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Advenella faeciporci sp. nov., a nitrite-denitrifying bacterium isolated from nitrifying–denitrifying activated sludge collected from a laboratory-scale bioreactor treating piggery wastewater

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Strain M-07^T was isolated from nitrifying–denitrifying activated sludge treating piggery wastewater. Phylogenetic analysis based on 16S rRNA gene sequences demonstrated that strain M-07^T belonged to the genus *Advenella*. 16S rRNA gene sequence similarity between M-07^T and *Advenella incenata* CCUG 45225^T, *Advenella mimigardefordensis* DPN7^T and *Advenella kashmirensis* WT001^T was 96.5, 97.3 and 96.9 %, respectively. The DNA G+C content of strain M-07^T was 49.5 mol%, which was approximately 5 mol% lower than the range for the genus *Advenella* (53.5–58.0 mol%). The predominant cellular fatty acids of strain M-07^T were $C_{16:0}$, summed feature 3 (comprising $C_{16:1}\omega 7c$ and/or iso-$C_{15:0}$ 2-OH), $C_{17:0}$ cyclo and summed feature 2 (comprising one or more of $C_{14:0}$ 3-OH, iso-$C_{16:1}$ I, an unidentified fatty acid with an equivalent chain-length of 10.928 and $C_{12:0}$ alde). The isoprenoid quinone was Q-8. On the basis of phenotypic characteristics, phylogenetic analysis and DNA–DNA relatedness, strain M-07^T should be classified as a novel species of the genus *Advenella*, for which the name *Advenella faeciporci* sp. nov. is proposed. The type strain is M-07^T (=JCM 17746^T =KCTC 23732^T).

The genus *Advenella* of the family *Alcaligenaceae* (De Ley *et al.*, 1986) was proposed by Coenye *et al.* (2005). At the time of writing, the genus *Advenella* comprised three species: the type species, *Advenella incenata* (Coenye *et al.*, 2005), *Advenella kashmirensis* (Ghosh *et al.*, 2005; Gibello *et al.*, 2009) and *Advenella mimigardefordensis* (Wübbeler *et al.*, 2006; Gibello *et al.*, 2009). *A. incenata* is a Gram-stain-negative, rod- to coccoid-shaped, oxidase-positive species that has been isolated from various human and veterinary clinical samples. *A. kashmirensis* is a mesophilic, neutrophilic, Gram-stain-negative, non-motile, oval- to coccoid-shaped species that occurs singly or in pairs, chains, branched chains or clusters that was isolated from temperate orchard soil on reduced sulfur compounds as energy and electron sources, and it can oxidize tetrathionate and thiosulfate. *A. mimigardefordensis* is Gram-stain-negative, motile, non-spore-forming coccoid rods, isolated from compost under mesophilic conditions, and it can utilize the organic disulfide 3,3′-dithiodipropionic acid.

During the course of screening denitrifiers with high N_2O reduction abilities, strain M-07^T was isolated from nitrifying–denitrifying activated sludge from a laboratory-scale bioreactor treating piggery wastewater. Strain M-07^T was isolated after N_2O enrichment. Activated sludge (1 mg) was inoculated into 9 ml pig manure (PM) medium, which resembles piggery wastewater, in a 27 ml L-shaped test tube. PM medium (pH 7.0) contained 20 : 80 (v/v) extracted pig manure and treated water from a laboratory-scale membrane sequencing batch bioreactor used for nitrogen removal during nitrification–denitrification processes (Miyahara *et al.*, 2010). After replacing the headspace gas with N_2O, the test tube was sealed with a double butyl rubber stopper and incubated at 30 °C with shaking at 150 r.p.m. N_2O consumption and N_2 production was

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain M-07^T is AB567741.

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observed. After N_2O reduction was complete, 90 μl enriched culture was inoculated into 9 ml fresh PM medium and incubated under the same conditions (second N_2O enrichment culture). This procedure was repeated four times. After 36 h incubation of the fifth N_2O enrichment culture, an aliquot was plated directly on PM medium containing 1.5 % gellan gum and incubated in closed jars under a 100 % (v/v) N_2O atmosphere at room temperature for 1 week. Strain M-07^T was obtained from a single colony after subcultivation on Luria–Bertani (LB; BD Biosciences) agar under aerobic conditions at 30 °C for 1 week. Denitrification activities were investigated using ^{15}N-labelled nitrate and nitrite. Strain M-07^T could denitrify nitrite, but not nitrate. The isolate was stored as a glycerol suspension (20 % v/v) at −80 °C.

Genomic DNA was prepared according to the method described by Saito & Miura (1963). The 16S rRNA gene of strain M-07^T was amplified by PCR using the bacterial universal primers 27F (5′-AGAGTTTGATCCTGGCTC-AG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Weisburg *et al.*, 1991). The amplification product was cloned into pT7Blue-2 (Novagen) and sequenced. The 16S rRNA gene sequence of strain M-07^T was compared against those in GenBank (http://www.ncbi.nlm.nih.gov). Multiple sequence alignments were performed using CLUSTAL X software version 1.83 (Thompson *et al.*, 1997). Phylogenetic trees were constructed with the neighbour-joining method (Saitou & Nei, 1987) using MEGA software version 3.1 (Kumar *et al.*, 2004) and the maximum-likelihood method (Felsenstein, 1981) using PhyML software version 2.4.4 (Guindon & Gascuel, 2003). Evolutionary distances were calculated according to Kimura's two-parameter model (Kimura, 1983). The stability of the groupings was estimated by bootstrap analysis of 1000 replicates (Felsenstein, 1985). As shown in the neighbour-joining tree (Fig. 1), strain M-07^T and members of the genus *Advenella* formed a monophyletic cluster with 100 % bootstrap support, which indicated that strain M-07^T belonged to the genus *Advenella*. The topology of the phylogenetic tree by the maximum-likelihood method was identical to that of the neighbour-joining tree. Strain M-07^T exhibited 96.5, 97.3 and 96.9 % 16S rRNA gene sequence similarity with *A. incenata* CCUG 45225^T, *A. mimigardefordensis* DPN7^T and *A. kashmirensis* WT001^T, respectively.

The DNA G+C content was determined as described by Tamaoka & Komagata (1984) and Mesbah *et al.* (1989) The DNA G+C content of strain M-07^T was 49.5 mol%, which was approximately 5 mol% lower than the range for the genus *Advenella* (53.5–58.0 mol%) (Coenye *et al.*, 2005; Ghosh *et al.*, 2005; Wübbeler *et al.*, 2006; Gibello *et al.*, 2009). DNA–DNA hybridization was performed with five replicates as described by Ezaki *et al.* (1989). Strain M-07^T exhibited low DNA–DNA relatedness to *A. incenata* LMG 22250^T (5.3 %), *A. mimigardefordensis* LMG 22922^T (7.2 %) and *A. kashmirensis* LMG 22695^T (1.1 %). Based on the phylogenetic analysis, DNA base composition and DNA–DNA relatedness, strain M-07^T was representative of a novel species of the genus *Advenella*.

Gram-staining was performed according to Murray *et al.* (1994) and showed that strain M-07^T was Gram-negative. Cell morphology and motility was determined by phase-contrast microscopy (Axioskop 2 plus; Zeiss). Catalase activity was determined by bubble production following the addition of 3 % H_2O_2 to colonies cultured on LB agar. Oxidase activity was determined by the oxidation of tetramethyl *p*-phenylenediamine on cytochrome *c* oxidase test paper (Nissui Pharmaceutical). The isolate exhibited both catalase and oxidase activities. Hydrolysis of casein, starch and DNA was examined on LB agar supplemented with 1 % substrate, as described by Hansen & Sørheim (1991). The isolate did not hydrolyse the substrates tested. Temperature and pH ranges for growth were investigated on LB agar. NaCl concentration for growth was investigated on LB agar containing 0–10 % (w/v) NaCl. The isolate grew at 10–45 °C (optimum, 25–35 °C), at pH 5.0–11.0 (optimum, 7.0–9.0). and in the presence of 0–4 % NaCl. API 20 NE and API ZYM (bioMérieux) and GN2 MicroPlates (Biolog) were used to determine the physiological and biochemical characteristics of the isolate, according to the manufacturers' instructions. The ability to denitrify nitrite was examined by

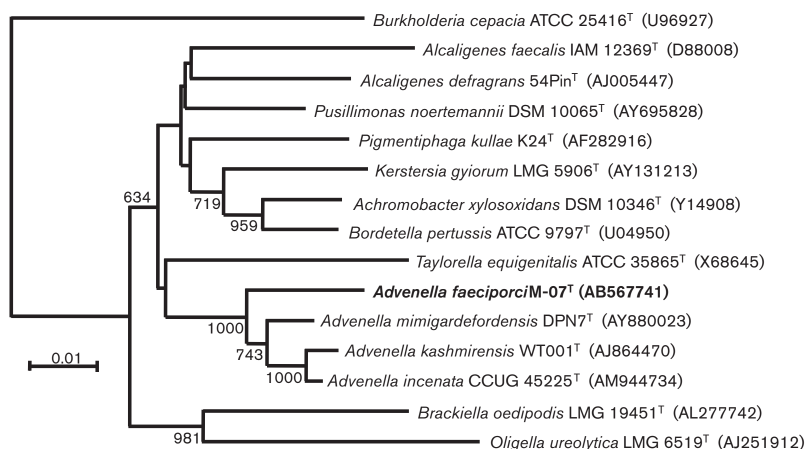

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships of strain M-07^T with members of the genus *Advenella* and related members of the family *Alcaligenaceae*. Bootstrap values (>600) based on 1000 replicates are shown at branch nodes. *Burkholderia cepacia* ATCC 25416^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

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anaerobic shaking with LB medium containing ^{15}N-labelled sodium nitrite. $^{15}N_2$ and $^{15}N_2O$ were periodically monitored by GC-MS (GCMS-GP5050; Shimadzu) equipped with a CP-PoraPLOT-Q column (Varian). The phenotypic characteristics that differentiated strain M-07^T from members of the genus *Advenella* are listed in Table 1. Differences were observed between strain M-07^T and the reference strains. The most distinguished characteristics of strain M-07^T were its ability to denitrify nitrite and assimilate phenylacetic acid, the absence of urease and acid phosphatase, its inability to assimilate L-arabinose and gluconate and its inability to utilize glycogen, Tween 80, D-galactose, α-D-glucose, D-mannose, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucuronic acid, malonic acid, quinic acid, L-asparagine, L-aspartic acid and L-phenylalanine.

The fatty acid composition was analysed according to the instructions of the Sherlock Microbial Identification System (MIDI) after 48 h incubation in tryptic soy broth agar at 30 °C (Sasser, 1990). The comparison of fatty acid compositions of strain M-07^T and the reference strains is shown in Table 2. The predominant cellular fatty acids of M-07^T were $C_{16:0}$, summed feature 3 ($C_{16:1}\omega 7c$ and/or iso-$C_{15:0}$ 2-OH), $C_{17:0}$ cyclo and summed feature 2 (one or more of $C_{14:0}$ 3-OH, iso-$C_{16:1}$ I, an unidentified fatty acid with an equivalent chain-length of 10.928 and $C_{12:0}$ alde). The reference strains had similar predominant fatty acids except that they also contained predominant amounts of $C_{18:1}\omega 7c$, which was present in a relatively small amount (8.1 %) in strain M-07^T. The isoprenoid quinones were determined by HPLC as described by Xie & Yokota (2003). The major isoprenoid quinone of strain M-07^T was Q-8.

On the basis of chemotaxonomic and physiological characteristics, 16S rRNA gene sequence analysis and DNA–DNA relatedness, strain M-07^T should be classified as a novel species of the genus *Advenella*, for which the name *Advenella faeciporci* sp. nov. is proposed.

Table 1. Phenotypic characteristics that differentiate strain M-07^T from members of the genus *Advenella*

Strains: 1, M-07^T; 2, *A. incenata* LMG 22250^T; 3, *A. kashmirensis* LMG 22695^T; 4, *A. mimigardefordensis* LMG 22922^T. All data were taken from this study. All strains are positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, naphthol-AS-BI-phosphohydrolase, assimilation of adipic acid, malic acid and citric acid and utilization of pyruvic acid methyl ester, succinic acid monomethyl ester, acetic acid, *cis*-aconitic acid, citric acid, α- and β-hydroxybutyric acid, itaconic acid, α-ketobutyric acid, DL-lactic acid, propionic acid, sebacic acid, succinic acid, bromosuccinic acid, succinamic acid, glucuronamide, alaninamide, D- and L-alanine, L-alanyl glycine, L-glutamic acid, L-leucine, L-ornithine and L-proline. All strains are negative for lipase (C14), trypsin, α-chymotrypsin, α- and β-galactosidase, β-glucuronidase, α- and β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, indole production, glucose fermentation, arginine dihydrolase, aesculin hydrolysis, gelatin hydrolysis, assimilation of D-mannose, D-mannitol, *N*-acetyl-D-glucosamine, maltose and capric acid, and utilization of α-cyclodextrin, dextrin, Tween 40, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, adonitol, L-arabinose, D-arabitol, cellobiose, *i*-erythritol, D-fructose, gentiobiose, *myo*-inositol, α-lactose, lactulose, maltose, D-mannitol, melibiose, methyl β-D-glucoside, D-psicose, raffinose, L-rhamnose, D-sorbitol, sucrose, trehalose, turanose, xylitol, D-glucosaminic acid, *p*-hydroxyphenylacetic acid, L-histidine, L-pyroglutamic acid, D-serine, DL-carnitine, urocanic acid, inosine, uridine, thymidine, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, DL-α-glycerol phosphate and glucose 1-phosphate.

Characteristic	1	2	3	4
Reduction of nitrate to nitrite (API 20 NE)	−	−	+	−
Denitrification from nitrite	+	−	−	−
Urease (API 20 NE)	−	+	+	+
Enzymes (API ZYM)				
Cystine arylamidase	−	+	−	−
Acid phosphatase	−	+	+	+
Utilization of (Biolog GN2):				
Glycogen, Tween 80, D-galactose, α-D-glucose, D-mannose, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucuronic acid, malonic acid, quinic acid, L-asparagine, L-aspartic acid, L-phenylalanine	−	+	+	+
L-Fucose, γ-aminobutyric acid	−	−	+	+
α-Ketoglutaric acid	+	+	−	+
α-Ketovaleric acid	+	−	−	+
Glycyl L-aspartic acid, glycyl L-glutamic acid	−	−	+	−
L-Serine, D-saccharic acid, hydroxy-L-proline	−	+	+	−
L-Threonine	+	+	−	−
Glycerol, glucose 6-phosphate, γ-hydroxybutyric acid	−	+	−	+
Assimilation of (API 20 NE):				
L-Arabinose, gluconate	−	+	+	+
Phenylacetic acid	+	−	−	−
DNA G+C content (mol%)	49.5	54.2	55.1	55.1

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Table 2. Fatty acid compositions of strain M-07^T and members of the genus *Advenella*

Strains: 1, M-07^T (data from this study); 2, *A. incenata* LMG 22250^T (Coenye *et al.*, 2005); 3, *A. kashmirensis* LMG 22695^T (Ghosh *et al.*, 2005); 4, *A. mimigardefordensis* LMG 22922^T (Wübbeler *et al.*, 2006).

Fatty acid (%)	1	2	3	4
Saturated				
$C_{12:0}$	2.9	4.6	3.1	4.5
$C_{16:0}$	33.3	21.7	21.7	18.2
$C_{17:0}$	1.2	–	–	–
$C_{18:0}$	–	1.2	1.9	2.1
Unsaturated				
$C_{18:1}\omega7c$	8.1	24.6	28.3	26.5
Hydroxy				
$C_{16:0}$ 3-OH	–	1.2	–	3.3
$C_{17:0}$ cyclo	16.8	9.2	3.5	4.8
$C_{19:0}\omega8c$ cyclo	1.4	4.0	1.3	2.6
Summed features*				
2	9.3	12.9	10.1	13.3
3	20.5	21.3	28.0	23.3

*Summed features represent two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 2 consisted of one or more of $C_{14:0}$ 3-OH, iso-$C_{16:1}$ I, an unidentified fatty acid with an equivalent chain-length of 10.928 and $C_{12:0}$ alde. Summed feature 3 consisted of $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH.

Description of *Advenella faeciporci* sp. nov.

Advenella faeciporci (fa.e.ci.por′ci. L. n. *faex*, *faecis* faeces; L. gen. n. *porci* of swine, pig; N.L. gen. n. *faeciporci* from pig faeces, associated with the isolation origin).

Cells are Gram-negative, non-motile cocci (1.5–2 µm). Colonies on LB agar are white, circular, flat, entire and rough. Grows at 10–45 °C (optimum, 25–35 °C), at pH 5–11 (optimum, pH 7–9) and with 0–4 % NaCl. Denitrifies nitrite, but not nitrate. Oxidase- and catalase-positive. Negative result in tests for hydrolysis of amylase, casein and aesculin; DNase; production of indole; and acidification of glucose, arginine dihydrolase, urease, gelatinase and β-galactosidase. In API 20NE tests assimilates adipic acid, malic acid, citric acid and phenylacetic acid. With GN2 MicroPlates, utilizes pyruvic acid methyl ester, succinic acid monomethyl ester, acetic acid, *cis*-aconitic acid, citric acid, α- and β-hydroxybutyric acid, itaconic acid, α-ketobutyric acid, α-ketoglutaric acid, α-ketovaleric acid, DL-lactic acid, propionic acid, sebacic acid, succinic acid, bromosuccinic acid, succinamic acid, glucuronamide, alaninamide, D- and L-alanine, L-alanyl glycine, L-glutamic acid, L-leucine, L-ornithine, L-proline and L-threonine. Additional physiological characteristics are shown in Table 1. The major fatty acids are $C_{16:0}$, summed feature 3 (comprising $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH), $C_{17:0}$ cyclo, summed feature 2 (comprising one or more of $C_{14:0}$ 3-OH, iso-$C_{16:1}$ I, an unidentified fatty acid with an equivalent chain-length of 10.928 and $C_{12:0}$ alde) and $C_{18:1}\omega7c$. The major isoprenoid quinone is Q-8.

The type strain, M-07^T (=JCM 17746^T =KCTC 23732^T), was isolated from nitrifying–denitrifying activated sludge from a laboratory-scale bioreactor treating piggery wastewater. The DNA G+C content of the type strain is 49.5 mol%.

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