Pseudomonas composti sp. nov., isolated from compost samples

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Two unusual, Gram-negative, catalase- and oxidase-positive rods, designated C2^T and C5, were isolated from compost samples. Comparative 16S rRNA gene sequencing studies demonstrated that both isolates were members of the genus *Pseudomonas* and belonged to the *Pseudomonas aeruginosa* group. Strain C2^T was most closely related to *Pseudomonas cuatrocienegasensis* 1N^T and *Pseudomonas borbori* R-20821^T (97.9 and 97.8% 16S rRNA gene sequence similarity, respectively). However, phylogenetic analysis based on *rpoD* gene sequences revealed that both isolates could be discriminated from members of the *P. aeruginosa* group that exhibited >97% 16S rRNA gene sequence similarity. The DNA G+C content of strain C2^T was 61.5 mol%. The major fatty acids of strain C2^T were a summed feature (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH), C_{18:1}ω7c/12t/9t, C_{16:0} and C_{12:0}, which supported the isolates' affiliation with the genus *Pseudomonas*. Moreover, strain C2^T could be distinguished from its closest phylogenetic neighbours of the genus *Pseudomonas* by DNA–DNA hybridization studies and biochemical tests. On the basis of both phenotypic and phylogenetic findings, it is proposed that the isolates be classified as a novel species, with the name *Pseudomonas composti* sp. nov. The type strain is C2^T (=CECT 7516^T=CCUG 59231^T).

The genus *Pseudomonas*, proposed by Migula (1894), comprises a group of rod-shaped, non-spore-forming and motile Gram-negative bacteria (Palleroni, 1984). This genus is highly heterogeneous and it has undergone several reclassifications on the basis of phenotypic, chemotaxonomic and genetic studies (Palleroni, 1993; Vancanneyt *et al.*, 1996; Anzai *et al.*, 2000). Currently, the genus *Pseudomonas* comprises more than 120 species with validly published names (Euzéby, 1997; <http://www.bacterio.cict.fr/>). Members of the genus are widespread in natural environments, such as soil, fresh and marine waters, plants and clinical specimens from human and animals. In

addition to the clinical relevance of some members of the genus (Las Heras *et al.*, 1999; Poirel *et al.*, 2004; Vela *et al.*, 2006; Jesús Cremades *et al.*, 2009), several species have been widely studied because of their metabolic ability to biodegrade xenobiotic compounds, such as herbicides and veterinary pharmaceutical products (Manickam *et al.*, 2008; Li *et al.*, 2009; Ma *et al.*, 2009). This versatile metabolism has motivated attempts to isolate *Pseudomonas* strains from diverse environments.

During the investigation of the bacterial populations of compost samples made with vegetable and animal waste in the province of Cuenca (central region of Spain), two Gram-negative organisms, designated C2^T and C5, were recovered on MacConkey agar after 48 h at 30 °C. The isolates were tested for growth on tryptic soy agar (TSA; bioMérieux), Luria–Bertani agar (LB; USB Corporation) and Columbia blood agar (bioMérieux). Cell morphology and the presence of flagella were observed using transmission electron microscopy after growth on LB agar at 30 °C

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains C2^T and C5 are FN429930 and FN652314, respectively, and those for the *rpoD* sequences of strains C2^T and C5 and *Pseudomonas cuatrocienegasensis* LMG 24676^T are FR716577, FR716576 and FR716578, respectively.

Two supplementary figures are available with the online version of this paper.

for 2 days. Oxidase activity was tested with oxidase test sticks (Oxoid). Growth was tested on TSA at 22, 30, 37 and 42 °C for 24 h and at 10 and 4 °C for 7–10 days. The ability of the isolates to produce a fluorescent pigment was tested after 8 days on King's B medium (King *et al.*, 1954) under UV light. Growth was also examined with 1.5, 3, 4.5, 6 and 6.5 % (w/v) NaCl in Luria–Bertani medium at 30 °C for 48 h. Biochemical properties were examined using the API 20 NE and API ZYM systems (bioMérieux), according to the manufacturer's instructions. Carbohydrate assimilation was assayed using the API 50 CH system inoculated with a 0.5 McFarland suspension of bacterial cells in AUX medium (bioMérieux), as described previously (Gibello *et al.*, 2009). The API 50 CH strips were read after up to 4 days of incubation at 30 °C.

Cells were catalase- and oxidase-positive rods. Both isolates were strictly aerobic and motile by means of a single polar flagellum (see Supplementary Fig. S1, available in IJSEM Online). They grew on MacConkey agar and were not haemolytic. Growth was observed between 4 and 37 °C, but not at 42 °C. Both isolates exhibited identical biochemical characteristics, grew with 0.5–6.5 % NaCl and did not produce fluorescent pigment on King's B medium. A detailed description of the physiological, biochemical and morphological characteristics of the isolates is given in the species description and Table 1. These phenotypic characteristics clearly differentiated the compost isolates from their closest phylogenetic neighbours.

A comparative 16S rRNA gene sequence analysis was performed as described previously (Vela *et al.*, 2006). A large continuous fragment (approximately 1466 bases) of the 16S rRNA gene of both isolates was sequenced bidirectionally using universal primers pA (5'-AGAGTTTG-ATCCTGGCTCAG-3'; positions 8–27, *Escherichia coli* numbering) and pH* (5'-AAGGAGGTGATCCAGCCGCA-3'; positions 1541–1522, *E. coli* numbering). The PCR product was sequenced directly by the dideoxynucleotide chain-termination method using a DNA sequencer (3730 DNA Analyzer; Applied Biosystems). Primers pA, pH*, antiKK (5'-CGTGCCAGCAGCCGCGTAAT; positions 517–537, *E. coli* numbering) and 3 (5'-GTTGCGCTCGTTGCGGGACT-3'; positions 1109–1090, *E. coli* numbering) were used in the sequencing reaction. Comparative sequence analysis revealed 100 % 16S rRNA gene sequence similarity between the two isolates. Sequence searches of GenBank using the FASTA program (Pearson, 1994) showed that the isolates were most closely related to members of the genus *Pseudomonas*. Strain C2^T showed high 16S rRNA gene sequence similarity with *Pseudomonas cuatrocieneegasensis* 1N^T (97.9 %), *P. borbori* R-20821^T (97.8 %), *P. peli* R-20805^T (97.6 %), *P. guineae* LMG 24016^T (97.6 %) and *P. anguilliseptica* NCIMB 1949^T (97.1 %). Strain C2^T also showed >97 % 16S rRNA gene sequence similarity with other members of the genus *Pseudomonas*, such as *P. alcaligenes* LMG 1224^T, *P. pseudoalcaligenes* LMG 1225^T, *P. mendocina* ATCC 25411^T, *P. resinovorans* ATCC 14235^T, *P. marincola* KMM 3042^T, *P. segetis* FR1439^T and *P. taeanensis* MS-3^T.

Table 1. Characteristics useful in differentiating strain C2^T from closely related members of the genus *Pseudomonas*

Strains: 1, *Pseudomonas composti* sp. nov. C2^T; 2, *P. cuatrocieneegasensis* LMG 24676^T; 3, *P. borbori* CCUG 53773^T; 4, *P. guineae* CECT 7231^T; 5, *P. peli* CCUG 53775^T; 6, *P. anguilliseptica* CCUG 35503^T; 7, *P. aeruginosa* CCUG 551^T. All data were taken from this study. +, Positive; w, weakly positive; –, negative.

Characteristic	1	2	3	4	5	6	7
Nitrate reduction	–	–	+	–	–	–	+
Assimilation of:							
D-Glucose	+	+	+	–	–	–	+
D-Mannose	+	–	–	–	–	–	–
D-Mannitol	+	–	–	–	–	–	+
Maltose	–	–	+	–	–	–	–
D-Fructose	+	+	–	–	–	–	+
Trehalose	–	–	–	+	+	–	–
L-Rhamnose	–	+	–	–	–	–	–
Caprate	+	+	–	–	–	+	+
Citrate	+	+	–	–	–	+	+
Glycerol	+	+	–	–	–	–	+
Gluconate	+	+	–	–	–	–	+
L-Malate	+	+	–	–	+	+	+
Sucrose	–	+	–	–	–	–	–
Production of:							
Alkaline phosphatase	+	+	–	–	–	+	–
Esterase (C4)	w	+	+	+	+	–	+
Ester lipase (C8)	+	+	+	+	+	–	+
Acid phosphatase	w	+	–	–	–	+	+
Leucine arylamidase	+	+	+	–	+	–	+
Trypsin	w	w	–	–	–	–	–
Naphthol-AS-BI-phosphohydrolase	w	+	–	+	+	+	–

Sequences of type strains of species of the genus *Pseudomonas* were retrieved from GenBank and aligned with the newly determined sequences using the SeqTools program (Rasmussen, 2002). Phylogenetic trees were constructed using three different algorithms: neighbour-joining (Saitou & Nei, 1987) using SeqTools and TreeView (Page, 1996; Rasmussen, 2002), maximum-likelihood using PHYML (Guindon & Gascuel, 2003) and maximum-parsimony using MEGA version 4.0 (Tamura *et al.*, 2007). Genetic distances for the neighbour-joining and maximum-likelihood algorithms were calculated using the Kimura two-parameter model (Kimura, 1980) and the maximum-parsimony analysis applied close-neighbour-interchange (search level=2, random additions=100). The stability of the groups was estimated by bootstrap analysis with 1000 replications. The 16S rRNA gene sequence neighbour-joining tree (Fig. 1) revealed a clear affiliation of the isolates with the *Pseudomonas aeruginosa* group of Anzai *et al.* (2000), which was also recovered in the maximum-parsimony and maximum-likelihood trees. The isolates formed a separate lineage within a subgroup containing *P. cuatrocieneegasensis* 1N^T, *P. borbori* R-20821^T, *P. peli* R-20805^T, *P. guineae* LMG 24016^T and *P. anguilliseptica* NCIMB 1949^T. The bootstrap resampling analysis showed

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences, showing the phylogenetic positions of strain C2^T and related members of the genus *Pseudomonas*. Bootstrap values (>50%) based on 1000 replications are shown at branch nodes. Filled circles indicate that the corresponding nodes were also recovered in the maximum-parsimony trees. Open circles indicate that the corresponding nodes were also recovered in the maximum-likelihood and maximum-parsimony trees. *E. coli* ATCC 11775^T (X80725) was used as an outgroup (not shown). Bar, 1% sequence divergence.

that the association of strain C2^T with this subgroup was not statistically significant, but both the neighbour-joining and maximum-parsimony algorithms showed that the association was relatively stable (Fig. 1).

DNA–DNA hybridization was performed between strains C2^T and C5 and between strain C2^T and members of the *P. aeruginosa* group that exhibited >97% 16S rRNA gene sequence similarity. Two independent determinations were performed for each experiment. DNA was isolated using a French pressure cell (Thermo Spectronic) and purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). DNA–DNA hybridization was carried out as described by De Ley & Tijtjat (1970) under consideration of the modifications described by Huß *et al.* (1983) using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with *in-situ* temperature probe (Varian). Strains C2^T and C5 showed 98.2 ± 1.5% DNA–DNA relatedness with each other, which confirmed that they

are members of the same species. DNA–DNA relatedness values between strain C2^T and *P. cuatrocieneegasensis* LMG 24676^T, *P. borbori* CCUG 53773^T, *P. peli* CCUG 53775^T, *P. guineae* CECT 7231^T and *P. anguilliseptica* CCUG 35503^T were 7.4 ± 2.2, 8.9 ± 1, 17.2 ± 4.3, 25.4 ± 1.6 and 33.2 ± 0.7%, respectively, which demonstrated a separate species status for strain C2^T (Wayne *et al.*, 1987).

To differentiate further between strains C2^T and C5 and their closest phylogenetic neighbours, *rpoD* gene sequences were analysed. This gene has been used for the phylogenetic characterization of *Pseudomonas* strains and it exhibits a higher discriminative power than other housekeeping genes (Mulet *et al.*, 2010). The *rpoD* gene was amplified by PCR using the primer set 70F and 70S as described by Yamamoto *et al.* (2000). Sequences and sequence similarities were determined and a phylogenetic tree (see Supplementary Fig. S2) was constructed as for the 16S rRNA gene sequence analysis. The two isolates showed 100% *rpoD* gene sequence similarity with each other.

Strains C2^T and C5 showed the highest *rpoD* gene sequence similarity with *Pseudomonas alcaliphila* LMG 23134^T and *P. mendocina* ATCC 25411^T (94.8 and 94.7 %, respectively) and <91.7 % *rpoD* gene sequence similarity with other members of the *P. aeruginosa* group. Divergence of *rpoD* gene sequences >5 % is exhibited between some members of established species of the genus *Pseudomonas* (Hirota *et al.*, 2011). Thus, phylogenetic analysis based on *rpoD* gene sequences differentiated strains C2^T and C5 from members of the *P. aeruginosa* group that exhibited >97 % 16S rRNA gene sequence similarity.

The DNA G+C content of strain C2^T was determined using HPLC, as described by Mesbah *et al.* (1989). The G+C content of strain C2^T was 61.5 mol%.

To further investigate the association of the compost isolates with the genus *Pseudomonas*, strain C2^T was subjected to cellular fatty acid analysis. Fatty acid methyl esters were prepared and analysed as described by Kämpfer & Kroppenstedt (1996) using 50–100 mg wet cells harvested from Columbia blood agar after growth for 24 h at 37 °C. The fatty acid profile of strain C2^T, together with those of its closest phylogenetic neighbours and the type strain of *P. aeruginosa*, the type species of genus *Pseudomonas*, are shown in Table 2. The major fatty acids were summed feature (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH), C_{18:1}ω7c/12t/9t, C_{16:0} and C_{12:0}, which together accounted for 84 % of the total fatty acids. The major fatty acid composition supported the affiliation of strain C2^T with the genus *Pseudomonas*, although some quantitative differences with the reference strains were observed.

It is apparent from this phenotypic, phylogenetic and chemotaxonomic study that strains C2^T and C5 should be considered as representing a novel species within the genus *Pseudomonas*, for which the name *Pseudomonas composti* sp. nov. is proposed.

Description of *Pseudomonas composti* sp. nov.

Pseudomonas composti (com.pos'ti. N.L. n. *compostum* -i compost; N.L. gen. n. *composti* of compost, from which strains were first isolated).

The description is based on two strains. Gram-negative and non-spore-forming rods (0.4–0.5 µm wide and 1.5–2 µm long). Catalase- and oxidase-positive, strictly aerobic and motile by a single polar flagellum. Colonies on Columbia blood agar are circular, creamy, brilliant and non-haemolytic. Colonies on LB agar are round, yellow and smooth with irregular edges (1.5–2 mm in diameter). Grows at 4–37 °C and with 6.5 % (w/v) NaCl. Fluorescent pigment is not produced on King's B medium. Grows on MacConkey agar. Does not reduce nitrate or nitrite and is negative for arginine dihydrolase, indole production, β-galactosidase and acid production from glucose. Does not hydrolyse gelatin, urea or aesculin. With API 50 CH and API 20 NE, assimilates D-glucose, D-mannose, D-mannitol, caprate, L-malate, citrate, glycerol, D-fructose, D-arabitol

Table 2. Cellular fatty acid compositions of strain C2^T and closely related members of the genus *Pseudomonas*

Strains: 1, *Pseudomonas composti* sp. nov. C2^T; 2, *P. cuatrocienezasensis* LMG 24676^T; 3, *P. borbori* CCUG 53773^T; 4, *P. guineae* CECT 7231^T; 5, *P. peli* CCUG 53775^T; 6, *P. anguilliseptica* CCUG 35503^T; 7, *P. aeruginosa* CCUG 551^T. All data were taken from this study except for columns 2 and 4 (Escalante *et al.*, 2009 and Bozal *et al.*, 2007, respectively). Fatty acids amounting to <1 % of the total in all strains are not shown. ECL, Equivalent chain-length; tr, trace (<1 %); –, not detected.

Fatty acid (%)	1	2	3	4	5	6	7
C _{10:0} 3-OH	5.2	3.1	3.9	3.5	4.5	3.6	3.9
C _{12:0}	11.4	8.1	4.8	4.0	7.1	3.9	2.9
C _{12:0} 2-OH	–	–	–	–	–	tr	4.4
C _{12:0} 3-OH	3.9	3.7	2.4	3.6	4.5	3.4	4.2
C _{14:0}	tr	–	tr	tr	1.7	tr	tr
C _{15:0}	1.5	–	2.7	tr	3.7	1.2	tr
C _{15:1} ω6c	tr	–	–	–	1.2	–	–
C _{16:0}	14.1	14.9	18.8	10.9	14.9	19.4	24.5
C _{16:1} ω7c	–	–	44.7	–	29.1	17.8	14.8
C _{17:0}	tr	–	1.0	tr	1.1	1.0	tr
C _{17:1} ω8c	1.7	tr	2.1	tr	1.9	tr	tr
C _{18:0}	–	1.0	tr	–	tr	1.1	tr
C _{18:1} ω7c/12t/9t	26.6	31.3	12.3	31.0	21.9	39.9	40.5
C _{19:0} ω8c cyclo	–	–	–	–	–	–	1.0
Unknown ECL	tr	–	3.1	3.7	4.6	3.8	–
11.798							
Summed features*							
a	31.9	–	–	37.8	–	–	–
b	–	34.8	–	–	–	–	–

*Summed features represent two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature a consisted of C_{16:1}ω7c and/or iso-C_{15:0} 2-OH. Summed feature b consisted of C_{16:1}ω7c and/or C_{16:1}ω6c.

and gluconate, but not *N*-acetyl-D-glucosamine, erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, amygdalin, arbutin, aesculin, salicin, cellobiose, lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, starch, D-adonitol, glycogen, xylitol, gentiobiose, turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, 2-ketogluconate, 5-ketogluconate, L-arabitol, methyl β-D-xylopyranoside, D-galactose, L-sorbose, L-rhamnose, dulcitol, inositol, D-sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, maltose, adipate or phenylacetate. With API ZYM, positive for ester lipase (C8), esterase (C4) (weakly), trypsin (type strain weakly), leucine arylamidase, alkaline phosphatase, acid phosphatase (type strain weakly) and naphthol-AS-BI-phosphohydrolase (type strain weakly); all other tests are negative. The major cellular fatty acids are a summed feature (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH), C_{18:1}ω7c/12t/9t, C_{16:0} and C_{12:0}.

The type strain, C2^T (=CECT 7516^T=CCUG 59231^T), was isolated from a compost sample. The DNA G+C content of the type strain is 61.5 mol%.

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