Acidovorax caeni sp. nov., a denitrifying species with genetically diverse isolates from activated sludge

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Four Gram-negative, rod-shaped, non-spore-forming, denitrifying isolates were obtained from the activated sludge of an aerobic–anaerobic wastewater treatment plant in Belgium. Analysis of repetitive sequence-based PCR showed that the four isolates were genetically different from each other. Results of 16S rRNA gene sequence analysis and DNA–DNA hybridization experiments indicated that these four isolates were affiliated to the genus *Acidovorax* and could be differentiated from all recognized species of the genus. Analysis of whole-cell proteins and results of physiological and biochemical tests allowed differentiation of the new isolates from their closest phylogenetic neighbours. These new isolates therefore represent a novel species of the genus *Acidovorax*, for which the name *Acidovorax caeni* sp. nov. is proposed. The type strain is R-24608^T (=LMG 24103^T =DSM 19327^T).

At the time of writing, the genus *Acidovorax* comprises eight recognized species, which can be separated into soil and water inhabitants, *Acidovorax facilis*, *Acidovorax delafieldii*, *Acidovorax temperans* (Willems *et al.*, 1990) and *Acidovorax defluvii* (Schulze *et al.*, 1999), and the phytopathogenic species *Acidovorax avenae* and its three subspecies, *Acidovorax konjaci* (Willems *et al.*, 1992), *Acidovorax anthurii* (Gardan *et al.*, 2000) and *Acidovorax valerianellae* (Gardan *et al.*, 2003). This separation based on occurrence and habitat is reflected in the 16S rRNA gene sequence phylogeny of these organisms, although separate phylogenetic clustering of the genus within the *Comamonadaceae* has been confirmed (Willems & Gillis, 2005).

A previous cultivation-dependent study on activated sludge from an aerobic–anaerobic wastewater treatment plant used different defined growth media for specific isolation of denitrifying bacteria (Heylen *et al.*, 2006). Nineteen denitrifiers were assigned to the genus *Acidovorax* based on partial 16S rRNA gene sequence analysis. The role of members of the *Comamonadaceae* in the removal of nitrogen in wastewater treatment plants has been recognized and described (Etchebehere *et al.*, 2001; Gumaelius *et al.*, 2001; Mechichi *et al.*, 2003; Hoshino *et al.*, 2005). Four *Acidovorax*-like isolates, R-24607, R-24608^T, R-24613 and R-24614, were retrieved from G1M1, a mineral

medium containing 15 mM sodium succinate, 3 mM potassium nitrite and different vitamins. Based on partial 16S rRNA gene sequence similarity data, these new isolates could possibly represent a novel species within the environmental cluster of *Acidovorax* species, and were analysed further in a polyphasic study. The type strain and a second representative strain of each recognized environmental species of the genus *Acidovorax*, i.e. *A. facilis*, *A. delafieldii*, *A. defluvii* and *A. temperans*, were re-examined for phenotyping, chemotaxonomy and biochemical analysis to guarantee comparable results.

To avoid studying duplicate isolates of the same strain, genotyping by random amplified polymorphism DNA PCR analysis (Coenye *et al.*, 2002) and repetitive sequence-based PCR analysis with REP and BOX primers (Heyrman *et al.*, 2005) were carried out. The three fingerprint methods generated different patterns for isolates R-24607, 24608^T, R-24613 and R-24614, indicating genetic differences between all four isolates (data not shown). The average DNA G + C content of the four isolates, determined singly by HPLC (Mesbah *et al.*, 1989), was 64.3 ± 0.8 mol%. The nearly complete 16S rRNA gene sequences of R-24607, R-24608^T, R-24613 and R-24614 were determined as described by Vanparrys *et al.* (2005). Phylogenetic analysis was performed by using TREECON (Van de Peer & De Wachter, 1994) and BioNumerics software version 4.6 after multiple alignment with CLUSTAL_X (Thompson *et al.*, 1997). Cluster analysis according to the neighbour-joining algorithm, with or without corrections for evolutionary distances as described by Jukes & Cantor (1969) and Kimura (1980), was in agreement with those based on the maximum-parsimony and maximum-likelihood methods.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain R-24608^T is AM084006.

A figure showing the grouping of normalized digitized SDS-PAGE patterns for members of the genus *Acidovorax* is available as supplementary material with the online version of this paper.

Strains R-24607, R-24608^T, R-24613 and R-24614 clustered together with *A. temperans* LMG 7169^T, *A. delafieldii* LMG 5943^T, *A. defluvii* DSM 12644^T and *A. facilis* LMG 2193^T, but clearly formed a separate group, supported by high bootstrap values (Fig. 1). Therefore, DNA–DNA hybridization experiments were performed within this cluster, by using a modification of the microplate method of Ezaki *et al.* (1989) as described by Willems *et al.* (2001). A hybridization temperature of 45 °C (calculated with correction for 50 % formamide) was used. First, strains R-24607, R-24608^T, R-24613 and R-24614 were hybridized among themselves to substantiate the hypothesis of their relatedness at the species level. Levels of DNA–DNA relatedness among the four new isolates ranged between 78.5 and 88.5 % (± 1.3 –10.9 %), suggesting that they represented a single species, but also confirming the genetic diversity within the strains. Strain R-24608^T was further hybridized with *A. temperans* LMG 7169^T (DNA–DNA relatedness of 26.7 ± 5.6 %), *A. delafieldii* LMG 5943^T (23.9 ± 5.9 %), *A. defluvii* DSM 12644^T (26.0 ± 0.6 %) and *A. facilis* LMG 2193^T (18.2 ± 4.8 %). These results confirmed that R-24607, R-24608^T, R-24613 and R-24614 belong to a novel genospecies.

Cell morphology and motility were investigated by electron microscopy (Fig. 2) and phase-contrast microscopy (at a magnification of $\times 1000$), respectively, for cells grown on tryptone soy agar (TSA; Oxoid) for 48 h at 28 °C. Cells were Gram stained and examined via light microscopy, and catalase and oxidase activity was determined. Utilization of carbon sources and enzyme production were tested with the API 20NE (48 h, 28 °C), API ZYM (4 h, 28 °C)

Fig. 1. Phylogenetic dendrogram obtained by neighbour-joining clustering of 16S rRNA gene sequences (without correction), showing the position of strains R-24608^T, R-24607, R-24613 and R-24614 among the type strains of recognized *Acidovorax* species. EMBL accession numbers are shown in parentheses. *Variovorax paradoxus* LMG 1797^T was used as the outgroup. Relevant bootstrap values (expressed as percentages of 1000 replicates) are shown at branch points. Bar, 0.02 changes per sequence position.

Fig. 2. Electron micrograph of cells of strain R-24608^T, showing peritrichously flagellated rods (cell size about 0.9×1.8 μm).

(bioMérieux) and Biolog (24 h, 28 °C) systems according to the manufacturers' recommendations. The temperature range (at 4, 15, 28, 37, 45 and 52 °C), pH range (pH 4.5–10.5 at 28 °C) and salinity range (0.5–5 % NaCl, w/v, at 28 °C) for growth were recorded after incubation for 48 h in tryptone soy broth (TSB; Oxoid). The ability to denitrify was tested, as described by Smibert & Krieg (1994), after growth for 1 week in TSB supplemented with 10 mM potassium nitrate at 37 °C and in liquid isolation medium G1M1 at 37 °C, and was confirmed with N₂O measurements, as described by Heylen *et al.* (2006). Lipolytic activity was determined after 72 h based on hydrolysis of Tween 80, as described by Sierra (1957). Differential biochemical characteristics among the test strains are given in Table 1.

After a pre-culture, all strains were incubated under identical conditions for 48 h at 28 °C on TSA. A loopful of well-grown cells was harvested and fatty acid methyl esters were prepared and extracted according to the standardized protocol of the Microbial Identification System (MIS; Microbial ID Inc.), and were identified by using MIDI with the TSBA database version 5.0. All strains of recognized *Acidovorax* species contained the characteristic fatty acids 3-hydroxyoctanoic acid (C_{8:0} 3-OH) and 3-hydroxydecanoic acid (C_{10:0} 3-OH) (Willems & Gillis, 2005). The dominant fatty acids for strains R-24607, R-24608^T, R-24613 and R-24614 were summed feature 3 (38–41 %), C_{18:1}ω7c (22–32 %) and C_{16:0} (25–26.5 %). Unfortunately, the Sherlock MIS software could not clearly resolve summed feature 3, referring to the peaks of C_{16:1}ω7c and/or iso-C_{15:0} 2-OH. However, Sherlock lists the closest to the observed ECL first, which was C_{16:1}ω7c.

Table 1. Differential physiological characteristics between strains R-24607, R-24608^T, R-24613 and R-24614 and their closest phylogenetic neighbours in the genus *Acidovorax*

Strains: 1, R-24607, R-24608^T, R-24613 and R-24614; 2, *A. defluvi* DSM 12644^T and DSM 12578; 3, *A. delafieldii* LMG 5943^T and LMG 8909; 4, *A. facilis* LMG 2193^T and LMG 6598; 5, *A. temperans* LMG 7169^T and LMG 7163. Data are from this study unless otherwise indicated. +, Positive; w, weakly positive; –, negative; v, variable (test result for the type strain given in parentheses). All were positive for oxidase, leucine arylamidase and assimilation of methyl pyruvate, monomethyl succinate, β -hydroxybutyric acid, α -ketovaleric acid and DL-lactic acid. All were negative for arginine dihydrolase, urease, lipase (C14), valine arylamidase, cystine arylamidase, α -chymotrypsin, acid phosphatase, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase (aesculin hydrolysis), *N*-acetyl- β -glucosamidase, α -mannosidase, α -fucosidase, indole production, glucose fermentation and assimilation of L-mannose, phenylacetic acid, *N*-acetyl-D-galactosamine, adonitol, cellobiose, L-fucose, gentiobiose, *myo*-inositol, α -D-lactose, lactulose, D-melibiose, methyl β -glucoside, D-raffinose, sucrose, trehalose, turanose, xylitol, citric acid, D-galactonic acid lactone, D-galacturonic acid, D-glucosaminic acid, D-glucuronic acid, saccharic acid, glucuronamide, L-histidine, inosine, uridine, thymidine, phenyl ethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, DL- α -glycerol phosphate and glucose 1-phosphate.

Characteristic	1	2	3	4	5
Catalase	+	v (+)	–, v*	+	+
Hydrolysis of Tween 40	+	v (–)	+	v (–)	+
Hydrolysis of Tween 80	+	†	+	v (–)	+
Nitrate reduction	+	v‡, +§	+	+	+
Denitrification	+	v‡	–	–	+
Alkaline phosphatase	w	–	+	w	+
Esterase (C4)	w	+	–	+	w
Esterase lipase (C8)	+	w	+	+	+
Trypsin	+	–	–	–	–
Naphthol-AS-BI-phosphohydrolase	+	–	–	–	–
Indole production	+	–	–	–	–
Gelatinase	–	–	–, v* v (–), +*	–	–
Assimilation of:					
L-Arabinose	–	–	+	–, +*	–
D-Mannose	–	–	+	–, +*	–
D-Mannitol	–	–	+	–, +*	+
N-Acetylglucosamine	–	–	–	+	–
Maltose	–	–	–	+	–
Potassium gluconate	–	–	+	–, +*	–
Capric acid	–	–	–, v*	–	–, v*
Adipic acid	–	+	–	–	–
Malate	+	–	v	–	+
Trisodium citrate	–	–	–, v*	–	–
α -Cyclodextrin	v (–)	–	v (–)	–	–
Dextrin	–	–	v (–)	–	–
Glycogen	+	–	–	–	+, –*
D-Arabitol	–	–	+	–	v (–)
i-Erythritol	v (–)	–	–	–	–
D-Fructose	–	–	+	v (–)	+
D-Galactose	–	v (+)	+	–	–

Characteristic	1	2	3	4	5
α -D-Glucose	–	–	v (v)II	–, +*	+
D-Psicose	–	–	v (–)	v (–)	+
D-Sorbitol	–	–	+	–	+
Acetic acid	v (+)	–	–	–	–
<i>cis</i> -Aconitic acid	v (+)	–	v (+)	–	–
Formic acid	+	–	v (+)	–	–
D-Gluconic acid	–	–	+	–	–
α -Hydroxybutyric acid	+	v (–)	+	–	+
γ -Hydroxybutyric acid	+	v (–)	+	–	+
<i>p</i> -Hydroxyphenylacetic acid	–	–	+	–	–
Itaconic acid	–	–	–	–	–
α -Ketobutyric acid	+	v (–)	+	v (–)	+
α -Ketoglutaric acid	+	v (+)	+	v (–)	+
Malonic acid	v (–)	–	–	v (–)	–
Propionic acid	+	–	v (+)	–	v (–)
Quinic acid	–	–	+	–	–
Sebacic acid	v (+)	+	+	v (–)	+
Succinic acid	+	+	+	v (–)	+
Bromosuccinic acid	+	–	v (+)	v (–)	+
Succinamic acid	v (–)	–	v (+)	v (–)	+
Alaninamide	v (–)	v (+)	+	v (–)	+
D-Alanine	+	–	+	v (–)	+
L-Alanine	+	+	+	v (–)	+
L-Alanyl glycine	+	v (+)	+	+	+
L-Asparagine	v (–)	–	+	–	+
L-Aspartic acid	+	–	+	–	+
L-Glutamic acid	+	–	+	–	+
Glycyl L-aspartic acid	–	–	+	v (–)	–
Glycyl L-glutamic acid	–	–	+	–	v (+)
Hydroxy L-proline	–	–	+	–	–
L-Leucine	+	–	+	v (–)	+
L-Ornithine	–	–	v (+)	–	v (–)
L-Phenylalanine	–	–	v (+)	+	+
L-Proline	+	–	+	+	+
L-Pyrogutamic acid	+	–	v (+)	+	+
D-Serine	v (–)	–	–	–	–
L-Serine	+	–	+	–, +*	–
L-Threonine	+	v (–)	+	–	v (–)
DL-Carnitine	–	–	v (+)	v (+)	–
γ -Aminobutyric acid	–	–	+	v (+)	v (–)
Urocanic acid	–	–	+	–	–
Glycerol	+	v (+)	+	–	+
Glucose 6-phosphate	v (–)	–	v (+)	–	–

*Data from Willems & Gillis (2005).

†DSM 12644^T scored negative for Tween 80 hydrolysis in the Biolog screening (after 24 h), but positive when grown on Sierra medium for 72 h.

‡In contrast to DSM 12578, DSM 12644^T does not reduce nitrate to nitrite or further (denitrification) under the tested growth conditions (in G1M1 and in supplemented TSB). With the API 20NE gallery, the two strains tested positive for nitrate reduction and denitrification.

§Data from Schulze *et al.* (1999).

||A. *delafieldii* LMG 5943^T tested positive for glucose assimilation with API 20NE but negative with Biolog.

In addition, comparison of the fatty acid data in the literature for the type strains of recognized species of the genus *Acidovorax* (Willems *et al.*, 1990; Schulze *et al.*, 1999) and our data on the same strains suggests C_{16:1}ω7c as the major fatty acid for this peak. No characteristic fatty acids for the novel genospecies were detected (Table 2).

SDS-PAGE analysis of whole-cell proteins was performed on aerobically grown cells after incubation at 28 °C for 40 h on phosphate-buffered nutrient agar (pH 6.8). An SDS-PAGE banding pattern for all strains was generated according to a standardized protocol (Pot *et al.*, 1994). Pearson's correlation similarity coefficients were clustered with UPGMA and analysed with the co-phenetic correlation method in BioNumerics version 4.6 (see Supplementary Fig. S1 in IJSEM Online). The different strains of each species grouped together, supported with high co-phenetic correlation values. Although strains R-24607, R-24608^T, R-24613 and R-24614 demonstrated significant variation in whole-cell protein profiles, they formed a distinct group, separate from recognized species of the genus *Acidovorax*.

Based on the polyphasic data presented here, strains R-24607, R-24608^T, R-24613 and R-24614 are considered to represent a novel species of the genus *Acidovorax*, for which the name *Acidovorax caeni* sp. nov. is proposed.

Description of *Acidovorax caeni* sp. nov.

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

After 48 h, colonies are round, smooth and yellow–brown. Cells are motile, non-spore-forming rods (0.9 × 1.8 µm). Gram-negative and catalase- and oxidase-positive. Growth is observed at 15–37 °C but not at 4 or 45–52 °C, at pH 5.5–10.5, but not at pH 4.5–5, and at salt concentrations of 0.5–2 % NaCl (w/v), but not at 3–5 % NaCl. Anaerobic respiration and growth are possible through denitrification. Positive for the following enzyme activities: hydrolysis of Tweens 40 and 80, esterase lipase, trypsin, naphthol-AS-BI-phosphohydrolase and production of indole. Malate, glycogen, formic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, α-ketobutyric acid, α-ketoglutaric acid, propionic acid, succinic acid, bromosuccinic

Table 2. Fatty acid content of strains R-24607, R-24608^T, R-24613 and R-24614 and their closest phylogenetic neighbours in the genus *Acidovorax*

Strains: 1, R-24607, R-24608^T, R-24613 and R-24614; 2, *A. defluvii* DSM 12644^T and DSM 12578; 3, *A. delafieldii* LMG 5943^T and LMG 8909; 4, *A. facilis* LMG 2193^T and LMG 6598; 5, *A. temperans* LMG 7169^T and LMG 7163. All data were obtained in the present study. Values are percentages of the total fatty acid content. –, Not present; tr, trace component (<1 %).

Fatty acid	1	2	3	4	5
C _{8:0} 3-OH	0.78–1.19	tr	tr	0.88–1.87	1.04–1.27
C _{9:0} 3-OH	–	0.32–1.14	–	–	tr
C _{11:0}	–	tr	–	–	tr
C _{10:0} 3-OH	2.81–4.32	3.13–3.90	3.16–3.59	2.78–6.78	3.96–4.25
Unknown 11.799	–	–	–	tr	–
C _{12:0}	2.64–3.24	2.18–2.39	2.77–2.89	2.91–5.48	5.52–5.72
iso-C _{11:0} 3-OH	–	tr	–	–	–
C _{11:0} 3-OH	–	tr	–	–	–
C _{13:0}	–	tr	–	–	1.08–3.46
C _{14:0}	tr	1.57–1.63	3.25–3.37	3.09–3.74	0.99–3.53
C _{15:1} ω6c	–	1.15–11.97	–	–	3.23–4.51
iso-C _{15:0}	–	tr	–	–	–
Summed feature 3*	38.24–41.16	40.57–48.05	39.35–41.32	43.68–43.71	46.92–47.15
C _{16:0}	24.90–26.41	17.70–22.78	26.33–27.99	25.51–29.24	19.35–20.99
iso-C _{17:1} ω9c	–	tr	–	–	–
iso-C _{17:0}	tr	tr	–	–	–
C _{17:0} cyclo	–	–	tr	tr	–
C _{17:1} ω8c	–	tr	–	–	–
C _{17:1} ω6c	–	1.41–2.09	–	–	tr
C _{17:0}	tr	4.87–6.27	tr	tr	2.10–3.7
C _{18:1} ω7c	22.24–31.96	8.07–11.04	19.42–22.73	11.78–16.56	9.12–9.64
C _{18:0}	tr	–	tr	tr	–
Methyl C _{18:1} ω7c	tr	tr	tr	–	–
Summed feature 7†	tr	–	–	–	–

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

†Summed feature 7 comprises C_{19:1}ω6c and/or C_{19:0} cyclo.

acid, D-alanine, L-alanine, L-alanyl glycine, L-aspartic acid, L-glutamic acid, L-leucine, L-proline, L-pyrogutamic acid, L-serine, L-threonine, methyl pyruvate, monomethyl succinate, β -hydroxybutyric acid, α -ketovaleric acid, DL-lactic acid and glycerol can be used as carbon sources. Can be differentiated from the type strains of its closest phylogenetic neighbours, *A. defluvi*, *A. delafieldii*, *A. facilis* and *A. temperans*, through SDS-PAGE analysis of whole-cell proteins, by the ability to produce indole and by the presence of trypsin and naphthol-AS-BI-phosphohydrolase.

The type strain, R-24608^T (=LMG 24103^T =DSM 19327^T), has a DNA G+C content of 65.7 mol% and was isolated from activated sludge from an aerobic-anaerobic wastewater treatment plant (Bourgoyen-Ossemeersen) in Gent, Belgium. Due to the genetic variation within this species, R-24607, R-24613 and R-24614 were also deposited in the BCCM/LMG collection with strain numbers LMG 24104, LMG 24105 and LMG 24106, respectively.

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