorata and Pseudomonas parafulva (Uchino et al., 2001) showed that strain IH5T forms a separate group with P. graminis DSM 11363T (see Supplementary Fig. II, available in IJSEM Online). Fig. 1 shows the phylogenetic placement of strain IH5T within the genus Pseudomonas. The same results were obtained when phylogenetic distances were calculated by using the Jukes–Cantor one-parameter, Kimura two-parameter, Tamura–Nei three-parameter and Tajima–Nei four-parameter models. Trees obtained with the neighbour-joining, maximum-likelihood and parsimony methods (using, with each one, the four models already mentioned to calculate phylogenetic distances) also showed the same results (data not shown). Sequence similarity, based on pairwise sequence comparisons, was investigated by using complete 16S rDNA sequences. The data obtained showed that the 16S rDNA sequence of strain IH5T shows 97–98% similarity to many species of the genus Pseudomonas sensu stricto, except in the case of P. graminis (to which it shows 99% similarity). Therefore, P. graminis and strain IH5T form a group within the genus Pseudomonas that is clearly distinguishable from the other species, based on 16S rRNA gene sequences. These results are in agreement with those
of phenotypic analysis, which showed that P. graminis and strain IH5T belong to a separate group of Pseudomonas species that do not produce oxidase or fluorescent pigments. Taking these results into account, DNA–DNA hybridization was performed between P. graminis DSM 11363T and strain IH5T.

Glucose oxidation and fermentation were tested in the medium described by Hugh & Leifson (1953). Catalase activity was detected by using N,N,N',N'-tetramethyl 1,4-phenylenediamine dihydrochloride (10 g l⁻¹). P. fluorescens DSM 50090T and P. graminis DSM 11363T were used as positive and negative controls, respectively. Casein activity was detected on skimmed-milk agar after 7 days incubation. Amylases were detected on plates that contained 0.3 % (w/v) starch as the only carbon source; after 7 days incubation at 28 °C, the plates were stained by using Lugol’s solution. For caseinase and amylases, Bacillus cereus DSM 31T and P. graminis DSM 11363T were used as positive and negative controls, respectively. Other physiological and biochemical tests were done by using the API 20NE and API 50CH strips (bioMérieux), following the manufacturer’s instructions. P. graminis DSM 11363T was included in these studies and analysed under the same conditions. Ability to solubilize phosphate was tested in YED-P medium as described previously (Peix et al., 2001). Mesorhizobium mediterraneum UPM-Ca36T and Sinorhizobium meliloti ATCC 9930T were used as positive and negative controls, respectively. Pigment analysis was performed according to the method described by Behrendt et al. (1999), using P. graminis DSM 11363T as the reference.

Strain IH5T showed a great ability to solubilize phosphates on YED-P plates. On the same medium and under the same culture conditions, P. graminis DSM 11363T showed a lower ability to solubilize phosphates: after 24 h incubation, phosphate solubilization was clearly positive in strain IH5T, but weak in P. graminis DSM 11363T. Optimal growth temperature was 25 °C on nutrient agar. The API 20NE and API 50CH systems were only used to characterize strain IH5T, because the identification of non-clinical isolates is often wrong. This fact was pointed out by Behrendt et al. (1999) and coincides with the results obtained in our laboratory for many Gram-negative, aerobic, non-enteric isolates (data not shown).

Table 1 shows differential phenotypic characteristics between strain IH5T and its closest phylogenetic relatives in the genus Pseudomonas sensu stricto: P. graminis (98.9 % 16S rRNA gene sequence similarity), Pseudomonas jessenii and Pseudomonas agarici ( > 98 % 16S rRNA gene sequence similarity for both). According to the data, strain IH5T is very similar to P. graminis, forming a different phenotypic group within the genus Pseudomonas that is characterized by lack of oxidase and fluorescent pigment production. On the other hand, strain IH5T differs from P. graminis in ascellin hydrolysis and assimilation of erythritol and rhamnose.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<tbody>
<tr>
<td>Size (μm)</td>
<td>0-8–0-9 × 1-2–2-4</td>
<td>0-5–1-0 × 3-5–5-0</td>
<td>ND</td>
<td>1-2 × 3-3</td>
</tr>
<tr>
<td>Number of polar flagella</td>
<td>1</td>
<td>1</td>
<td>1 or 2</td>
<td>1</td>
</tr>
<tr>
<td>Fluorescent pigments</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Growth at:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 °C</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>40 °C</td>
<td>–</td>
<td>–</td>
<td>ND</td>
<td>–</td>
</tr>
<tr>
<td>Acid from glucose</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Phosphate solubilization* (24 h)</td>
<td>+</td>
<td>W</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>Utilization of:</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Erythritol</td>
<td>+</td>
<td>–</td>
<td>ND</td>
<td>–</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>ND</td>
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<tr>
<td>Hydrolysis of:</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Aesculin</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td>Casein</td>
<td>–</td>
<td>+†</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Gelatin</td>
<td>–</td>
<td>+†</td>
<td>ND</td>
<td>–</td>
</tr>
</tbody>
</table>

*Data from this study (only the type strain of P. graminis, DSM 11363T, was tested).
†Some strains give a negative result.
**Table 2.** Percentage cellular fatty acid composition of *Pseudomonas rhizosphaerae* and *P. graminis*

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1</th>
<th>2</th>
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<tbody>
<tr>
<td>C10:0 3-OH</td>
<td>2.9</td>
<td>ND</td>
</tr>
<tr>
<td>C12:0 2-OH</td>
<td>2.7</td>
<td>0-1-2-3</td>
</tr>
<tr>
<td>C12:0 3-OH</td>
<td>4.1</td>
<td>0-5-2-6</td>
</tr>
<tr>
<td>C12:0</td>
<td>4.3</td>
<td>0-3-4</td>
</tr>
<tr>
<td>C14:0</td>
<td>0-3</td>
<td>0-2-0-3</td>
</tr>
<tr>
<td>C16:1</td>
<td>38.6</td>
<td>50-1-61-4</td>
</tr>
<tr>
<td>C16:0</td>
<td>23-3</td>
<td>27-4-35-5</td>
</tr>
<tr>
<td>C17:0</td>
<td>0-2</td>
<td>ND</td>
</tr>
<tr>
<td>C18:1</td>
<td>23-2</td>
<td>2-4-17-6</td>
</tr>
<tr>
<td>C18:0</td>
<td>0-5</td>
<td>ND</td>
</tr>
</tbody>
</table>

Analyses of non-polar and hydroxy fatty acids were performed on a culture of strain IH5\textsuperscript{T} grown for 24 h in TSA medium (Merck) at 28 °C (Behrendt *et al.*, 1999; Rivas *et al.*, 2003b). The results of the chemotaxonomic analyses are shown in Table 2. The main non-polar fatty acids detected were hexadecenoic acid (C\textsubscript{16:1} \(\Delta\)), hexadecanoic acid (C\textsubscript{16:0}) and octadecenoic acid (C\textsubscript{18:1} \(\Delta\)). The hydroxy fatty acids detected were 3-hydroxydecanoic acid (C\textsubscript{10:0} 3-OH), 3-hydroxydodecanoic acid (C\textsubscript{12:0} 3-OH) and 2-hydroxydodecanoic acid (C\textsubscript{12:0} 2-OH). This fatty acid profile is characteristic of strains from rRNA group 1 (Oyaizu & Komagata, 1983). According to published data, the cellular fatty acid pattern of *P. graminis* (Table 2) is similar to that of strain IH5\textsuperscript{T} (Behrendt *et al.*, 1999). The main difference is the presence of 3-hydroxydecanoic acid (C\textsubscript{10:0} 3-OH) in the novel species; this fatty acid was not detected in *P. graminis* DSM 11363\textsuperscript{T} (Behrendt *et al.*, 1999).

For base composition analysis, DNA was prepared according to Chun & Goodfellow (1995). DNA G+C content was determined by using the thermal denaturation method (Mandel & Marmur, 1968). The G+C content was 61 mol%; this value is similar to those obtained for *P. graminis* (Behrendt *et al.*, 1999).

For DNA–DNA hybridization analyses, DNA was isolated by chromatography on hydroxyapatite by following the procedure of Cashion *et al.* (1977), which was carried out as described by De Ley *et al.* (1970) with the modification described by Huss *et al.* (1983) and Escara & Hutton (1980), using a model 2600 spectrophotometer equipped with a model 2527-R thermoprogrammer and plotter (Gilford Instrument Laboratories). Renaturation rates were computed with the TRANSFER.BAS program by Jahnke (1992). DNA–DNA homology was tested in 2× SSC + 10% (v/v) DMSO at 68 °C; the results showed 23% hybridization between strain IH5\textsuperscript{T} and *P. graminis* DSM 11363\textsuperscript{T}. When the recommended threshold value of 70% DNA–DNA similarity for species definition is considered (Wayne *et al.*, 1987), these results indicate that strain IH5\textsuperscript{T} does not belong to *P. graminis*.

Therefore, on the basis of phylogenetic, chemotaxonomic and phenotypic data, strain IH5\textsuperscript{T} should be classified as a novel species, for which we propose the name *Pseudomonas rhizosphaerae* sp. nov.

**Differentiation from *P. graminis***

From the phenotypic, chemotaxonomic and phylogenetic results of this study, we can conclude that *P. rhizosphaerae* IH5\textsuperscript{T} forms a separate group within the genus *Pseudomonas*, together with *P. graminis*. Nevertheless, the novel species can be differentiated from *P. graminis* by phenotypic characteristics, such as ascusin hydrolysis and growth in the presence of rhamnose or erythritol as the sole carbon source. Fatty acid analysis revealed the presence of 3-hydroxydecanoic acid (C\textsubscript{10:0} 3-OH) in strain IH5\textsuperscript{T}, which was absent in *P. graminis* [according to the data of Behrendt *et al.* (1999)]. 16S rRNA gene sequence similarity between *P. rhizosphaerae* and *P. graminis* was 98.8% and DNA–DNA relatedness was 23%.

**Description of *Pseudomonas rhizosphaerae* sp. nov.**

*Pseudomonas rhizosphaerae* (rhi. z.o. sphae.‘rae. Gr. fem. n. rhiza root; L. fem. n. sphaera from Gr. fem. n. sphaira ball, globe, sphere; N.L. gen. fem. n. rhizosphaerae of the rhizosphere).

Gram-negative, strictly aerobic, non-spore-forming, rod-shaped cells, 1.2–2.4 µm in length and 0.8–0.9 µm in diameter. Motile with one polar flagellum. Colonies on YED agar are circular, convex, yellow, weakly translucent and usually 1–2 mm in diameter after 2 days growth at 28 °C. Strain IH5\textsuperscript{T} is able to oxidize glucose in medium that contains ammonium nitrate as the nitrogen source and neutral red as a pH indicator, but is unable to ferment glucose in the same medium. Catalase is produced, but oxidase, gelatinase, caseinase, urease, arginine dehydroylase, tryptophan deaminase, β-galactosidase, indole and H\textsubscript{2}S are not. Aesculin is not hydrolysed. Strain IH5\textsuperscript{T} utilizes L-arabinose, D-arabinose, D-xylose, ribose, mannose, galactose, D-fructose, L-sorbose, D-fucose, L-fucose, rhamnose, dulcitol, inositol, sorbitol, mannitol, adonitol, glycerol, erythritol, D-arabitol, gentiobiose, D-turanose, D-tagatose, caprate, malate, gluconate, 2-ketogluconate, 5-ketogluconate and citrate as sole carbon sources. By contrast, it does not grow in N-acetylglucosamine, maltose, adipate or phenylacetate. DNA G+C content of the type strain is 61 mol%.

The type strain is IH5\textsuperscript{T} (=LMG 21640\textsuperscript{T} = CECT 5726\textsuperscript{T}).

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References


(2003b). Description of Devosia neptuniae sp. nov. that nodulates and fixes nitrogen in symbiosis with Neptunia natans, an aquatic legume from India. Syst Appl Microbiol 26, 47–53.


