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

Alvaro Peix,¹ Raúl Rivas,² Pedro F. Mateos,² Eustoquio Martínez-Molina,² Claudino Rodríguez-Barrueco¹ and Encarna Velázquez²

Correspondence

Encarna Velázquez
evp@gugu.usal.es

¹Instituto de Recursos Naturales y Agrobiología, CSIC, Salamanca, Spain

²Departamento de Microbiología y Genética, Lab. 209, Edificio Departamental de Biología, Campus M. Unamuno Universidad de Salamanca, 37007 Salamanca, Spain

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 *et al.*, 1973; Palleroni, 1992). *Pseudomonas putida* (Viveganandan & Jauhri, 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 Simone *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 insoluble 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 μ l 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

Published online ahead of print on 27 June 2003 as DOI 10.1099/ij.s.0.02703-0.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain IH5^T is AY152673.

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 IH5^T 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 grew 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 IH5^T (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* (Bařda *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 IH5^T forms a separate group with *P. graminis* DSM 11363^T (see Supplementary Fig. II, available in IJSEM Online). Fig. 1 shows the phylogenetic placement of strain IH5^T 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 IH5^T 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 IH5^T 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

Fig. 1. Comparative sequence analysis of 16S rDNA from *Pseudomonas rhizosphaerae* IH5^T and species from the genus *Pseudomonas sensu stricto* with validly published names. Significance of each branch is indicated by a bootstrap value, calculated for 1000 subsets. Bar, 2 substitutions per 100 nt.

of phenotypic analysis, which showed that *P. graminis* and strain IH5^T 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 11363^T and strain IH5^T.

Glucose oxidation and fermentation were tested in the medium described by Hugh & Leifson (1953). Catalase production was assayed by using 0.3 % (v/v) hydrogen peroxide with a colony taken from YED agar plates. Oxidase activity was detected by using *N,N,N',N'*-tetramethyl 1,4-phenylenediamine dihydrochloride (10 g l⁻¹). *P. fluorescens* DSM 50090^T and *P. graminis* DSM 11363^T were used as positive and negative controls, respectively. Casein activity was detected on skimmed-milk agar after 7 days incubation. Amylases were detected in plates that contained 0.7 % (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 31^T and *P. graminis* DSM 11363^T 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 11363^T 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-Ca36^T and *Sinorhizobium meliloti*

ATCC 9930^T 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 11363^T as the reference.

Strain IH5^T showed a great ability to solubilize phosphates on YED-P plates. On the same medium and under the same culture conditions, *P. graminis* DSM 11363^T showed a lower ability to solubilize phosphates: after 24 h incubation, phosphate solubilization was clearly positive in strain IH5^T, but weak in *P. graminis* DSM 11363^T. Optimal growth temperature was 25 °C on nutrient agar. The API 20NE and API 50CH systems were only used to characterize strain IH5^T, 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 IH5^T 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 IH5^T 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 IH5^T differs from *P. graminis* in aesculin hydrolysis and assimilation of erythritol and rhamnose.

Table 1. Differential characteristics between *Pseudomonas rhizosphaerae* sp. nov. and closely related species

Species: 1, *P. rhizosphaerae* IH5^T (=LMG 21640^T); 2, *P. graminis*; 3, *P. agarici*; 4, *P. jessenii*. Data from Holt *et al.* (1994), Behrendt *et al.* (1999) and Verhille *et al.* (1999). +, Positive; –, negative; w, weak; ND, no data available.

Characteristic	1	2	3	4
Size (µm)	0.8–0.9 × 1.2–2.4	0.5–1.0 × 3.5–5.0	ND	1.2 × 3.3
Number of polar flagella	1	1	1 or 2	1
Fluorescent pigments	–	–	+	+
Oxidase	–	–	+	+
Growth at:				
4 °C	+	+	ND	+
41 °C	–	–	ND	–
Acid from glucose	–	–	+	+
Phosphate solubilization* (24 h)	+	w	ND	ND
Utilization of:				
Erythritol	+	–	ND	–
Rhamnose	+	–	–	ND
Hydrolysis of:				
Aesculin	–	+	–	ND
Casein	–	+†	ND	ND
Gelatin	–	+†	ND	–

*Data from this study (only the type strain of *P. graminis*, DSM 11363^T, was tested).

†Some strains give a negative result.

Table 2. Percentage cellular fatty acid composition of *Pseudomonas rhizosphaerae* and *P. graminis*

Species: 1, *P. rhizosphaerae* IH5^T (=LMG 21640^T); 2, *P. graminis* DSM 11363^T. Data for *P. graminis* are from Behrendt *et al.* (1999). The results were obtained by using comparable methods. ND, Not detected.

Fatty acid	1	2
C _{10:0} 3-OH	2.9	ND
C _{12:0} 2-OH	2.7	0.1–2.3
C _{12:0} 3-OH	4.1	0.5–2.6
C _{12:0}	4.3	0–3.4
C _{14:0}	0.3	0.2–0.3
C _{16:1}	38.6	50.1–61.4
C _{16:0}	23.3	27.4–35.5
C _{17:0}	0.2	ND
C _{18:1}	23.2	2.4–17.6
C _{18:0}	0.5	ND

Analyses of non-polar and hydroxy fatty acids were performed on a culture of strain IH5^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_{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). This fatty acid profile is characteristic of strains from rRNA group I (Oyaizu & Komagata, 1983). According to published data, the cellular fatty acid pattern of *P. graminis* (Table 2) is similar to that of strain IH5^T (Behrendt *et al.*, 1999). The main difference is the presence of 3-hydroxydecanoic acid (C_{10:0} 3-OH) in the novel species; this fatty acid was not detected in *P. graminis* DSM 11363^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^T and *P. graminis* DSM 11363^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^T does not belong to *P. graminis*.

Therefore, on the basis of phylogenetic, chemotaxonomic and phenotypic data, strain IH5^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^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 aesculin 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_{10:0} 3-OH) in strain IH5^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.zo.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^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 dehydrolase, tryptophan deaminase, β-galactosidase, indole and H₂S are not. Aesculin is not hydrolysed. Strain IH5^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^T (= LMG 21640^T = CECT 5726^T).

Acknowledgements

This work was supported by CAICYT-DGES and JCyL (Spanish Government) to E.V. and E.M.-M. and by CLUE and TLINKS

European Research Projects to C. R.-B. We are grateful to Dr J. González and M. Ortiz-Aranda for their help with electron microscopy preparations and to M. Sánchez for his help with 16S rDNA sequencing. We are also grateful to Dr Kroppenstedt and Dr Schumann (DSMZ) for their help with the fatty acid methyl ester and DNA–DNA homology analysis, respectively. The authors thank I. Geldart for revising the English version of the manuscript.

References

- Anzai, Y., Kim, H., Park, J.-Y., Wakabayashi, H. & Oyaizu, H. (2000). Phylogenetic affiliation of the pseudomonads based on 16S rRNA sequence. *Int J Syst Evol Microbiol* **50**, 1563–1589.
- Baïda, N., Yazourh, A., Singer, E. & Izard, D. (2002). *Pseudomonas grimontii* sp. nov. *Int J Syst Evol Microbiol* **52**, 1497–1503.
- Behrendt, U., Ulrich, A., Schumann, P., Erler, W., Burghardt, J. & Seyfarth, W. (1999). A taxonomic study of bacteria isolated from grasses: a proposed new species *Pseudomonas graminis* sp. nov. *Int J Syst Bacteriol* **49**, 297–308.
- Cashion, P., Holder-Franklin, M. A., McCully, J. & Franklin, M. (1977). A rapid method for the base ratio determination of bacterial DNA. *Anal Biochem* **81**, 461–466.
- Chun, J. & Goodfellow, M. (1995). A phylogenetic analysis of the genus *Nocardia* with 16S rRNA sequences. *Int J Syst Bacteriol* **45**, 240–245.
- De Ley, J., Cattoir, H. & Reynaerts, A. (1970). The quantitative measurement of DNA hybridization from renaturation rates. *Eur J Biochem* **12**, 133–142.
- Delorme, S., Lemanceau, P., Christen, R., Corberand, T., Meyer, J.-M. & Gardan, L. (2002). *Pseudomonas lini* sp. nov., a novel species from bulk and rhizospheric soils. *Int J Syst Evol Microbiol* **52**, 513–523.
- Deubel, A., Gransee, A. & Merbach, W. (2000). Transformation of organic rhizodepositions by rhizosphere bacteria and its influence on the availability of tertiary calcium phosphate. *J Plant Nutr Soil Sci* **163**, 387–392.
- Di Simone, C. D., Sayer, J. A. & Gadd, G. M. (1998). Solubilization of zinc phosphate by a strain of *Pseudomonas fluorescens* isolated from a forest soil. *Biol Fertil Soils* **28**, 87–94.
- Doetsch, R. N. (1981). Determinative methods of light microscopy. In *Manual of Methods for General Bacteriology*, pp. 21–33. Edited by P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg & G. B. Phillips. Washington, DC: American Society for Microbiology.
- Escara, J. F. & Hutton, J. R. (1980). Thermal stability and renaturation of DNA in dimethyl sulfoxide solutions: acceleration of the renaturation rate. *Biopolymers* **19**, 1315–1327.
- Felsenstein, J. (1983). Parsimony in systematics: biological and statistical issues. *Annu Rev Ecol Syst* **14**, 313–333.
- Gardan, L., Shafik, H., Belouin, S., Broch, R., Grimont, F. & Grimont, P. A. D. (1999). DNA relatedness among the pathovars of *Pseudomonas syringae* and description of *Pseudomonas tremiae* sp. nov. and *Pseudomonas cannabina* sp. nov. (ex Sutic and Dowson 1959). *Int J Syst Evol Microbiol* **49**, 469–478.
- Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T. & Williams, S. T. (editors) (1994). *Bergey's Manual of Determinative Bacteriology*, 9th edn. Baltimore: Williams & Wilkins.
- Hugh, R. & Leifson, E. (1953). The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various gram-negative bacteria. *J Bacteriol* **66**, 24–26.
- Huss, V. A. R., Festl, H. & Schleifer, K.-H. (1983). Studies on the spectrophotometric determination of DNA hybridization from renaturation rates. *Syst Appl Microbiol* **4**, 184–192.
- Jahnke, K.-D. (1992). Basic computer program for evaluation of spectroscopic DNA renaturation data from GILFORD system 2600 spectrometer on a PC/XT/AT type personal computer. *J Microbiol Methods* **15**, 61–73.
- Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.
- Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.
- Kumar, V. & Singh, K. P. (2001). Enriching vermicompost by nitrogen fixing and phosphate solubilizing bacteria. *Bioresour Technol* **76**, 173–175.
- Kumar, S., Tamura, K., Jakobsen, I. B. & Nei, M. (2001). MEGA2: molecular evolutionary genetic analysis software. *Bioinformatics* **17**, 1244–1245.
- Mandel, M. & Marmur, J. (1968). Use of ultraviolet absorbance temperature profile for determining the guanine plus cytosine content of DNA. *Methods Enzymol* **12B**, 195–206.
- Manna, M. C., Ghosh, P. K., Ghosh, B. N. & Singh, K. N. (2001). Comparative effectiveness of phosphate-enriched compost and single superphosphate on yield, uptake of nutrients and soil quality under soybean-wheat rotation. *J Agri Sci* **137**, 45–54.
- Musarrat, J., Bano, N. & Rao, R. A. K. (2000). Isolation and characterization of 2,4-dichlorophenoxyacetic acid-catabolizing bacteria and their biodegradation efficiency in soil. *World J Microbiol Biotechnol* **16**, 495–497.
- Oyaizu, H. & Komagata, K. (1983). Grouping of *Pseudomonas* species on the basis of cellular fatty acid composition and the quinone system with special reference to the existence of 3-hydroxy fatty acids. *J Gen Appl Microbiol* **29**, 17–40.
- Palleroni, N. J. (1992). Present situation of the taxonomy of aerobic pseudomonads. In *Pseudomonas: Molecular Biology and Biotechnology*, pp. 105–115. Edited by E. Galli, S. Silver & B. Witholt. Washington, DC: American Society for Microbiology.
- Palleroni, N. J., Kunisawa, R., Contopoulou, R. & Doudoroff, M. (1973). Nucleic acid homologies in the genus *Pseudomonas*. *Int J Syst Bacteriol* **23**, 333–339.
- Pandey, A. & Palni, L. M. S. (1998). Isolation of *Pseudomonas corrugata* from Sikkim Himalaya. *World J Microbiol Biotechnol* **14**, 411–413.
- Pearson, W. & Lipman, D. (1988). Improved tools for biological sequence comparison. *Proc Natl Acad Sci U S A* **85**, 2444–2448.
- Peix, A., Rivas-Boyer, A. A., Mateos, P. F., Rodríguez-Barrueco, C., Martínez-Molina, E. & Velázquez, E. (2001). Growth promotion of chickpea and barley by a phosphate solubilizing strain of *Mesorhizobium mediterraneum* under growth chamber conditions. *Soil Biol Biochem* **33**, 103–110.
- Rivas, R., Velázquez, E., Valverde, A., Mateos, P. F. & Martínez-Molina, E. (2001). A two primers random amplified polymorphic DNA procedure to obtain polymerase chain reaction fingerprints of bacterial species. *Electrophoresis* **22**, 1086–1089.
- Rivas, R., Sánchez, M., Trujillo, M. E., Zurdo-Piñeiro, J. L., Mateos, P. F., Martínez-Molina, E. & Velázquez, E. (2003a). *Xylanimonas cellulolytica* gen. nov., sp. nov., a xylanolytic bacterium isolated from a decayed tree (*Ulmus nigra*). *Int J Syst Evol Microbiol* **53**, 99–103.
- Rivas, R., Willems, A., Subba-Rao, N. S., Mateos, P. F., Dazzo, F. B., Kroppenstedt, R. M., Martínez-Molina, E., Gillis, M. & Velázquez, E.

- (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.
- Rodríguez, H. & Fraga, R. (1999). Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotechnol Adv* **17**, 319–339.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Sikorski, J., Stackebrandt, E. & Wackernagel, W. (2001). *Pseudomonas kilonensis* sp. nov., a bacterium isolated from agricultural soil. *Int J Syst Evol Microbiol* **51**, 1549–1555.
- Singh, S. & Kapoor, K. K. (1994). Solubilization of insoluble phosphates by bacteria isolated from different sources. *Environ Ecol* **12**, 51–55.
- Tajima, F. & Nei, M. (1984). Estimation of evolutionary distance between nucleotide sequences. *Mol Biol Evol* **1**, 269–285.
- Tamura, K. & Nei, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol Biol Evol* **10**, 512–526.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.
- Uchino, M., Shida, O., Uchimura, T. & Komagata, K. (2001). *Pseudomonas parafulva* sp. nov. and *Pseudomonas cremoricolorata* sp. nov. In *Validation of the Publication of New Names and New Combinations Previously Effectively Published Outside the IJSEM*, List no. 85. *Int J Syst Evol Microbiol* **52**, 685–690.
- Vázquez, P., Holguin, G., Puente, M. E., López-Cortez, A. & Bashan, Y. (2000). Phosphate-solubilizing microorganisms associated with the rhizosphere of mangroves in a semiarid coastal lagoon. *Biol Fertil Soils* **30**, 460–468.
- Verhille, S., Baida, N., Dabboussi, F., Izard, D. & Leclerc, H. (1999). Taxonomic study of bacteria isolated from natural mineral waters: proposal of *Pseudomonas jessenii* sp. nov. and *Pseudomonas mandelii* sp. nov. *Syst Appl Microbiol* **22**, 45–58.
- Villegas, J. & Fortin, J. A. (2002). Phosphorus solubilization and pH changes as a result of the interactions between soil bacteria and arbuscular mycorrhizal fungi on a medium containing NO₃⁻ as nitrogen source. *Can J Bot* **80**, 571–576.
- Viveganandan, G. & Jauhri, K. S. (2000). Growth and survival of phosphate-solubilizing bacteria in calcium alginate. *Microbiol Res* **155**, 205–207.
- Wayne, L. G., Brenner, D. J., Colwell, R. R. & 9 other authors (1987). International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.
- Yang, Z. (1997). PAML: a program package for phylogenetic analysis by maximum likelihood. *Comput Appl Biosci* **13**, 555–556.