Pseudomonas argentinensis sp. nov., a novel yellow pigment-producing bacterial species, isolated from rhizospheric soil in Córdoba, Argentina

Alvaro Peix,^{1†} Odile Berge,² Raúl Rivas,³ Adriana Abril⁴ and Encarna Velázquez³

Correspondence

Alvaro Peix
alvarp@usal.es

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

²CEA/Cadarache, DSV-DEVM-LEMIR, Laboratoire d'Écologie Microbienne de la Rhizosphère, UMR6191 CNRS-CEA-Univ, Méditerranée, F-13108 Saint-Paul-Lez-Durance, France

³Departamento de Microbiología y Genética, Universidad de Salamanca, Spain

⁴Microbiología Agrícola, Facultad de Ciencias Agropecuarias, Universidad Nacional de Córdoba, Argentina

During a study in the Argentinian region of Chaco (Córdoba), some strains were isolated from the rhizosphere of grasses growing in semi-desertic arid soils. Two of these strains, one isolated from the rhizospheric soil of *Chloris ciliata* (strain CH01^T) and the other from *Pappophorum caespitosum* (strain PA01), were Gram-negative, strictly aerobic rods, which formed yellow round colonies on nutrient agar. They produced a water-insoluble yellow pigment, and a fluorescent pigment was also detected. A polyphasic taxonomic approach was used to characterize the strains. Comparison of the 16S rRNA gene sequences showed a similarity of 99.3 % between them, and phylogenetic analysis revealed that the strains belong to the genus *Pseudomonas*, within the γ -subclass of the *Proteobacteria*. The closest related species is *Pseudomonas straminea* IAM 1598^T (similarity of 99.0 % to strain CH01^T and 98.8 % to strain PA01), clustering in a separate branch with the various methods of tree building used. Strains CH01^T and PA01 both had a single polar flagellum, like other yellow pigment-producing pseudomonads related to them. Both strains produced catalase and oxidase. Similar to *P. straminea*, they did not hydrolyse gelatin or casein. The G + C DNA contents determined were 57.5 mol% for CH01^T and 58.0 mol% for PA01. DNA–DNA hybridization results showed 81 % relatedness between them, and only 40–44 % relatedness with respect to the type strain of *P. straminea*. These results, together with other phenotypic characteristics, support the conclusion that both isolates belong to the same species, and should be described as representing a novel species within the genus *Pseudomonas*, for which the name *Pseudomonas argentinensis* sp. nov. is proposed. The type strain is CH01^T (=LMG 22563^T=CECT 7010^T).

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[†]Present address: UMR 1229 INRA-Université, de Bourgogne 'Microbiologie et Géochimie des Sols', INRA, 17 Rue Sully, BP 86510, 21065 Dijon CEDEX, France.

Abbreviation: rep-PCR, repetitive extragenic palindromic DNA–PCR.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains CH01^T and PA01 are AY691188 and AY691189, respectively.

A phylogenetic tree for *Pseudomonas argentinensis* sp. nov. and related species of the genus *Pseudomonas sensu stricto*, rep-PCR patterns, similarity matrix data and reciprocal values for percentage DNA–DNA hybridization are available as supplementary material in IJSEM Online.

The arid Chaco ecoregion occupies the western-most, driest portion of the vast South American Chaco woodlands and savannas in central Argentina. Vegetation is dominated by xerophyllous woody species such as *Aspidosperma quebracho-blanco* and *Prosopis flexuosa*. The dense 2–3 m high shrub layer is dominated by species of *Larrea*, *Celtis*, *Mimozyanthus* and *Acacia*. Intermingled grassland patches are dominated by *Aristida*, *Setaria*, *Trichloris*, *Chloris* and *Pappophorum* species. Grass species in these patches play a central role in cattle ranching, the dominant economic activity in the region (Bucher, 1982). For this reason, the aim of the study was the screening and isolation of plant growth-promoting rhizobacteria strains that are

well-adapted to arid and dry-warm environments, which could be used to inoculate introduced pastures in the Chaco region.

The rhizosphere of grasses contains complex populations of bacteria, and pseudomonads are very commonly found in these environments. Moreover, several *Pseudomonas* species producing yellow pigments have been isolated and described, mostly from plant materials. These include *Pseudomonas oryzae* (Kodama *et al.*, 1985; Anzai *et al.*, 1997), *Pseudomonas fluorescens* (Hildebrand *et al.*, 1994), *Pseudomonas straminea* (Uchino *et al.*, 2000), *Pseudomonas graminis* (Behrendt *et al.*, 1999), and the very recently described *Pseudomonas rhizosphaerae* and *Pseudomonas lutea* (Peix *et al.*, 2003, 2004). According to the 16S rRNA gene sequences, all of these species of *Pseudomonas* are included in rRNA group I (Palleroni *et al.*, 1973; Palleroni, 1992).

Material was collected from the Chancaní Provincial Reserve, Córdoba, Argentina (31° 24' S, 65° 33' W). The local soils (Mollic Ustifluent) are of alluvial origin. Vegetation is typical Chaco woodland (Bucher, 1982). Roots from flowering grass specimens in the study area, including *Pappophorum caespitosum* and *Chloris ciliata*, were collected. Rhizospheric soil was obtained by vigorous shaking in sterile water. Strains were isolated by cultivation in nitrogen-free medium according to Döbereiner (1995), and were characterized for their ecological interest and possible practical applications. Among efficient strains, isolates CH01^T and PA01 were also found to be interesting from a taxonomic point of view, during an initial identification by partial 16S rRNA gene sequencing.

The cells were stained according to the Gram procedure described by Doetsch (1981). Motility was checked by phase-contrast microscopy after growth on nutrient agar medium at 22 °C for 48 h. Strains CH01^T and PA01 are Gram-negative, rod-shaped (0.5–0.7 × 1.3–1.9 µm), motile bacteria, with a single polar flagellum. Cells grew as round translucent yellow-coloured colonies on nutrient agar. The yellow colour was slightly darker in strain PA01, and in both cases became darker with culture age, as observed previously for *P. straminea* by Uchino *et al.* (2000).

For pigment analysis, cells were grown on King's B agar and nutrient agar, and testing for pigment production and spectral characteristics was performed by extraction with methanol, according to Hildebrand *et al.* (1994), using a visible–UV Kontron Uvikon 860 spectrophotometer. Both strains produced a water-insoluble yellow pigment with a major peak at 442 nm, whereas the positions of the major peak of the closest related species are 445.8 nm for *P. straminea* and 446 nm for *P. fluorescens* (Hildebrand *et al.*, 1994; Uchino *et al.*, 2000). In the novel species, a fluorescent pigment was also detected, similar to *P. fluorescens*, and by contrast with *P. straminea*, in which pigments that were not always fluorescent were found (Uchino *et al.*, 2000). *P. straminea* was isolated from Japanese paddy fields and was

first described by Iizuka and Komagata in 1963, but was later emended by Uchino *et al.* (2000), who recharacterized the species and reclassified other strains originally assigned to species such as *Pseudomonas ochracea* as belonging to *P. straminea*.

The isolates CH01^T and PA01 were subjected to repetitive extragenic palindromic DNA–PCR (rep-PCR) fingerprinting using primers REP1R-I and REP2-I (Versalovic *et al.*, 1991), as already reported by Frey *et al.* (1997). Different electrophoretic patterns were obtained for each isolate (see Supplementary Figure A in IJSEM Online), from which it was concluded that they correspond to different strains.

For 16S rRNA gene sequencing and comparative analysis, DNA extraction, amplification and sequencing of 16S rRNA genes were performed as already reported (Rivas *et al.*, 2003). Nearly complete 16S rRNA gene sequences for isolates CH01^T and PA01 were obtained (1532 and 1530 nucleotides, respectively). They were compared with sequences retrieved from GenBank by using the BLAST program (Altschul *et al.*, 1990), and aligned by using CLUSTAL_X software (Thompson *et al.*, 1997). The sequences were analysed by using the parameters and methods previously used for *P. rhizosphaerae* (Peix *et al.*, 2003). Bootstrap analysis was based on 1000 resamplings. The MEGA 2.1.0 package (Kumar *et al.*, 2001) was used for all the analyses.

The 16S rRNA gene sequence of strain CH01^T showed 99.3 % similarity with that of strain PA01. The closest relative of both CH01^T and PA01 was the type strain of *P. straminea*, IAM 1598^T (= CIP 106745^T = ATCC 33636^T = JCM 2783^T = NRIC 0164^T), with a sequence similarity of 99.0 and 98.8 %, respectively.

A complete phylogenetic analysis was performed. The distances were calculated according to Jukes & Cantor (1969), Kimura (1980), Tajima & Nei (1984) and Tamura & Nei (1993). Phylogenetic trees were inferred using the neighbour-joining method (Saitou & Nei, 1987), maximum-likelihood (Yang, 1997) and parsimony analysis (Felsenstein, 1983). The trees were rooted using *Pseudomonas pertucinogena* as an outgroup. All the methods used gave the same results (data not shown). Together with the closest relative, *P. straminea* IAM 1598^T, all the type strains of species of the genus *Pseudomonas sensu stricto*, according to Anzai *et al.* (2000), other species of *Pseudomonas* with validly published names listed in Peix *et al.* (2003), including the recently described novel species *P. rhizosphaerae* and *P. lutea* (Peix *et al.*, 2003, 2004), and the closely related species previously reclassified from *Pseudomonas fulva* (Iizuka & Komagata, 1963) by Uchino *et al.* (2001), *P. fulva*, *Pseudomonas parafulva* and *Pseudomonas cremoricolorata*, were included in the analysis. Fig. 1 shows a reduced phylogenetic tree obtained by using the Kimura two-parameter model (Kimura, 1980) and the neighbour-joining method (Saitou & Nei, 1987), showing the phylogenetic placement of strains CH01^T and PA01 within the genus *Pseudomonas sensu stricto*,

Fig. 1. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences of *P. argentinensis* sp. nov. and related species of the genus *Pseudomonas sensu stricto*. The significance of each branch is indicated by a bootstrap value (%) calculated for 1000 subsets. Bar, 0.005 estimated substitution per 100 base positions.

clustering separately in a group together with *P. straminea* IAM 1598^T (a more complete tree and a similarity matrix for the strains considered in the phylogenetic tree are available as Supplementary Figure B and Supplementary Table A, respectively, in IJSEM Online). As can be seen, strains CH01^T and PA01 form a separate branch together with *P. straminea*. The closest related species to this group is *P. flavescens*, with similarities of 98.4 and 98.2 % with respect to strain CH01^T and strain PA01, respectively. Other phenotypic characteristics, such as yellow pigment production, fatty acid pattern, and others detailed later in this report, are also shared.

DNA–DNA hybridization was carried out by using the method of Ezaki *et al.* (1989), following the recommendations of Willems *et al.* (2001). Strains CH01^T and PA01, and the type strain of the closest relative, *P. straminea* IAM 1598^T, were included in the assay. The results of the DNA–DNA hybridization showed a relatedness of 81 % between strains CH01^T and PA01; with respect to *P. straminea* IAM 1598^T, relatedness values of 40 and 44 % were obtained, respectively (the values given are mean values; for reciprocal values see Supplementary Table B in IJSEM Online). Taking into account the recommendation of a threshold

value of 70 % DNA–DNA relatedness for the definition of a species (Wayne *et al.*, 1987), these results indicate that strains CH01^T and PA01 do not belong to the species *P. straminea*. For the same reason, strains CH01^T and PA01 were confirmed as belonging to the same species.

For base composition analysis, DNA was prepared according to Chun & Goodfellow (1995). The mol% G + C DNA content was determined by using the thermal denaturation method (Mandel & Marmur, 1968). The G + C content was 57.5 mol% for CH01^T and 58.0 mol% for PA01. These values are similar to those obtained for *P. straminea* and related species (Uchino *et al.*, 2000).

The phenotypic analyses were performed as described previously (Peix *et al.*, 2003), using *P. straminea* IAM 1598^T as a reference. According to the results obtained, neither strain was able to solubilize bi-calcium phosphate in YED-P plates (7 g glucose l⁻¹, 3 g yeast extract l⁻¹, 3 g bi-calcium phosphate l⁻¹ and 17 g agar l⁻¹), similar to *P. straminea* IAM 1598^T. The optimal growth temperature was 25 °C on nutrient agar. None of the strains grew at 4 or 41 °C. Similar to *P. straminea* and *P. flavescens* (Hildebrand *et al.*, 1994; Uchino *et al.*, 2000), the novel species was oxidative

Table 1. Differential characteristics among *P. argentinensis* sp. nov. and the phylogenetically and phenotypically closely related species *P. straminea* and *P. flavescens*

Species: 1, *P. argentinensis* sp. nov.; 2, *P. straminea*; 3, *P. flavescens*. Data are from this study. +, Positive; –, negative; w, weak; SD, strain-dependent.

Characteristic	1	2	3
Fluorescent pigments	+	–	+
Growth at 4 °C	–	+	+
Nitrate reduction to nitrite	+	–	–
Assimilation of:			
2-Ketogluconate	+	–	–
5-Ketogluconate	–	–	+
Trehalose	+	–	+
D-Ribose	SD	–	–
D-Xylose	SD	–	–
D-Arabitol	SD	w	–
Inositol	–	+	–
Phenylacetate	–	–	+
Glycerol	SD	+	+
Erythritol	–	w	–
Melibiose	SD	–	–
Sucrose	–	–	+

for the O–F test, was not fermentative, did not produce arginine dihydrolase or gelatinase, and did not hydrolyse casein in 10 % skimmed milk agar plates. Oxidase and catalase were produced. Some differential phenotypic characteristics among strains CH01^T and PA01 and the closely related species *P. straminea* and *P. flavescens* are given in Table 1. As can be seen, by contrast with *P. straminea* and *P. flavescens*, both strains of the novel species reduce nitrate to nitrite, and assimilate 2-ketogluconate but not 5-ketogluconate. Other differential characteristics were found in the assimilation of D-arabitol, trehalose, phenylacetate, erythritol and D-ribose. The two strains of the novel species differed in the assimilation of glycerol, D-ribose, D-xylose, melibiose and D-arabitol, which was positive for CH01^T and negative for PA01.

The analyses of non-polar and hydroxy fatty acids were performed with cultures of strains CH01^T and PA01 that were grown for 24 h in TSA medium (Merck) at 28 °C, as previously reported (Peix *et al.*, 2003). The type strains of the closely related species *P. straminea* and *P. flavescens* were also included in the analyses. There were no significant differences in the cellular fatty acid composition between the two strains of the novel species. The results of the chemotaxonomic analyses are shown in Table 2. The main non-polar fatty acids detected were dodecanoid acid (12:0), hexadecanoic acid (16:0) and octadecenoic acid (18:1). The main hydroxy fatty acids detected were 3-hydroxydecanoic acid (3-OH 10:0), 3-hydroxyundecanoic acid (3-OH 11:0), 3-hydroxydodecanoic acid (3-OH 12:0) and 2-hydroxydodecanoic acid (2-OH 12:0). This profile

Table 2. Cellular fatty acid content (%) of the type strains of *P. argentinensis* sp. nov., *P. straminea* and *P. flavescens*

Strains: 1, *P. argentinensis* sp. nov. CH01^T (=LMG 22563^T); 2, *P. straminea* IAM 1598^T; 3, *P. flavescens* DSM 12071^T. Data are from this study. ND, Not detected.

Fatty acids	1	2	3
3-OH 10:0	2·4	3·91	3·74
3-OH 11:0	0·10	ND	ND
2-OH 12:0	0·09	0·21	ND
3-OH 12:0	2·58	3·57	3·55
10:0	0·09	0·20	ND
11:0	0·09	ND	ND
12:0	7·88	9·58	9·23
13:0	0·08	ND	ND
14:0	0·69	0·78	0·71
15:1	0·14	ND	ND
15:0	0·97	ND	ND
16:0	19·69	17·63	19·75
17:1	0·73	0·54	0·31
17:0	0·52	0·36	ND
18:1	41·52	39·73	38·51
18:0	0·51	0·52	0·78
Summed feature 3*	21·34	22·40	22·39

*16:1 ω 7c and 2-OH iso 15:0.

corresponds to the typical fatty acid profile of species from rRNA group I (Oyaizu & Komagata, 1983). According to our data, the fatty acid composition of the novel species is similar to that of *P. straminea* and *P. flavescens*; it is also in agreement with results obtained previously by other authors (Hildebrand *et al.*, 1994; Uchino *et al.*, 2000). The main differences between the novel species and its closest relatives are in the fatty acids undecanoic acid (11:0), tridecanoic acid (13:0), pentadecanoic acid (15:0), pentadecenoic acid (15:1) and 3-hydroxyundecanoic acid (3-OH 11:0), which are present in the novel species but were not detected in *P. straminea* or in *P. flavescens*.

Therefore, from the analysis of all the phylogenetic, chemotaxonomic and phenotypic data, it can be concluded that CH01^T and PA01 are different strains from the same species, and that they should be classified as representing a novel species within the genus *Pseudomonas*, for which the name *Pseudomonas argentinensis* sp. nov. is proposed.

Description of *Pseudomonas argentinensis* sp. nov.

Pseudomonas argentinensis (ar.gen'tin.en.sis. N.L. fem. adj. *argentinensis* pertaining to the Argentine, of the Argentine).

Gram-negative, strictly aerobic, non-spore-forming, rod-shaped cells of 1·3–1·9 µm in length and 0·5–0·7 µm in diameter; motile with a single polar flagellum. Colonies on nutrient agar are circular, convex, yellow, translucent and

usually 0.5–2.5 mm in diameter, within 2 days of growth at 25 °C. Does not grow at 4 or 41 °C. The metabolism is oxidative, and no sugars are fermented in peptone media. A water-insoluble yellow pigment is produced on nutrient agar, with a major absorbance peak at 442 nm, and a fluorescent pigment is also produced at a low rate on King's B medium. Oxidase- and catalase-positive, and does not hydrolyse casein, aesculin or gelatin. The arginine dihydrolase system is not present. Urease, tryptophan deaminase and β -galactosidase are not produced. Nitrate is reduced to nitrite. Assimilates L-arabinose, galactose, glucose, D-fructose, mannose, mannitol, trehalose, D-fucose, β -gentiobiose, gluconate, 2-ketogluconate, caprate, malate and citrate. Utilization of glycerol, D-ribose, D-xylose, melibiose and D-arabitol as sole carbon source is strain-dependent. Does not assimilate adipate, phenylacetate, D-arabinose, L-fucose, inositol, adonitol, L-arabitol, L-lyxose, xylitol, L-xylose, L-sorbose, methyl β -D-xyloside, methyl α -D-mannoside, methyl α -D-glucoside, L-rhamnose, amygdalin, arbutin, salicin, cellobiose, lactose, sucrose, inulin, melezitose, D-raffinose, starch, glycogen, erythritol, sorbitol, dulcitol, N-acetylglucosamine, maltose, D-turanose, D-tagatose or 5-ketogluconate. The fatty acid pattern is shown in Table 2.

The type strain is CH01^T (=LMG 22563^T=CECT 7010^T), which has a G+C content of 57.5 mol%.

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