Pseudomonas caeni sp. nov., a denitrifying bacterium isolated from the sludge of an anaerobic ammonium-oxidizing bioreactor

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Two Gram-negative, rod-shaped, motile, aerobic bacterial strains designated HY-14^T and HY-24 were isolated from the sludge of an anaerobic ammonium-oxidizing bioreactor. The strains could not grow with 5% (w/v) NaCl, did not produce acid from D-glucose or utilize D-glucose, gluconate or citrate as a sole carbon source. Summed feature 3 and C_{16:0} were the most abundant fatty acids; hydroxyl fatty acids C_{12:0} 3-OH and C_{10:0} 3-OH were also present. Fatty acid C_{12:0} 2-OH was absent. The DNA G + C contents of strains HY-14^T and HY-24 were 50.6 ± 0.5 mol%. Phylogenetic analyses based on 16S rRNA gene sequences showed that strains HY-14^T and HY-24 formed a monophyletic clade within the genus *Pseudomonas*. The highest sequence similarities were to *Pseudomonas pseudoalcaligenes* DSM 50188^T (95.9%). On the basis of phenotypic and phylogenetic properties, strains HY-14^T and HY-24 are proposed as a novel species of the genus *Pseudomonas*, for which the name *Pseudomonas caeni* sp. nov. is proposed. The type strain is HY-14^T (=KCTC 22292^T=CCTCC AB208156^T).

The genus *Pseudomonas* was first proposed by Migula (1894). Members of the genus *Pseudomonas* are widely distributed in a variety of environments such as soil, water, wastewater plants and clinical specimens.

Strain HY-14^T was isolated from the sludge of an anaerobic ammonium-oxidizing (Anammox) bioreactor by the dilution-plating technique on R2A (Difco) agar plates under aerobic conditions. Strain HY-24 was isolated from the sludge of the Anammox bioreactor on modified R2A (containing 5 mM NaNO₃ and 0.1 mM Na₂S) agar plates in an anaerobic test tube using an AnaeroGen kit (Oxoid). Cell morphology was examined by light microscopy (YS-2; Nikon) and transmission electron microscopy (H600; Hitachi) after negative staining with 1% (w/v) phosphotungstic acid, using cells grown on tryptic soy agar (TSA, Difco) after 2 days incubation at 30 °C. Motility was determined by phase-contrast microscopy (Eclipse TS 100; Nikon). Growth in the presence of 0, 0.5, 1, 2, 3 and 5% (w/v) NaCl and at pH 5.0–11.0 (in increments of 1 pH

unit) and at 4, 10, 20, 30, 37 and 42 °C was tested on TSA for up to 1 week. The production of water-soluble fluorescent pigments on King's B agar was determined as described by King *et al.* (1954). Catalase activity was determined by bubble production in 3% (v/v) hydrogen peroxide solution; oxidase activity was determined by oxidation of 1% (w/v) tetramethyl *p*-phenylenediamine (Merck). Gram staining, hydrolysis of starch, gelatin and Tween 80, endospore formation and utilization of arabinose and malate as the sole carbon resource were determined as described by Dong & Cai (2001). The denitrification rate was measured as nitrite consumed over time under anaerobic conditions, according to the methods described by Gumaelius *et al.* (1996, 2001). Other physiological and biochemical tests and enzyme activities were conducted using API 20NE and API ZYM strips (bioMérieux) according to the manufacturer's instructions. *Pseudomonas aeruginosa* KCTC 1750^T, *Pseudomonas chlororaphis* DSM 50083^T, *Pseudomonas fluorescens* KCTC 12453^T, *Pseudomonas mendocina* DSM 50017^T, *Pseudomonas pseudoalcaligenes* KACC 10304^T, *Pseudomonas putida* KCTC 1751^T and *Pseudomonas stutzeri* KCTC 1066^T were included in these studies and analysed under the same conditions.

Strains HY-14^T and HY-24 had the same cultural, physiological and biochemical characteristics. The two

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains HY-14^T and HY-24 are EU620679 and EU620680, respectively.

A transmission electron micrograph of a negatively stained cell of strain HY-14^T, showing a polar flagellum, is available with the online version of this paper.

novel strains grew faster on R2A agar under aerobic conditions than on modified R2A agar under anaerobic conditions and had essentially the same denitrification rate [$2.2 \pm 0.2 \text{ mg NO}_2^- \text{-N h}^{-1} (\text{g wet cells})^{-1}$] under anaerobic conditions. Additional cultural, physiological and biochemical characteristics of strains HY-14^T and HY-24 are given in the species description and in Table 1. The two novel strains could be readily differentiated from other pseudomonads on the basis of several phenotypic properties such as no growth with 5 % NaCl, the acidification of glucose and the utilization of D-glucose, gluconate and citrate as the sole carbon source.

For quantitative analysis of the cellular fatty acid composition, 40 mg wet cell material was harvested from TSA plates after 2 days incubation at 28 °C. The cellular fatty acids were extracted, saponified and methylated according to the protocol of the Sherlock Microbial Identification System (MIDI). The fatty acids were

analysed by GC (Hewlett Packard 6890) and identified by the Microbial Identification software package (Sasser, 1990). Chromosomal DNA was isolated and purified using a Cell Culture DNA Midi kit (Qiagen) according to the manufacturer's protocol. The G+C content of the DNA was determined by the fluorescence monitoring method (Xu *et al.*, 2000; Gonzalez & Saiz-Jimenez, 2002) with a real-time thermal cycler (Mx3000p; Stratagene). The genomic DNA of *Escherichia coli* strain B (Sigma-Aldrich) and *P. aeruginosa* KCTC 1750^T were used as the calibration references. The tests were repeated five times.

The cellular fatty acid profiles of strains HY-14^T and HY-24 and related species are presented in Table 2. Strains HY-14^T and HY-24 contained high amounts of summed feature 3 (37.4–37.6 %; comprising C_{16:1}ω7c and/or iso-C_{15:0} 2-OH), C_{16:0} (28.1–28.3 %) and C_{18:1}ω7c (13.4–13.7 %), with lesser amounts of C_{12:0} (7.9–8.1 %), C_{10:0} 3-OH (4.3–4.4 %), C_{12:0} 3-OH (3.1–3.3 %) and C_{14:0} (1.4–1.6 %). The

Table 1. Differential characteristics of strains HY-14^T and HY-24 and related species of the genus *Pseudomonas*

Taxa: 1, strains HY-14^T and HY-24; 2, *P. pseudoalcaligenes* KACC 10304^T; 3, *P. stutzeri* KCTC 1066^T; 4, *P. mendocina* DSM 50017^T; 5, *P. aeruginosa* KCTC 1750^T; 6, *P. chlororaphis* DSM 50083^T; 7, *P. fluorescens* KCTC 12453^T; 8, *P. putida* KCTC 1751^T. Data are from this study. All strains are positive for catalase and oxidase activities, motility and utilization of caprate as sole carbon source. All strains are negative for the following characteristics: urease, β-galactosidase and β-glucosidase activities, indole production and utilization of phenylacetate as sole carbon source. +, Positive; –, negative.

Characteristic	1	2	3	4	5	6	7	8
Fluorescent pigments	–	–	–	–	+	+	+	+
Arginine dihydrolase	–	–	–	+	+	+	+	+
Lipase	–	–	–	+	+	+	+	–
Growth with 5 % NaCl	–	+	+	+	+	+	+	+
Growth at:								
4 °C	+	–	–	–	–	+	+	+
42 °C	–	+	+	+	+	–	–	–
Hydrolysis of:								
Gelatin	–	–	–	–	+	+	+	–
Starch	–	–	+	–	–	–	–	–
Tween 80	–	–	+	+	+	+	+	–
Denitrification	+	–	+	+	+	+	–	–
Acid produced from:								
D-Glucose	–	–	+	+	+	+	+	+
Utilization of:								
N-Acetylglucosamine	–	–	–	–	+	+	+	–
Adipate	–	–	–	–	+	–	–	–
Arabinose	–	–	–	–	+(–*)	+(–*)	–	–
Citrate	–	–	+	+	+	+	+	+
Gluconate	–	–	+	+	+	+	+	+
D-Glucose	–	–	+	+	+	+	+	+
Malate	+	+	+	+	+	+	–(+*)	+
Maltose	–	–	+	–	–	–	–	–
D-Mannitol	–	–	–	–	+	+	+	–
Mannose	–	–	–	–	–	+	+	–
DNA G+C content (mol%)	50.1–51.1	61.9–63.7	62.5–64.3	62.3–63.5	66.6†	62.5–63.9	59.5–61.1	61.5–62.3

*Data from API 20NE tests.

†Data from Gonzalez & Saiz-Jimenez (2002).

Table 2. Fatty acid content (%) of strains HY-14^T and HY-24 and related species in the genus *Pseudomonas*

Taxa: 1, strain HY-14^T; 2, strain HY-24; 3, *P. pseudoalcaligenes* KACC 10304^T; 4, *P. stutzeri* KCTC 1066^T; 5, *P. mendocina* DSM 50017^T; 6, *P. aeruginosa* KCTC 1750^T; 7, *P. chlororaphis* DSM 50083^T; 8, *P. fluorescens* KCTC 12453^T; 9, *P. putida* KCTC 1751^T. Data are from this study. ND, Not detected; tr, trace (<1 %). The fatty acids amounting to <1 % in all strains studied are not listed.

Fatty acid	1	2	3	4	5	6	7	8	9
Straight-chain saturated									
C _{10:0}	tr	tr	tr	tr	tr	tr	tr	tr	tr
C _{10:0} 3-OH	4.3	4.4	4.6	3.9	4.6	3.6	4.7	5.4	3.3
C _{12:0}	8.1	7.9	6.9	8.7	8.0	4.8	2.3	5.2	2.9
C _{12:0} 2-OH	ND	ND	ND	tr	tr	3.7	4.9	5.4	4.1
C _{12:0} 3-OH	3.1	3.3	3.3	4.0	4.3	4.5	4.6	6.1	3.7
C _{14:0}	1.6	1.4	ND	1.7	1.0	1.3	1.3	1.0	1.1
C _{15:0}	tr	tr	2.5	ND	1.6	tr	tr	tr	tr
C _{16:0}	28.1	28.3	11.2	16.3	12.7	20.5	26.7	19.1	27.2
C _{17:0}	tr	ND	2.3	ND	tr	tr	tr	tr	tr
C _{18:0}	tr	tr	1.4	tr	tr	tr	tr	tr	tr
Branched saturated									
iso-C _{16:0}	tr	tr	ND	ND	ND	tr	ND	ND	ND
iso-C _{17:0}	ND	ND	1.5	tr	tr	tr	ND	ND	ND
C _{17:0} cyclo	ND	ND	ND	tr	4.3	tr	5.5	2.9	1.6
Summed feature 3*	37.4	37.6	15.0	31.5	23.2	20.0	34.5	38.5	30.5
Mono-unsaturated									
C _{17:1} ω5c	ND	ND	4.1	ND	ND	ND	ND	ND	ND
C _{17:1} ω6c	ND	ND	2.3	ND	ND	ND	ND	ND	ND
C _{17:1} ω8c	ND	ND	3.0	ND	2.1	tr	tr	tr	ND
C _{18:1} ω7c	13.7	13.4	38.3	31.5	34.0	38.9	13.8	14.9	22.3

*Summed feature 3 comprised C_{16:1}ω7c and/or iso-C_{15:0} 2-OH.

two novel strains had similar fatty acid profiles to *P. chlororaphis* DSM 50083^T, *P. fluorescens* KCTC 12453^T and *P. putida* KCTC 1751^T, containing high amounts of summed feature 3 and C_{16:0}. The novel strains also had the typical hydroxyl fatty acids C_{12:0} 3-OH and C_{10:0} 3-OH but did not contain fatty acid C_{12:0} 2-OH. The fatty acid profiles suggested that strains HY-14^T and HY-24 belonged to the genus *Pseudomonas* (Oyaizu & Komagata, 1983). The DNA G + C contents of the two strains (with five replications) were 50.6 ± 0.5 mol%. This level of G + C was greatly below the range (58–69 mol%) previously described for members of the genus *Pseudomonas* (Palleroni, 2005), however, *Pseudomonas duriflava* HR2^T, a recently described species, has also been reported to have a low DNA G + C content (55.2 mol%) (Liu *et al.*, 2008). The DNA G + C contents of *P. aeruginosa* KCTC 1750^T, *P. chlororaphis* DSM 50083^T, *P. fluorescens* KCTC 12453^T, *P. mendocina* DSM 50017^T, *P. pseudoalcaligenes* KACC 10304^T, *P. stutzeri* KCTC 1066^T and *P. putida* KCTC 1751^T were also determined by the fluorescence monitoring method in this study and the results were consistent with those given in the literature (Palleroni, 2005) (Table 1).

The 16S rRNA genes of strains HY-14^T and HY-24 were amplified by PCR using the universal primer pair 9F and 1512R as described previously (Quan *et al.*, 2005). The

PCR products were purified with a QIAquick PCR Purification kit (Qiagen) and sequenced using an Applied Biosystems 3730X1 DNA Analyzer and the primers 519F, 536R, 907F, 1100R (Quan *et al.*, 2005). The typical 16S rRNA gene sequences of related taxa were obtained from EzTaxon (Chun *et al.*, 2007) and multiple alignments were performed using the CLUSTAL_X program (Thompson *et al.*, 1997). Gaps at the 5' and 3' ends of the alignment were omitted from further analysis. Phylogenetic trees were constructed based on the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Swofford, 1993) algorithms using the MEGA4 program (Tamura *et al.*, 2007) with bootstrap values based on 1000 replications (Felsenstein, 1985). The evolutionary distances were calculated using the method of Jukes & Cantor (1969).

The nearly complete 16S rRNA gene sequences of strains HY-14^T and HY-24 (1508 bp) were obtained. Strain HY-24 showed 99.9 % 16S rRNA gene sequence similarity to strain HY-14^T. Phylogenetic trees based on the neighbour-joining and maximum-parsimony methods indicated that the novel strains formed a monophyletic clade within the genus *Pseudomonas* (Fig. 1) with the genus *Cellvibrio* of the family *Pseudomonadaceae* used as the outgroup. On the basis of 16S rRNA gene similarity, the closest relative was *P. pseudoalcaligenes* DSM 50188^T (95.9 %), suggesting that the

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences (1325 bp) showing the relationship of *Pseudomonas caeni* sp. nov. strains HY-14^T and HY-24 and related species in genus *Pseudomonas*. *Cellvibrio ostraviensis* LMG 19434^T and *Cellvibrio fibrivorans* LMG 18561^T were used as the outgroup. Filled circles indicate branches that were also recovered by using the maximum-parsimony algorithm. Bootstrap values (expressed as percentages of 1000 replications) greater than 50 are shown at the branching points. Bar, 1 substitution per 100 nucleotide positions.

new strains represent a novel genomic species, without the need for DNA–DNA hybridization characterization (Stackebrandt & Goebel, 1994).

On the basis of the phylogenetic tree and fatty acid profiles, it is proposed that strains HY-14 and HY-24 belong to the genus *Pseudomonas*. Due to the large phylogenetic distance and different phenotypic characteristics of the new strains when compared with those of the type strains of members of the genus *Pseudomonas*, strains HY-14^T and HY-24 should be classified as a novel species of the genus *Pseudomonas*, for which the name *Pseudomonas caeni* sp. nov. is proposed.

Description of *Pseudomonas caeni* sp. nov.

Pseudomonas caeni (cae'ni. L. gen. neut. n. *caeni* of sludge).

Cells are Gram-negative, aerobic, non-spore-forming rods approximately 0.4–0.7 × 1.0–2.0 µm in size. Cells are motile by means of a polar flagellum (see Supplementary Fig. S1 in IJSEM Online). Colonies on TSA are translucent, pale yellow, circular and usually 1–2 mm in diameter after 2 days incubation at 30 °C. Cells do not produce fluorescent pigment on King's B media. Growth occurs at 4–37 °C (optimum, 30 °C), but not at 42 °C. Growth occurs at pH 6.0–10.0 (optimum, pH 7.0–8.0). Growth occurs in the presence of 0–3 % (w/v) NaCl (optimum, 0.5–1 %), but not in the presence of 5 % (w/v) NaCl. Catalase- and oxidase-positive. Positive for esterase (C4),

esterase lipase (C8) and leucine arylamidase, but negative for activities of urease, α-glucosidase, β-glucosidase, lipase (C14), arginine dihydrolase, α-chymotrypsin, α-galactosidase, β-galactosidase, naphthol-AS-BI-phosphohydrolase, alkaline phosphatase, acid phosphatase, valine arylamidase, cystine arylamidase, β-glucuronidase, α-fucosidase, α-mannosidase and N-acetyl-β-glucosaminidase. Negative for acidification of D-glucose, indole production and hydrolysis of starch, aesculin, gelatin, trypsin and Tween 80. Nitrate and nitrite are reduced under anaerobic conditions. Caprate and malate are utilized as sole carbon sources, but the following substrates are not utilized as a sole carbon source: adipate, arabinose, citrate, D-glucose, gluconate, maltose, mannitol, mannose, N-acetylglucosamine or phenylacetate. The major fatty acids are summed feature 3, C_{16:0} and C_{18:1ω7c}, with smaller amounts of C_{12:0}, C_{10:0} 3-OH, C_{12:0} 3-OH and C_{14:0}. The G+C content of the genomic DNA is 50.6 ± 0.5 mol%.

The type strain, HY-14^T (=KCTC 22292^T=CCTCC AB208156^T) and an additional strain, HY-24 (=KCTC 22293=CCTCC AB208157), were isolated from the sludge of an anaerobic ammonium-oxidizing bioreactor.

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