Caenimonas koreensis gen. nov., sp. nov., isolated from activated sludge

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A Gram-negative, rod-shaped bacterium, designated strain EMB320^T, was isolated from activated sludge performing enhanced biological phosphorus removal in a sequencing batch reactor. The isolate was strictly aerobic and non-motile. Growth was observed between 10 and 35 °C (optimum 30 °C) and between pH 6.0 and 9.0 (optimum pH 7.0–8.0). The predominant cellular fatty acids of strain EMB320^T were C_{16:0}, C_{18:1ω7c} and summed feature 3 (C_{16:1ω7c} and/or iso-C_{15:0} 2-OH). The major polar lipids were phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol. Strain EMB320^T contained ubiquinone-8 (Q-8) as the major respiratory quinone system and 2-hydroxyputrescine and putrescine as the major polyamines, which suggests that it belongs to the *Betaproteobacteria*. The G+C content of the genomic DNA was 62.7 mol%. Comparative 16S rRNA gene sequence analysis showed that strain EMB320^T formed a phyletic lineage distinct from other genera within the family *Comamonadaceae*. On the basis of chemotaxonomic data and molecular properties, strain EMB320^T represents a novel genus and species within the family *Comamonadaceae*, for which the name *Caenimonas koreensis* sp. nov. is proposed. The type strain of *Caenimonas koreensis* is EMB320^T (=KCTC 12616^T =DSM 17982^T).

The family *Comamonadaceae* belonging to the *Beta-proteobacteria* (Stackebrandt *et al.*, 1988) was described to include the genera *Comamonas*, *Acidovorax*, *Hydrogenophaga*, *Xylophilus* and *Variovorax* (described to include the former *Alcaligenes paradoxus*), as well as phylogenetically misnamed [*Aquaspirillum*] and phytopathogenic [*Pseudomonas*] species (Willems *et al.*, 1991). Since then, several species have been transferred to other genera or reclassified as members of novel genera by the application of molecular and improved phenotypic approaches (Willems *et al.*, 1992; Hiraishi, 1994; Wen *et al.*, 1999;

Ding & Yokota, 2004), and new genera such as *Rhodoferrax*, *Xenophilus*, *Polaromonas*, *Alicyclophilus* and *Ramlibacter* have been added to the family (Hiraishi *et al.*, 1991; Irgens *et al.*, 1996; Blümel *et al.*, 2001; Heulin *et al.*, 2003; Mechichi *et al.*, 2003).

Activated sludge processes with cyclic changes of anaerobic and aerobic conditions have been used to remove phosphate from wastewater and are becoming more important for reducing eutrophication of lakes. An understanding of the microbial community responsible for phosphorus removal is a prerequisite for understanding the mechanism of enhanced biological phosphorus removal (EBPR). Therefore, efforts to isolate bacteria performing EBPR have been made in our laboratory (Lu *et al.*, 2006) and here we describe the taxonomic characterization of a strain isolated from activated sludge that represents a novel genus belonging to the family *Comamonadaceae*.

Strain EMB320^T was isolated from activated sludge performing EBPR in a lab-scale sequencing batch reactor (SBR). Sodium acetate was supplied as a sole carbon source

Abbreviation: EBPR, enhanced biological phosphate removal.

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The GenBank/EMBL/DBJ accession number for the 16S rRNA gene sequence of strain EMB320^T is DQ349098.

A transmission electron micrograph of cells and results of TLC of polar lipids of strain EMB320^T and maximum-likelihood and maximum-parsimony 16S rRNA gene sequence-based phylogenetic trees are available as supplementary material with the online version of this paper.

and the operation of the SBR has been described elsewhere (Jeon *et al.*, 2003). A sludge sample was diluted serially with 1% (w/v) saline solution and spread on R2A agar (Difco) and incubated at 20 °C for 7 days for isolation. Subcultivation was done on R2A agar at 30 °C for 5 days. The type strains of some other related taxa, *Rhodoferrax ferrireducens* DSM 15236^T, *Curvibacter delicatus* DSM 11558^T, *Xylophilus ampelinus* DSM 7250^T, *Ramlibacter tataouinensis* DSM 14655^T, *Variovorax paradoxus* KCTC 12459^T and *Polaromonas naphthalenivorans* CJ2^T, were used as reference strains for biochemical tests.

Gram staining was performed using the bioMérieux Gram stain kit according to the manufacturer's instructions. Cell morphology and motility were studied using phase-contrast microscopy and transmission electron microscopy (JEM-1010; JEOL) as described by Jeon *et al.* (2005). Physiological characteristics of strain EMB320^T were examined by growing the isolate on R2A medium at different temperatures and pH. R2A medium was prepared with different pH values as described previously (Gomori, 1955). Oxidase activity was tested by oxidation of 1% (w/v) tetramethyl-*p*-phenylenediamine (Merck) and catalase activity was evaluated by production of oxygen bubbles in 3% (v/v) aqueous hydrogen peroxide solution. The hydrolysis of compounds was assessed on R2A agar after 5 days of incubation according to methods described previously (Lányi, 1987; Smibert & Krieg, 1994). Nitrate reduction was performed according to the method of Lányi (1987) and acid production from carbohydrates was tested as described by Leifson (1963). Utilization of thiosulfate was tested in R2A broth supplemented with 10 mM Na₂S₂O₃ · 5H₂O as described by Spring *et al.* (2004) and the concentration of sulfate, the end product of thiosulfate oxidation, was quantified in spent R2A medium using anion chromatography (Dionex; ICS-1000). Carbon source utilization was tested in mineral medium as described by Kämpfer *et al.* (1991). The utilization of D-fructose, D-glucose, glycerol, malonate, D-mannitol, maleate and D- and L-tryptophan was tested. Chemolithoautotrophic growth of strain EMB320^T with hydrogen gas was tested on medium 81 (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) agar under the conditions described by Malik & Schlegel (1981). More enzyme activities and biochemical features were determined by using API kits (API ZYM, API 20E and API 20NE) as recommended by the manufacturer (bioMérieux).

Strain EMB320^T on R2A agar formed white, glistening, translucent, slightly sticky and slightly raised circular colonies when grown at 30 °C for 5 days. Growth was observed at temperatures between 10 and 35 °C, with an optimum growth temperature of 30 °C. The strain grew at the range of pH 6.0–9.0 with optimum growth between pH 7.0 and 8.0. Cells of the isolate were non-motile rods (0.4–0.6 µm wide and 0.8–2.0 µm long) without flagella (Supplementary Fig. S1, available in IJSEM Online), Gram-negative and oxidase- and catalase-positive. The isolate did not grow on R2A agar with the removal of yeast extract and

peptone under aerobic conditions, but it grew well following the addition of vitamin mixture (Wolin *et al.*, 1963), meaning that it was possible to replace these complex nutrients (yeast extract and peptone) with vitamins. Anaerobic growth was not observed after incubation for 10 days at 30 °C on R2A agar or on R2A agar containing 10 mM nitrate.

Analysis of fatty acid methyl esters was performed according to the instructions of the Microbial Identification System (MIDI Inc.) after cultivation for 5 days on R2A agar at 30 °C. Analyses of polar lipids and isoprenoid quinones were carried out using the methods described by Komagata & Suzuki (1987). Polyamines were analysed using an HPLC (Shimadzu LC-10A) equipped with a fluorescence detector (Shimadzu RF-10AXL) and a reversed-phase column [Akzo Nobel; Kromasil ODS (250 × 4.6 mm)] as described previously (Busse & Auling, 1988; Busse *et al.*, 1997). The DNA G + C content of strain EMB320^T was determined using an HPLC fitted with a reversed-phase column [GROM; GROM-SIL 100 ODS-2FE (250 × 4.6 mm)] according to the method of Tamaoka & Komagata (1984). The major respiratory lipoquinone of strain EMB320^T was ubiquinone-8 (Q-8). The cellular fatty acids of the strain were summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH; 41.8%), C_{16:0} (25.9%), C_{18:1}ω7c (15.8%), C_{18:1}ω7c 11-methyl (4.9%), C_{10:0} 3-OH (3.5%), C_{17:0} (2.0%), C_{15:0} (1.9%), C_{14:0} (1.5%), C_{12:0} (0.9%), C_{18:0} (0.9%) and summed feature 7 (C_{19:0} cyclo ω10c and/or C_{19:1}ω6c; 0.9%). The strain exhibited a polar lipid profile consisting of major lipid phosphatidylethanolamine, moderate amounts of phosphatidylglycerol and diphosphatidylglycerol and a small amount of an unknown amino group-containing lipid (Supplementary Fig. S2). The presence of phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol and the lack of glycolipids were in agreement with the polar lipid characteristics reported for the related species *Polaromonas naphthalenivorans*, *Polaromonas aquatica* and *Xenophilus azovorans*. Strain EMB320^T was characterized by the presence of the betaproteobacteria-specific diamine 2-hydroxyputrescine [55 µmol (g dry weight)⁻¹] and putrescine [16 µmol (g dry weight)⁻¹]. The content of 2-hydroxyputrescine was a little higher than in any other species analysed so far, such as *P. naphthalenivorans* and *P. aquatica* (Busse & Auling, 1988; Kämpfer *et al.*, 2006). Other polyamines were only detected in trace amounts. The G + C content of the genomic DNA of strain EMB320^T was 62.7 mol%. The phenotypic characteristics of strain EMB320^T are presented in the genus and species descriptions and are compared with those of closely related taxa in Table 1.

Sequencing and assembly of the 16S rRNA gene were carried out as described previously (Lane, 1991). The resultant 16S rRNA gene sequence (1457 nucleotides) of strain EMB320^T was compared with available 16S rRNA gene sequences from GenBank using the BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST/>) to determine an approximate phylogenetic affiliation and was aligned with closely related members using the CLUSTAL W software

Table 1. Characteristics of strain EMB320^T (*Caenimonas koreensis* gen. nov., sp. nov.) and related members of the family Comamonadaceae

Data for reference genera were taken from Heulin *et al.* (2003) (*Ramlibacter*), Willems *et al.* (1991) and Yoon *et al.* (2006) (*Variovorax*), Hiraishi *et al.* (1991) and Finneran *et al.* (2003) (*Rhodoferrax*), Ding & Yokota (2004) (*Curvibacter*), Irgens *et al.* (1996), Jeon *et al.* (2004), Kämpfer *et al.* (2006) and Sizova & Panikov (2007) (*Polaromonas*) and Willems *et al.* (1987) (*Xylophilus*) unless indicated. NA, No data available; RQ, rhodoquinone.

Characteristic	Strain EMB320 ^T	<i>Ramlibacter</i>	<i>Variovorax</i>	<i>Rhodoferrax</i>	<i>Curvibacter</i>	<i>Polaromonas</i>	<i>Xylophilus</i>
Morphology	Rods	Rods or cysts	Rods	Curved rods	Curved rods	Rods or cocci	Rods
Flagella	None	None	Peritrichous	One polar	None or polar	None or polar	One polar
Oxidase*	+	+	+	+	+	+	–
Urease*	–	–	+	–	–	–	+
Nitrate reduction*	+	+	+	+	–	+	–
Pigments on R2A*	–	+	+	–	–	–	+
Growth at 4 °C*	–	–	–	–	–	+	–
Chemolithotrophic growth with H ₂ *	–	–	+	–	+	+	–
Glucose fermentation*	–	–	–	+	–	–	–
β-Galactosidase*	–	–	–	+	+	–	–
Hydrolysis of:*							
Aesculin	–	+	–	+	–	+	–
Gelatin	+	–	–	–	–	+	–
Assimilation of:*							
D-Glucose, L-arabinose, D-mannose, D-mannitol	–	–	+	–	–	–	–
Gluconate, adipic acid	–	–	+	–	+	–	–
Quinone(s)	Q-8	NA	Q-8	Q-8, RQ-8	Q-8	Q-8	Q-8, RQ-8
Major cellular fatty acids	16:0, 16:0 ω 7 α , iso-15:0 2-OH, 18:1 ω 7 α	NA	16:0, 16:1, 18:1	16:0, 16:1	16:0, 16:1, 18:1	16:0, 16:1 ω 7 α , 16:1 ω 6 α , 18:1 ω 7 α , 17:0 cyclo†	NA
Major 3-OH acid	10:0	NA	10:0	8:0	8:0	10:0‡	NA
DNA G + C content (mol%)	62.7	67–70	66–69	59–62	62–66	52–63	68–69

*Data obtained in this study for *Ramlibacter tataouinensis* TTB310^T, *Variovorax paradoxus* IAM 12373^T, *Rhodoferrax ferrireducens* T118^T, *Curvibacter delicatus* LMG 4328^T, *Polaromonas naphthalenivorans* CJ2^T and *Xylophilus ampelinus* DSM 7250^T. All of these strains and strain EMB320^T were negative for indole and arginine dihydrolase production and assimilation of *N*-acetylglucosamine, maltose, capric acid, malic acid, trisodium citrate and phenylacetic acid.

†C_{17:0} cyclo is present only in *P. aquatica* CCUG 39402^T (37.3%) and *P. naphthalenivorans* CJ2^T (7.2%) (Jeon *et al.*, 2004; Sizova & Panikov, 2007).

‡Only *P. hydrogenivorans* DSM 17735^T and *P. naphthalenivorans* CJ2^T contain C_{10:0} 3-OH as the major hydroxylated fatty acid (Jeon *et al.*, 2004; Sizova & Panikov, 2007).

(Thompson *et al.*, 1994). Phylogenetic trees were constructed using three different methods, the neighbour-joining (NJ), maximum-likelihood (ML) and maximum-parsimony (MP) algorithms, which are available in the PHYLIP software, version 3.6 (Felsenstein, 2002). Sequence similarity values were computed using Similarity Matrix version 1.1 (Ribosomal Database Project II; <http://35.8.164.52/html/>) (Cole *et al.*, 2003). A bootstrap analysis was performed according to the algorithm of Kimura's two-parameter model (Kimura, 1980) of the NJ method in the PHYLIP package. Comparative analysis of 16S rRNA gene sequences showed that the isolate was closely related to *Variovorax paradoxus* IAM 12373^T, *Curvibacter delicatus* LMG 4328^T, *Curvibacter gracilis* 7-1^T, *Xylophilus ampelinus* DSM 7250^T and *Ramlibacter tataouinensis* TTB310^T, with similarities of

96.8, 96.8, 96.8, 96.7 and 96.0%, respectively. However, the phylogenetic analysis indicated that strain EMB320^T formed a phyletic lineage with the genera *Curvibacter*, *Rhodoferrax* and *Polaromonas* within the family Comamonadaceae with a low bootstrap value (44%), meaning that the phylogenetic topology is not stable (Fig. 1). Additional phylogenetic analyses also showed that the phylogenetic topologies varied remarkably by the addition or removal of just one species. The topologies of phylogenetic trees built using the ML and MP algorithms also supported the notion that there is no genus group that shows a clear phylogenetic relationship with strain EMB320^T in the family Comamonadaceae (Supplementary Fig. S3), which was confirmed using the Ribosomal Database Project Classifier program (Wang *et al.*, 2007).

Fig. 1. Neighbour-joining tree showing phylogenetic relationships based on 16S rRNA gene sequences of strain EMB320^T and related taxa. Bootstrap values are shown as percentages of 1000 replicates when greater than 50 %. *Burkholderia cepacia* ATCC 25416^T was used as an outgroup. Bar, 0.01 changes per nucleotide position.

Description of *Caenimonas* gen. nov.

Caenimonas (Cae'ni.mo'nas. L. n. *caenum* mud, sludge; N.L. fem. n. from Gr. fem. n. *monas* a unit, monad; N.L. fem. n. *Caenimonas* monad isolated from sludge).

Cells are Gram-negative, strictly aerobic, non-motile rods, 0.4–0.6 µm wide and 0.8–2.0 µm long, at 30 °C on R2A agar. Catalase- and oxidase-positive. Nitrate is reduced to nitrite and thiosulfate is oxidized to sulfate. No aerobic chemoautotrophic growth with hydrogen as substrate. Contain phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol and an unknown amino group-containing lipid as polar lipids. The major isoprenoid quinone is ubiquinone-8 (Q-8). Major polyamines are 2-hydroxyputrescine and putrescine. The major fatty acids are C_{16:1}ω7c and/or iso-C_{15:0} 2-OH, C_{16:0} and C_{18:1}ω7c. The DNA G+C content of the type strain of the type species is 62.7 mol% (HPLC). Phylogenetically, the genus belongs to the family *Comamonadaceae*. The type species is *Caenimonas koreensis*.

Description of *Caenimonas koreensis* sp. nov.

Caenimonas koreensis (ko.re.en'sis. N.L. fem. adj. *koreensis* pertaining to Korea, where the type strain was isolated).

Displays the following properties in addition to those given in the genus description. Colonies are white, glistening,

translucent, slightly sticky, raised and circular on R2A agar. Growth occurs optimally at 30 °C and pH 7.0–8.0. Negative for the production of indole, H₂S and acetoin and negative for citrate utilization (API 20E). Hydrolyses urea, gelatin and Tween 20 but does not hydrolyse casein, Tween 80, tyrosine, aesculin or starch. Acid is produced from raffinose, *myo*-inositol, D-lactose, L-arabinose and D-fructose, but not from D-glucose, sorbitol, sucrose, rhamnose, amygdalin, melibiose, D-galactose, D-mannose, D-mannitol, arbutin or salicin. Negative for assimilation of D-glucose, D-mannose, D-mannitol, *N*-acetylglucosamine, maltose, L-arabinose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate and phenylacetic acid (API 20NE). Utilizes D-fructose, D-glucose, malonate and maleate as sole carbon sources, but does not utilize glycerol, D-mannitol or D- or L-tryptophan. Produces alkaline phosphatase, leucine arylamidase and urease, but not lipase (C14), cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, arginine dihydrolase, lysine decarboxylase or ornithine decarboxylase. Weak activity is observed for esterase (C4), esterase lipase (C8), valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and tryptophan deamidase.

The type strain is EMB320^T (=KCTC 12616^T =DSM 17982^T), isolated from activated sludge that performed EBPR.

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