Pseudomonas xiamenensis sp. nov., a denitrifying bacterium isolated from activated sludge

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A taxonomic study was carried out on strain C10-2^T, a moderately halophilic denitrifier isolated from activated sludge samples collected in China. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain C10-2^T belonged to the genus *Pseudomonas*, with highest sequence similarity of 98.1 and 97.7% to '*Pseudomonas denitrificans*' IAM 12023 and *Pseudomonas pertucinogena* JCM 11590^T, respectively. The *gyrB*, *rpoD* and *rpoB* gene sequence similarity between strain C10-2^T and *P. pertucinogena* JCM 11590^T was respectively 84.1, 79.0 and 88.2%. Phylogenetic trees based on these housekeeping genes showed that strain C10-2^T and *P. pertucinogena* JCM 11590^T form a clade at the periphery of the genus *Pseudomonas*. The DNA–DNA hybridization value between strain C10-2^T and *P. pertucinogena* JCM 11590^T was 30–32%. The G+C content of the chromosomal DNA was 61.2 mol%. The combined genotypic and phenotypic data show that strain C10-2^T represents a novel species of the genus *Pseudomonas*, for which the name *Pseudomonas xiamenensis* sp. nov. is proposed, with the type strain C10-2^T (=CGMCC 1.6446 =JCM 13530^T =MCCC 1A00089^T).

Pseudomonads are well-known and widespread micro-organisms that have been isolated from a variety of natural sources including soil, plants and mineral waters and from clinical specimens, and they are characterized by a high level of metabolic diversity (Rosselló *et al.*, 1991; Moore *et al.*, 1996). During the screening of denitrifying bacteria, a *Pseudomonas*-like bacterium, strain C10-2^T, was isolated from activated sludge samples collected at the Qianpu sewage treatment plant, Xiamen, Fujian, China, in 2003, and selected for further characterization by a polyphasic approach, including genotypic, chemotaxonomic and classical phenotypic characteristics. Based on these results, we consider that strain C10-2^T represents a novel species in the genus *Pseudomonas*.

Samples used in this study were loaded into sterile sampling cups and processed on the same day. Medium C (designed in this study) used for enrichment contained

The GenBank/EMBL/DDBJ accession numbers for the nucleotide sequences reported in this study are DQ088664 (strain C10-2^T 16S rRNA gene), DQ350612 (strain C10-2^T partial *gyrB* gene), EF596884 (strain C10-2^T partial *rpoD* gene), EF667149 (strain C10-2^T partial *rpoB* gene), EF673695 (*P. pertucinogena* JCM 11590^T 16S rRNA gene), DQ350613 (*P. pertucinogena* JCM 11590^T partial *gyrB* gene) and EF596883 (*P. pertucinogena* JCM 11590^T partial *rpoD* gene).

Transmission electron micrographs of cells of strain C10-2^T and dendrograms showing the phylogenetic position of the strain plus related species based on 16S rRNA, *gyrB*, *rpoD* and *rpoB* gene sequences, detailed cellular fatty acid profiles and DNA–DNA hybridization results are available as supplementary material with the online version of this paper.

(per litre distilled water) 24.0 g NaCl, 7.0 g MgSO₄·7H₂O, 7.0 g sodium acetate, 1.0 g yeast extract, 1.0 g NaNO₃, 3.0 g KH₂PO₄, 2.0 g K₂HPO₄ and 10 ml trace element solution (pH 7.2). The trace element solution contained (per litre distilled water) 90 mg FeSO₄·7H₂O, 4 mg CuSO₄·5H₂O, 24 mg MnSO₄·H₂O, 5 mg ZnSO₄·7H₂O and 50 mg Na₂MoO₄·2H₂O (Labbe *et al.*, 2003). The enrichment was incubated anaerobically in tubes and subjected to five transfers to fresh medium at intervals of 7 days. HLB medium was a modification of Luria–Bertani (LB) medium (Sambrook *et al.*, 1989), with the concentration of NaCl increased to 30 g l⁻¹. The enrichment was diluted tenfold and aliquots (0.1 ml) of the 10⁻⁴–10⁻⁷ dilutions were spread on HLB plates. Plates were incubated aerobically for 3 days. Individual colonies were picked off and purified by successive streaking on HLB plates. Each isolate was then inoculated into medium F (designed in this study; same composition as medium C except for replacement of 1.0 g NaNO₃ by 1.0 g NaNO₂) under anoxic conditions in order to detect its denitrifying ability. Isolate C10-2^T, which showed the most rapid growth in medium C, was used for the present study. Isolated strains were stored at –80 °C in HLB supplemented with 16% (v/v) glycerol for maintenance. LB was used for routine cultivation of the isolates and most phenotypic tests. All cultures were incubated at 28 °C unless noted otherwise.

Genomic DNA was prepared according to the method of Ausubel *et al.* (1995) and the 16S rRNA gene was amplified by PCR using primers that have been described previously (Liu & Shao, 2005). Sequences of related taxa were

obtained from the GenBank database. Phylogenetic analysis was performed using MEGA version 3.1 after multiple alignment of data by DNAMAN (version 5.1; Lynnon Biosoft). Distances (distance options according to Kimura's two-parameter model) and clustering with the neighbour-joining (Saitou & Nei, 1987) and minimum-evolution (Rzhetsky & Nei, 1992, 1993) methods were determined by using bootstrap values based on 1000 replications. The full neighbour-joining tree is available as Supplementary Fig. S1(a) in IJSEM Online; the result of the minimum-evolution clustering was similar to that obtained with the neighbour-joining method, so the data are not shown.

A nearly full-length 16S rRNA gene sequence (1498 nt) of strain C10-2^T was determined. Phylogenetic analysis of strain C10-2^T indicated that it belonged to the *Gammaproteobacteria*, forming a robust clade with the *Pseudomonas pertucinogena* group (Anzai *et al.*, 2000) within the genus *Pseudomonas* (Fig. 1; the full version of this tree is available as Supplementary Fig. S1a). 16S rRNA gene sequence comparisons showed that strain C10-2^T was most closely related to '*Pseudomonas denitrificans*' IAM 12023 (98.1 %) and *P. pertucinogena* JCM 11590^T (97.7 %); we determined the sequence of strain JCM 11590^T, since the sequence of *P. pertucinogena* NBRC 14163^T available under GenBank accession no. AB021380 contained a number of unknown bases; similarity to all other sequences was below 95 %. Since the epithet *denitrificans* in '*Pseudomonas denitrificans*' (Christensen) Bergey *et al.* 1923 has been rejected by Judicial Commission [Opinion 54; Appendix 5 of the International Code of Nomenclature of Bacteria; Lapage *et al.*, 1992], this study just compared strain C10-2^T with *P. pertucinogena* JCM 11590^T.

For further comparison of strain C10-2^T with *P. pertucinogena* JCM 11590^T based on housekeeping gene sequences, the *gyrB*, *rpoD* and *rpoB* genes of the two strains were sequenced using the method described by Yamamoto *et al.* (2000) and Ait Tayeb *et al.* (2005). The *gyrB*, *rpoD* and *rpoB* gene sequence similarity between strain C10-2^T and *P. pertucinogena* JCM 11590^T was respectively 84.1, 79.0 and 88.2 %. As shown in Supplementary Fig. S1(b–d), phylogenetic trees based on the three housekeeping gene sequences all showed that

strain C10-2^T and *P. pertucinogena* JCM 11590^T formed an independent monophyletic cluster on the periphery of the genus *Pseudomonas*, similar to the tree based on 16S rRNA gene sequences. These results differentiated strain C10-2^T from all species of the genus *Pseudomonas* with validly published names.

DNA–DNA hybridization experiments were performed with genomic DNA from strain C10-2^T and *P. pertucinogena* JCM 11590^T using a previously described method (Liu & Shao, 2005). Genomic DNA from *Escherichia coli* DH5 α was used as an outgroup sample. Salmon-sperm DNA was used as a negative control. The results are shown in Supplementary Table S1. Values reported in the table are means from two independent hybridization experiments. Strain C10-2^T and *P. pertucinogena* JCM 11590^T possessed low DNA–DNA relatedness (30–32 %).

General cell morphology was studied under an Olympus inverted microscope using 1-day-old cultures grown on LB agar. For electron microscopy, exponential phase cells were harvested, suspended and absorbed on a Formvar–carbon-coated grid and then stained with phosphotungstic acid. The Gram reaction, catalase and oxidase activities, the methyl red test, lipase (Tween 80) and amylase were examined according to Dong & Cai (2001). Fluorescent pigment production was tested on King's medium B (King *et al.*, 1954) and *Pseudomonas* agar F (PAF; Difco). The optimal growth temperature was determined over the temperature range 4–55 °C on LB. Tolerance of NaCl was tested by using LB medium supplemented with NaCl concentration of 0, 0.5, 1, 3, 5, 7, 8, 9, 10, 12 and 15 % (w/v). Other biochemical tests were carried out by using API 20NE test kits (bioMérieux) according to the manufacturer's instructions and the Biolog GN2 MicroPlate panel as described by Ivanova *et al.* (1998). Tests for other substrates as sole carbon sources at a concentration of 0.1 % (w/v) were performed in 5 ml artificial seawater (ASW) medium with the NaCl concentration adjusted to 1 % (Liu & Shao, 2005). *P. pertucinogena* JCM 11590^T was tested at the same time as a comparison.

Strain C10-2^T was a Gram-negative, non-pigmented, rod-shaped bacterium that was motile by means of a single polar flagellum (Supplementary Fig. S2). The differences in

Fig. 1. Neighbour-joining tree showing the phylogenetic positions of strain C10-2^T and representatives of some other related taxa, based on 16S rRNA gene sequences. Bootstrap values (expressed as percentages of 1000 replications) are shown at branch points. Bar, 0.01 nucleotide substitution rate (K_{nuc}) units. The full version of this tree is available as Supplementary Fig. S1.

physiological, biochemical and chemotaxonomic characteristics between strain C10-2^T and *P. pertucinogena* JCM 11590^T are given in Table 1.

Antibiotic susceptibility tests were performed by the disc-diffusion method as described by Shieh *et al.* (2003). Strain C10-2^T and *P. pertucinogena* JCM 11590^T were tested at the same time. Both were sensitive to ciprofloxacin (5 µg per disc; Oxoid), doxycycline (30 µg), gentamicin (10 µg), kanamycin (30 µg), minocycline (30 µg), neomycin (10 µg), norfloxacin (10 µg), ofloxacin (5 µg), polymyxin B (300 U) and rifampicin (5 µg) and resistant to cefoperazone (30 µg), cephalexin (30 µg), cephalosin (30 µg), clindamycin (2 µg), lincomycin (2 µg), metronidazole (5 µg), oxacillin (1 µg), penicillin G (10 µg), piperacillin (100 µg), sulfamethoxazole (25 µg) and vancomycin (30 µg). Differential susceptibility of the two strains to nine other antibiotics is detailed in Table 1.

Fatty acids of cells grown aerobically in LB broth at 28 °C for 48 h were extracted, freeze-dried, saponified and esterified according to the methods described by Mrozik *et al.* (2004). Analysis of fatty acid methyl esters was performed on a GC-MS (QP2010; Shimadzu) equipped with an RTX-5MS column. The fatty acid profile of *P. pertucinogena* JCM 11590^T was determined in parallel with strain C10-2^T. The fatty acid profiles of the two strains are

shown in Supplementary Table S2. The major fatty acids in both strains were C_{16:0}, C_{16:1}ω9c, C_{18:1}ω7c and C_{18:0}, which together accounted for >73 % of the total fatty acids. Similar fatty acid components (C_{16:0}, C_{16:1}ω9c and C_{18:1}ω7c) predominate in species of the genus *Pseudomonas* (Oyaizu & Komagata, 1983) such as *Pseudomonas stutzeri* (Rainey *et al.*, 1994), *Pseudomonas alcaliphila* (Yumoto *et al.*, 2001) and *Pseudomonas pachastrellae* (Romanenko *et al.*, 2005). As the fatty acid profiles of strain C10-2^T and *P. pertucinogena* JCM 11590^T were determined under the same conditions, the considerable differences in the proportions of individual components could distinguish strain C10-2^T from *P. pertucinogena* JCM 11590^T.

The G+C content of the chromosomal DNA was determined according to the methods described by Mesbah & Whitman (1989) using a reversed-phase HPLC. The DNA G+C content of the new isolate C10-2^T was 61.2 mol%, which is within the range reported for most *Pseudomonas* species (Palleroni, 1984).

On the basis of morphological, physiological and chemotaxonomic characteristics, together with phylogenetic analysis based on 16S rRNA, *gyrB*, *rpoD*, *rpoB* gene sequences and DNA–DNA hybridization, strain C10-2^T should be placed into a novel species of genus

Table 1. Physiological characteristics of strain C10-2^T and the type strain of *P. pertucinogena*

Data for *P. pertucinogena* were taken from Kawai & Yabuuchi (1975) unless indicated. w, Weak; +, positive; –, negative.

Characteristic	Strain C10-2 ^T	<i>P. pertucinogena</i> NBRC 14163 ^T
Cell size (µm)	0.6–0.8 × 1.1–1.3	0.4 × 1.1
Colony colour	White	Grey
Tolerance to NaCl (%)	0–8	0–5
Acidification of glucose	–	+
Lipase (Tween 80 hydrolysis)	+	–
DNA G+C content (mol%)	61.2	60.0
Susceptibility to antimicrobial agents*		
Ampicillin (10 µg), carbenicillin (100 µg), ceftriaxone (30 µg), cephradine (30 µg), streptomycin (10 µg), tetracycline (30 µg)	+	–
Chloramphenicol (30 µg), erythromycin (15 µg)	+	–†
Furazolidone (15 µg)	–	+
API 20NE tests*		
Reduction of nitrate to nitrite	+	–
Denitrification	+	–
Utilization of capric acid and trisodium citrate	+	–
Utilization of adipic acid	–	+
Biolog GN2 tests*		
α-Ketobutyric acid	–	+
L-Threonine, γ-aminobutyric acid	–	w
α-Hydroxybutyric acid, cis-aconitic acid, citric acid, quinic acid, DL-lactic acid, D-serine	+	–
Glycogen, α-ketovaleric acid, L-arabinose, L-alaninamide, L-proline	w	–
Utilization of xylose and ethanol as sole carbon sources in ASW medium*	+	–

*Determined in this study for *P. pertucinogena* JCM 11590^T.

†Reported as susceptible by Kawai & Yabuuchi (1975).

Pseudomonas, for which the name *Pseudomonas xiamenensis* sp. nov. is proposed.

Description of *Pseudomonas xiamenensis* sp. nov.

Pseudomonas xiamenensis (xia.men.en'sis. N.L. fem. adj. *xiamenensis* of Xiamen, a district in Fujian, China, where the type strain was isolated).

Cells are straight rods, 0.6–0.8 µm wide and 1.1–1.3 µm long, motile by a single polar flagellum. Positive for oxidase, catalase and lipase (Tween 80), but negative for the Gram reaction, methyl red reaction, H₂S, indole and acetoin production, urease, amylase, gelatinase, β-glucosidase, β-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and tryptophan deaminase activity. On LB agar plates, produces smooth white colonies with regular edges, 1–2 mm in diameter after 72 h incubation at 30 °C, non-pigmented and slightly raised in the centre. Moderately halophilic, growing in 0–8 % of NaCl (optimum 1–3 %). Grows at 10–45 °C (optimum 25–30 °C), but not at 4 or 50 °C. Capable of denitrification and unable to ferment glucose. Principal fatty acids are C_{12:0} (4.6 %), C_{16:1ω9c} (8.0 %), C_{16:0} (18.8 %), C_{18:1ω7c} (52.9 %) and C_{18:0} (8.8 %). Of the 95 carbon sources in the Biolog GN2 system, the type strain can utilize acetic acid, *cis*-aconitic acid, L-alaninamide, D-alanine, L-alanine, L-arabinose, bromosuccinic acid, citric acid, glycogen, α-hydroxybutyric acid, β-hydroxybutyric acid, α-ketovaleric acid, DL-lactic acid, L-proline, methyl pyruvate, propionic acid, quinic acid, sebacic acid, D-serine, monomethyl succinate, succinic acid, Tween 40 and Tween 80. The type strain is sensitive to ampicillin (10 µg per disc; Oxoid), carbenicillin (100 µg), ceftriaxone (30 µg), cephradine (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), doxycycline (30 µg), erythromycin (15 µg), gentamicin (10 µg), kanamycin (30 µg), minocycline (30 µg), neomycin (10 µg), norfloxacin (10 µg), ofloxacin (5 µg), polymyxin B (300 U), rifampicin (5 µg), streptomycin (10 µg) and tetracycline (30 µg) and resistant to cefoperazone (30 µg), cephalixin (30 µg), cephalozin (30 µg), clindamycin (2 µg), furazolidone (15 µg), lincomycin (2 µg), metronidazole (5 µg), oxacillin (1 µg), penicillin G (10 µg), piperacillin (100 µg), sulfamethoxazole (25 µg) and vancomycin (30 µg). The G+C content of the DNA of the type strain is 61.2 mol%. Table 1 shows characteristics that distinguish strain C10-2^T from related species.

The type strain, C10-2^T (=CGMCC 1.6446^T =JCM 13530^T =MCCC 1A00089^T), was isolated from activated sludge samples collected in 2003 at Qianpu sewage treatment plant, Xiamen, Fujian, China.

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