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NOTE

Arenimonas aquaticum sp. nov., a Member of the Gammaproteobacterium, Isolated from a Freshwater Reservoir[§]

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A novel bacterial strain, designated NA-09^T, was isolated from a freshwater sample collected from the Cheonho reservoir, Republic of Korea. Colonies were creamy-white pigmented, translucent, and circular with convex shape. The isolate was Gram-staining negative, strictly aerobic, motile, and rod-shaped. The 16S rRNA gene sequence analysis revealed that strain NA-09^T belonged to the genus *Arenimonas* and showed the highest sequence similarities with *Arenimonas malthae* CC-JY-1^T (95.4%), *A. oryziterrae* YC6267^T (94.9%), *A. composti* P2-12-1^T (94.8%), and *A. donghaensis* H03-R19^T (94.1%). The major fatty acids were iso-$C_{16:0}$ (20.8%), iso-$C_{15:0}$ (16.9%), summed feature 1 (13.2%), and iso-$C_{16:1}\omega7c$ alcohol (10.2%). The major isoprenoid quinone of the isolate was ubiquionone-8. On the basis of the data from the polyphasic characterization, the strain NA-09^T represents a novel species, for which the name *Arenimonas aquaticum* sp. nov. is proposed (type strain NA-09^T =KACC 14663^T =NBRC 106550^T).

Keywords: gammaproteobacteria, *Arenimonas*, freshwater

The genus *Arenimonas* was proposed by Kwon *et al.* (2007) and currently this genus comprises four species with validly published names: *A. composti*, *A. donghaensis*, *A. malthae*, and *A. oryziterrae* (http://www.bacterio.cict.fr/a/arenimonas.html). The genus *Arenimonas* belongs to a member of the class gammaproteobacteria comprising several scientifically and medically important groups of bacteria (Olsen *et al.*, 2005). The genus *Arenimonas* has the following characteristics: cells are aerobic, Gram-staining negative, non-spore-forming, and rod-shaped. The catalase- and oxidase-positive characteristics represent an aerobic metabolism. Nitrate and nitrite are not reduced. Major isoprenoid quinone is ubiquinone-8 and predominant cellular fatty acids are iso-$C_{16:0}$, iso-$C_{15:0}$, and iso-$C_{17:1}\omega9c$ (Kwon *et al.*, 2007). Members of the genus have been isolated from diverse environments, such as oil-contaminated seashore sand (Kwon *et al.*, 2007), compost samples (Jin *et al.*, 2007), diesel-oil-contaminated soil samples (Young *et al.*, 2007), and a field of rice (Aslam *et al.*, 2009). In this study, we report taxonomic characterization of *Arenimonas*-like bacterial strain NA-09^T recovered from a freshwater environment.

Strain NA-09^T was isolated from a freshwater reservoir (36° 49′ N 127° 10′ E) of Cheonan, Korea, using the standard dilution plating technique at 25°C on nutrient agar (NA; BD, USA). After primary isolation and purification, the isolate was cultivated at 25°C on NA and stored at -80°C with 20% (v/v) glycerol. Reference strains, *A. composti* KACC 11312^T, *A. donghaensis* KACC 11381^T, *A. malthae* KACC 14618^T, and *A. oryziterrae* KACC 14607^T were obtained from the Korean Agricultural Culture Collection (KACC, Korea).

Gram staining was performed using a commercial Gram staining kit (Sigma-Aldrich, USA). Cell morphology and dimensions were examined by using phase-contrast microscopy with a Axio Imager (Zeiss, Germany), with the cells grown for 2 days at 25°C on NA medium. Growth was tested on R2A agar (BD, USA), tryptic soy agar (TSA; BD), Luria-Bertani agar (LB; BD), Eosin-methylene blue agar (EMB; BD), and MacConkey agar (BD). Catalase and oxidase activities were determined using 3% (v/v) hydrogen peroxide and Kovacs' reagent. Motility test was carried out on NA medium containing 0.5% of agar. Growth at various temperatures was examined in NA medium at 5–50°C with 5°C intervals. The pH range for growth was determined in NA medium from pH 4.0 to 10.0 (increments of 1 pH units). The requirement for NaCl (1–10%, w/v, with 1% intervals) was tested using NA medium. Hydrolysis of casein, starch, CM-cellulose, chitin, DNA, pectin, Tween 80, tyrosine, xanthine, and hypoxanthine were determined using the methods of Smibert and Krieg (1994). Additional biochemical tests were performed using the API 20NE (bioMérieux, France). Enzyme activity tests were conducted by using the API ZYM galleries, according to the manufacturer's instructions (bioMérieux, France). The API 20NE was read after 24 and 48 h of incubation at 25°C and the API ZYM galleries were read after 3 h of incubation at 37°C. Bacterial genomic DNA was extracted and purified as described (Moore and Dowhan, 1995) and enzymically degraded into

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nucleosides. As a reference strain for G+C content analysis, *E. coli* KACC 10005 was obtained from KACC. Determination of DNA G+C content was carried out by high-performance liquid chromatography analysis of deoxyribonucleosides as described by Mesbah *et al.* (1989), using a reverse-phase column (Supelcosil LC-18 S; Supelco). After incubation for 2 days on NA medium, colonies of strain NA-09^T were creamy white, translucent, circular, and convex shape. Cells were Gram-staining negative, strictly aerobic, motile, and rod-shaped (0.4–0.5 μm wide, 1.4–2.6 μm long). The isolate and reference strains were able to grow on NA, R2A, TSA, LB, and EMB agars, but not on MacConkey agar. The strain NA-09^T grew at 10–45°C (optimum 25°C), at pH 7–9 (optimum at pH 8.0), and in 0–2% NaCl (optimum 0%, w/v). The genomic DNA G+C content of the type strain is 70.0 mol%. Interestingly, the novel bacterial strain NA-09^T has lipase (C14) activity whereas it has not been found in other reference strains. Phenotypic characteristics of strain NA-09^T and closely related species are shown in Table 1.

Genomic DNA preparation, 16S rRNA gene amplification and sequencing reactions for the 16S rRNA gene analysis were performed as previously described (Lee *et al.*, 2009). The resulting 16S rRNA gene sequence (1,456 nucleotides) was compared with 16S rRNA gene sequences available from EzTaxon server ver. 2.1 (Chun *et al.*, 2007). After multiple sequence alignments of data using the software Bioedit version 7.0.9.0 (Hall, 1999), MEGA ver. 5.03 (Tamura *et al.*, 2011) was used to construct phylogenetic trees using the neighbor-joining (NJ) and maximum-parsimony (MP) methods. The robustness of the topologies for the NJ and MP trees was evaluated by means of bootstrap analysis (Felsenstein, 1985) based on 1,000 resamplings of the sequences. The bootstrap analysis was performed according to the Kimura 2-parameter model (Kimura, 1980). Furthermore, we performed a median-joining network approach (Bandelt *et al.*, 1999) as implemented in the software NETWORK version 4.6.0.0 (http://www.fluxus-engineering.com/sharenet.htm) to rigorously examine the phylogenetic relationships of the strain NA-09^T and closely related strains. *Escherichia coli* KCTC 2441^T (GenBank accession no. EU014689) was used as an out-group. The network was generated using

Table 1. Differential characteristics of strain NA-09^T and related members of the genus *Arenimonas*

Strains: 1, strain NA-09^T (this study); 2, *A. malthe* KACC 14618^T (data from Young *et al.*, 2007); 3, *A. donghaensis* KACC 11381^T (data from Kwon *et al.*, 2007); 4, *A. composti* KACC 11312^T (data from Jin *et al.*, 2007); 5, *A. oryziterrae* KACC 14607^T (data from Aslam *et al.*, 2009). CW, creamy-white; TB, transparent to brownish; YW, yellowish-white; +, positive; -, negative; w, weakly positive. All strains were negative for the following characteristics (API 20NE and ZYM): nitrate reduction, indole production, glucose fermentation, L-arginine, urea, esculin ferric citrate, 4-nitrophenyl-βD-galctopyranoside, D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetyl-glucosamine, D-maltose, potassium gluconate, adipic acid, malic acid, trisodium citrate, phenylacetic acid, crystine arylamidase, α-galactosidase, β-glucuronidase, β-glucosidase (naphthol-AS-BI-βD-glucuronide), α-glucosidase, β-glucosidase (6-Br-2-naphthyl-βD-glucopyranoside), N-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase. All strains were positive for alkaline phosphatase, esterase Lipase (C8), and acid phospatase (API ZYM).

Characteristic	1	2	3	4	5
Colonies color	CW	TB	YW	CW	CW
Motility	+	+	-	+	+
Range (optimum) of:					
Temperature (°C)	10–45 (25)	4–50 (28)	15–37 (28)	20–42 (30)	5–45 (30)
NaCl (%, w/v)	0–2 (0)	0–2 (0)	0–3 (1-2)	0–2 (0)	0 (0)
Growth at:					
pH 6.0	-	+	+	+	+
pH 10.0	-	+	+	-	+
Hydrolysis of gelatin (API 20NE)[a]	w	+	+	-	w
Enzymatic activities (API ZYM)[a]					
Esterase (C4)	+	-	+	+	+
Lipase (C14)	+	-	-	-	-
Valine arylamidase	-	-	-	-	+
Trypsin	-	+	-	-	+
α-Chymotrypsin	+	+	-	+	-
Naphtol-AS-BI-phosphohydrolase	+	+	-	+	+
Polar lipids[a,b]					
DPG	+	+	+	-	+
PE	+	+	+	+	+
PG	+	+	+	-	+
PL	+	-	+	-	-
APL	-	-	+	-	-
GE	-	-	-	+	-
PME	+	-	-	+	-
DNA G+C content (mol%)	70.0	70.4	65.0	70.8	65.8

[a] Data from this study.
[b] DPG, Diphosphatidylglycerol; PE, Phosphatidylethanolamine; PG, Phosphatidylglycerol; PL, Phospholipid; APL, Aminolipid; GE, Glycerolether; PME, Phosphatidylmonomethylethanolamine

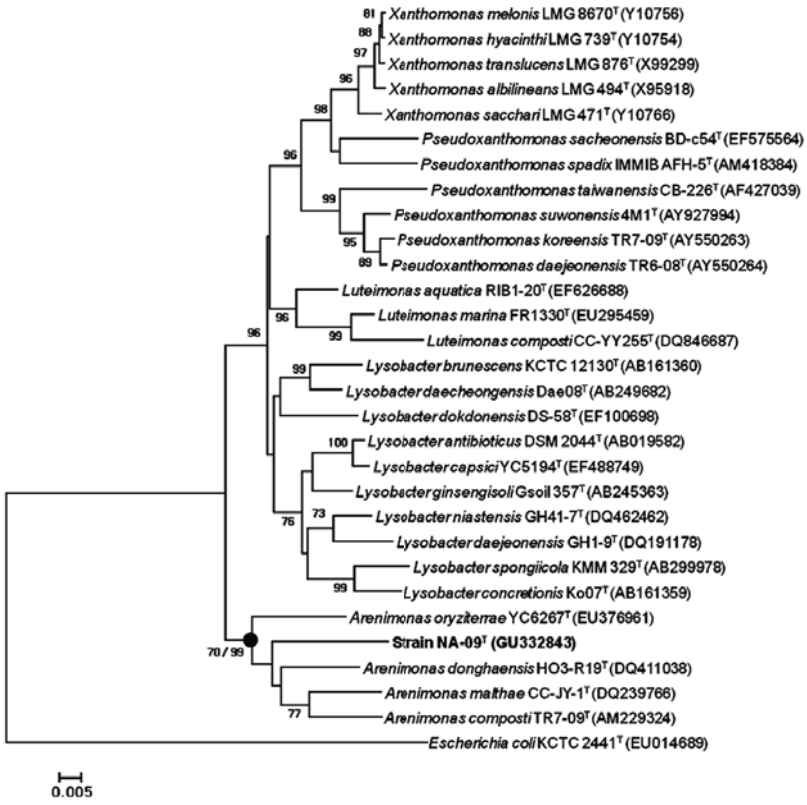

Fig. 1. Neighbor-joining tree showing the phylogenetic position of novel species based on 16S rRNA gene sequences. A filled circle represents that the corresponding branches were also recovered in the maximum-parsimony tree. Evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980). Bootstrap values (>70%) based on 1,000 replications are shown. *E. coli* KCTC 2441^T was used as an out-group. Bar, 0.005 nucleotide substitutions per nucleotide position.

partial 16S rRNA gene sequences (969 bp). The 16S rRNA gene similarity values between the strain NA-09^T and its closest neighbors, *A. malthae*, *A. oryziterrae*, *A. composti*, and *A. donghaensis* were 95.4%, 94.8%, 94.7%, and 94.1%, respectively. A NJ tree showed that strain NA-09^T was placed in the genus *Arenimonas*, and the MP tree showed essentially the same topology (Fig. 1). In addition, we applied the median-joining network method (Bandelt *et al.*, 1999) using partial 16S rRNA gene sequences of five *Arenimonas* species which belong to a single clade (Supplementary data Fig. S1). This network shows the comprehensive sequential order in which the species diversified, according to the number of substitutions. As shown in Supplementary data Fig. S1 the difference between the 16S rRNA gene sequences of strain NA-09^T and *A. oryziterrae* KACC 14607 ^{T} was 60 nucleotides. The result of the network study was congruent with the phylogenetic inference based on the NJ and MP studies.

Fatty acid methyl esters were obtained from fresh wet biomass, grown on R2A at 28°C for two days, by saponification, methylation, and extraction as described by Aslam *et al.* (2009), and the fatty acids were separated, identified, and quantified according to the protocol of the Sherlock Microbial Identification System (MIDI) version and the RTSBA6 6.10 database. The gas chromatograph was Agilent 87 Technologies 7890A GC system. Isoprenoid quinones were analyzed by HPLC as describe by Groth *et al.* (1996). Polar lipids were analyzed according to Minnikin *et al.* (1984). The main isoprenoid quinone was ubiquinone-8. The fatty acid profile of strain NA-09^T was composed of iso-$C_{16:0}$ (20.8%), iso-$C_{15:0}$ (16.9%), summed feature 1 (13.2%), iso-$C_{16:1}\omega7c$ alcohol (10.2%), iso-$C_{14:0}$ (8.0%), iso-$C_{11:0}$ 3-OH (4.9%), iso-$C_{15:1}\omega9c$ (4.0%), iso-$C_{11:0}$ (2.9%), iso-$C_{17:1}\omega9c$ (2.9%), anteiso-$C_{15:0}$ (2.3%), iso-$C_{17:1}\omega10c$ (2.3%), iso-$C_{17:0}$ (2.0%), and summed feature 3 (1.9%). The fatty acid profile of strain NA-09^T was similar to that of *Arenimonas* type species, but it also contains large amounts of a iso-$C_{15:1}\omega9c$, iso-$C_{16:1}\omega7c$ alcohol, iso-$C_{17:1}\omega10c$, and summed feature 1 (Table 2). The following polar lipids are present: diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylmethylethanolamine, and unknown phospholipid (Table 1 and Supplementary data Fig. S2).

Based on the genetic, phenotypic, and chemotaxonomic features, we propose that strain NA-09^T represents a novel species of the genus *Arenimonas*, for which the name *Arenimonas aquaticum* sp. nov. is suggested.

Description of *Arenimonas aquaticum* sp. nov.

Arenimonas aquaticum (a.qua'ti.cum. L. neut. adj. aquaticum, living, growing, or found in or by the water, aquatic).

Cells are Gram-staining negative, motile, catalase- and oxidase-positive, and rod-shaped (0.4–0.5×1.4–2.6 μm). Colonies are circular, convex, translucent and creamy-white on R2A at 25°C for 2 days. Growth occurs at 10–45°C (optimum 25°C), at pH 7.0–9.0 (optimum pH 8.0), and presence of 0–2% (w/v) NaCl. The isolate hydrolyses starch, casein, tyrosine, and Tween 80 but does not hydrolyze xanthine, hypoxanthine, chitin, CM-cellulose, and DNA. In the API 20NE system, reactions are obtained for hydrolysis of gelatin and assimilation of caprate. In the API ZYM strips, the cells are

Table 2. Fatty acid composition (%) of strain NA-09^T and the type strains of members of the genus *Arenimonas*
Strains: 1, strain NA-09^T; 2, *A. malthe* KACC 14618^T; 3, *A. donghaensis* KACC 11381^T; 4, *A. composti* KACC 11312^T; 5, *A. oryziterrae* KACC 14607^T. All data from this study. Fatty acids amounting to less than 0.5% in all strains are not shown. Tr, traces (<0.5%); -, not detected.

Fatty acid	1	2	3	4	5
Saturated					
$C_{14:0}$	Tr	Tr	0.5	Tr	0.6
$C_{16:0}$	Tr	Tr	0.5	Tr	1.0
Branched					
iso-$C_{11:0}$	2.9	2.9	3.0	2.8	-
iso-$C_{13:0}$	1.0	0.5	Tr	Tr	0.6
iso-$C_{14:0}$	8.0	11.5	6.9	8.7	16.8
iso-$C_{15:0}$	16.9	26.5	30.3	27.9	33.3
iso-$C_{15:1}$ F	-	6.0	3.3	-	4.6
iso-$C_{16:0}$	20.8	21.0	24.5	28.0	13.2
iso-$C_{16:0}$ G	-	-	-	0.6	-
iso-$C_{16:1}$ H	0.8	1.9	1.1	1.2	Tr
iso-$C_{17:0}$	2.0	Tr	1.1	1.4	1.6
anteiso-$C_{15:0}$	2.3	1.4	0.7	1.7	2.0
Unsaturated					
$C_{14:1}\omega5c$	Tr	0.6	Tr	Tr	-
$C_{15:1}\omega5c$	0.5	0.9	Tr	1.0	Tr
iso-$C_{15:1}\omega9c$	4.0	-	-	-	-
$C_{16:1}\omega5c$	0.9	0.8	Tr	0.8	Tr
$C_{16:1}\omega7c$ alcohol	10.2	-	-	-	4.0
$C_{16:1}\omega11c$	0.8	-	-	-	Tr
iso-$C_{17:1}\omega9c$	2.9	16.0	17.3	11.3	14.3
iso-$C_{17:1}\omega10c$	2.3	-	-	-	-
Hydroxy					
iso-$C_{11:0}$ 3-OH	4.9	3.7	3.9	4.0	5.7
Summed feature[a]					
1	13.2	2.1	2.2	5.8	-
3	1.9	2.0	2.5	2.5	1.3

[a] Summed features are groups of two fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 1 contains iso-$C_{15:1}$ H and/or $C_{13:0}$ 3-OH. Summed feature 3 contains $C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$.

positive for phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, α-chymotrypsin, acid phosphatase, and Naphtol-AS-BI-phosphohydrolase. The major cellular fatty acids (>10% of the total fatty acids) are iso-$C_{16:0}$, iso-$C_{15:0}$, summed feature 1, and iso-$C_{16:1}\omega7c$ alcohol. The predominant quinone is ubiquinone-8. The polar lipid profile of strain NA-09^T consisted of the predominant compounds diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phospholipid, and phosphatidylmonomethylethanolamine. The G+C content is 70.0 mol%. The type strain, NA-09^T (=KACC 14663^T =NBRC 106550^T), was isolated from a Cheonho reservoir from Cheonan, Korea.

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