Arenibacter echinorum sp. nov., isolated from the sea urchin *Strongylocentrotus intermedius*

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Two marine, heterotrophic, aerobic, pigmented and gliding bacteria, isolated from the sea urchin *Strongylocentrotus intermedius* were subjected to a polyphasic taxonomy study. The 16S rRNA gene sequence analysis revealed that strains KMM 6032^T and KMM 6047 formed a distinct branch within the genus *Arenibacter*, a member of the family *Flavobacteriaceae*. The level of sequence similarity between the novel isolates and members of the genus *Arenibacter* was 94.5–98.9%. The DNA G+C content was 39–40 mol%. The dominant fatty acids were iso-C_{15:1}, iso-C_{15:0}, C_{15:0}, C_{15:1}ω6c, iso-C_{15:0} 3-OH, iso-C_{17:1}ω9c, iso-C_{17:0} 3-OH and summed feature 3 (comprising iso-C_{15:0} 2-OH and/or C_{16:1}ω7c). The results of DNA–DNA hybridization experiments supported by phenotypic data indicated that the isolates represent a novel species within the genus *Arenibacter*, for which the name *Arenibacter echinorum* sp. nov. is proposed. The type strain is KMM 6032^T (=KCTC 22013^T=LMG 22574^T).

The genus *Arenibacter*, a member of the family *Flavobacteriaceae* (Bernardet *et al.*, 2002), was proposed for accommodating Gram-negative, aerobic, heterotrophic and dark-orange pigmented marine bacteria, isolated from a sediment sample, from the edible holothurian *Apostichopus japonicus* and from the brown alga *Chorda filum* (Ivanova *et al.*, 2001). Recently, the description of the genus *Arenibacter* was emended to include new data on gliding motility and halotolerance (Nedashkovskaya *et al.*, 2006). To date, the genus includes four species of marine bacteria, *Arenibacter certesi*, *Arenibacter latericius*, *Arenibacter palladensis* and *Arenibacter troitsensis* (Ivanova *et al.*, 2001; Nedashkovskaya *et al.*, 2003, 2004, 2006).

During a taxonomic survey of microbiota of sea urchins, strains KMM 6032^T and KMM 6047 were isolated from a sea urchin *Strongylocentrotus intermedius* collected in the Troitsa Bay, Gulf of Peter the Great, East Sea. For strain isolation, 0.1 ml homogenates of the sea urchin tissues was transferred onto plates of marine agar 2216 (Difco). After primary isolation and purification, strains were cultivated

at 28 °C on the same medium and stored at –80 °C in marine broth (Difco) supplemented with 20% (v/v) glycerol. The two novel sea urchin isolates were studied using a polyphasic taxonomy approach. Phylogenetic analysis based on 16S rRNA gene sequences indicated that the sea urchin isolates formed a distinct branch within the genus *Arenibacter*. On the basis of the data obtained, we assigned the two isolates to a novel species of the genus *Arenibacter*, for which the name *Arenibacter echinorum* sp. nov. is proposed.

The phylogenetic position of strains KMM 6032^T and KMM 6047 was determined from their almost-complete 16S rRNA gene sequences (1431 and 1430 bp, respectively). Genomic DNA extraction, PCR and sequencing of 16S rRNA genes were performed by using previously described procedures (Cho *et al.*, 2006). The sequences obtained were aligned with those of representative members of selected genera belonging to the family *Flavobacteriaceae* by using PHYDIT version 3.1 (<http://plaza.snu.ac.kr/~jchun/phydit/>). Phylogenetic trees were inferred by using suitable programs of the PHYLIP package (Felsenstein, 1993). Phylogenetic distances were calculated from the two-parameter model (Kimura, 1980), and the trees were constructed on the basis of the neighbour-joining (Saitou & Nei, 1987), maximum-

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains KMM 6032^T and KMM 6047 are EF536748 and EF536749, respectively.

likelihood (Felsenstein, 1993) and maximum-parsimony (Kluge & Farris, 1969) algorithms. Bootstrap analysis was performed with 1000 resampled datasets, using SEQBOOT and CONSENSE programs of the PHYLIP package.

Phylogenetic analysis revealed that strains KMM 6032^T and KMM 6047 were members of the family *Flavobacteriaceae* and formed a distinct branch within the genus *Arenibacter* (Fig. 1). The two isolates shared 99.9% 16S rRNA gene sequence similarity and the levels of sequence similarity between strains KMM 6032^T and KMM 6047 and *A. certesii* KMM 3941^T, *A. latericius* KMM 426^T, *A. palladensis* KMM 3961^T and *A. troitsensis* KMM 3674^T were 94.6–94.7, 94.5–94.6, 98.8–98.9 and 98.8%, respectively.

DNA was isolated by the method of Marmur (1961) and the DNA G+C content was determined by using the thermal denaturation method (Marmur & Doty, 1962). The DNA G+C contents of strains KMM 6032^T and KMM 6047 were 40.0 and 39.2 mol%, respectively. DNA–DNA

hybridization was performed spectrophotometrically and initial renaturation rates were recorded as described by De Ley *et al.* (1970). The level of DNA–DNA reassociation between strains KMM 6032^T and KMM 6047 was 96%, demonstrating that the two novel isolates belong to the same species according to Wayne *et al.* (1987). The levels of DNA–DNA binding between strain KMM 6032^T and *A. palladensis* KMM 3961^T and *A. troitsensis* KMM 3674^T were 34 and 40%, respectively. Based on these data, the sea urchin isolates can be affiliated to the genus *Arenibacter* as a distinct and novel species.

In order to determine its whole-cell fatty acid profile, strain KMM 6032^T was grown at 25 °C for 48 h on marine agar 2216 (Difco). The analysis of fatty acid methyl esters was carried out according to the standard protocol of the Microbial Identification System (Microbial ID). The predominant cellular fatty acids of strain KMM 6032^T were the straight-chain unsaturated and unsaturated and branched-chain saturated and unsaturated fatty acids iso-C_{15:1}, iso-C_{15:0}, C_{15:0}, C_{15:1}ω6c, iso-C_{15:0} 3-OH, iso-C_{17:1}ω9c, iso-C_{17:0} 3-OH and summed feature 3 (comprising iso-C_{15:0} 2-OH and/or C_{16:1}ω7c) (Table 1). The fatty acid composition of strain KMM 6032^T is in accordance with those reported for the species of the genus *Arenibacter* (Nedashkovskaya *et al.*, 2006).

Physiological and biochemical properties of strains KMM 6032^T and KMM 6047 were examined as described by Nedashkovskaya *et al.* (2003, 2004) and using API 20E, API 20NE and API ZYM galleries (bioMérieux) and the Biolog GN2 Microplate system according to the manufacturer's instructions.

Physiological, morphological and biochemical characteristics of the strains studied are listed in the species description and in Table 2. Strains KMM 6032^T and KMM 6047 had many phenotypic features in common with the *Arenibacter* species (Table 2): they were aerobic, rod-shaped and dark-orange pigmented organisms, producing oxidase, catalase and alkaline phosphatase. However, the novel isolates could be distinguished from described *Arenibacter* species by acetoin production, by the absence of α-chymotrypsin activity and the ability to utilize citrate, malate and mannitol. Combinations of several phenotypic traits may be used for the discrimination of strains KMM 6032^T and KMM 6047 from other *Arenibacter* species (Table 2). The novel isolates differ from their close phylogenetic neighbour, *A. palladensis*, by their inability to grow with 10% NaCl, to form acid from D-galactose and N-acetyl-D-glucosamine, and to produce lipase (C14). The isolates are motile by gliding, they grow without NaCl or seawater and with 8% NaCl, and oxidize D-glucose, D-melibiose, L-rhamnose and DL-xylose, in contrast with their other close relative, *A. troitsensis* (Table 2). Gliding motility, requirement of Na⁺ ions for growth, nitrate reduction, urea degradation, acid production from D-galactose and DL-xylose, and chymotrypsin and β-glucuronidase activities clearly differentiate the

Fig. 1. Phylogenetic tree of *Arenibacter* species and related taxa based on the 16S rRNA gene sequences. The tree was constructed using Kimura's two-parameter model and the neighbour-joining algorithm. Identical tree topologies were also obtained using the maximum-likelihood and maximum-parsimony algorithms. The numbers at nodes indicate the levels of bootstrap support (%) when >50. Bar, 0.01 nucleotide substitutions per position.

Table 1. Cellular fatty acid composition (%) of *Arenibacter* species

Taxa: 1, *A. echinorum* KMM 6032^T; 2, *A. certesii* KMM 3941^T; 3, *A. latericius* (four strains; results for the type strain in parentheses); 4, *A. palladensis* KMM 3961^T; 5, *A. troitsensis* KMM 3674^T. All strains were cultivated under the same conditions. Only those fatty acids amounting to more than 1.0% in one of the strains listed are indicated. Summed feature 3 consisted of one or more of the following fatty acids which could not be separated by the Microbial Identification System: iso-C_{15:0} 2-OH and/or C_{16:1}ω7c.

Fatty acid	1	2	3	4	5
iso-C _{15:0}	6.4	7.7	6.9–15.8 (8.1)*	8.7	6.8
anteiso-C _{15:0}	1.8	6.3	4.8–13.5 (9.3)	3.3	3.2
iso-C _{15:1}	13.1	7.2	4.9–14.0 (14.0)	12.7	12.2
anteiso-C _{15:1}	0.5	0.8	0.6–2.9 (2.9)	0.5	0.6
C _{15:0}	22.0	11.5	4.2–16.0 (14.2)	15.0	13.6
C _{15:1} ω6c	4.2	1.9	1.0–2.3 (2.3)	2.6	1.2
iso-C _{16:0}	1.0	1.7	0.5–1.3 (0.5)	0.2	0.3
C _{16:0}	0.6	1.0	1.1–2.7 (1.9)	0.6	1.5
iso-C _{17:1} ω9c	3.9	4.7	2.2–4.6 (2.9)	4.0	5.3
C _{17:1} ω8c	1.1	2.4	0.5–2.0 (1.3)	0.5	0.9
C _{17:1} ω6c	2.9	3.0	0.7–2.9 (2.4)	1.4	1.1
C _{15:0} 2-OH	0.6	0.6	0.7–1.0 (0.6)	0.4	0.4
iso-C _{15:0} 3-OH	3.5	3.5	4.6–5.7 (5.6)	5.3	5.1
C _{15:0} 3-OH	2.0	0.6	0.0–1.4 (0)	2.2	1.6
iso-C _{16:0} 3-OH	1.0	7.2	2.1–5.7 (2.1)	1.6	2.2
C _{16:0} 3-OH	1.0	0.8	0.6–1.3 (1.3)	2.0	2.2
iso-C _{17:0} 3-OH	10.5	13.3	6.9–14.4 (6.9)	17.4	21.9
C _{17:0} 2-OH	0.3	3.8	2.1–5.1 (2.1)	1.0	1.7
Summed feature 3	10.7	13.5	9.8–11.9 (9.8)	11.1	9.6

*The values of fatty acids for *Arenibacter latericius* KMM 426^T are indicated in parentheses.

novel isolates from strains of *A. latericius* and *A. certesii* (Table 2).

Differentiating phenotypic characteristics (Table 2) taken together with genomic distinctiveness support the discrimination of the strains studied from recognized *Arenibacter* species. Consequently, strains KMM 6032^T and KMM 6047 can be considered to be members of a novel species of the genus *Arenibacter*, for which the name *Arenibacter echinorum* sp. nov. is proposed.

Description of *Arenibacter echinorum* sp. nov.

Arenibacter echinorum (e.chi.no'rum. L. gen. pl. n. *echinorum* of sea urchins; N.L. gen. n. *echinorum* bacterium isolated from sea urchins).

The main characteristics are the same as those given for the genus. In addition, cells range from 0.4 to 0.5 µm in width and from 1.5 to 2.7 µm in length and move by means of gliding. On marine agar, colonies are 2–4 mm in diameter, circular, convex with entire edges and dark-orange in

colour. Growth is observed at 4–35 °C and with 0–8 % NaCl. Optimum growth occurs at 24–26 °C and with 1 % NaCl. Tween 40 is hydrolysed. Hydrolysis of Tween 20 is strain-dependent. Agar, casein, gelatin, alginate, starch, Tween 80, DNA, urea, cellulose (CM-cellulose and filter paper) and chitin are not decomposed. Acid is produced from D-cellobiose, L-fucose, D-glucose, D-lactose, maltose, D-melibiose, L-rhamnose, sucrose, DL-xylose and amygdalin, but not from L-arabinose, D-galactose, L-sorbose, N-acetyl-D-glucosamine, citrate, adonitol, dulcitol, glycerol, inositol or mannitol. Acid production from D-raffinose is strain-dependent. L-Arabinose, glucose, lactose, D-mannose, sucrose, N-acetyl-D-glucosamine, citrate, malate and mannitol are utilized, but inositol and sorbitol are not. Nitrate is not reduced. Acetoin (Voges–Proskauer reaction) is produced, but indole and H₂S are not. Arginine dihydrolase, lysine- and ornithine-decarboxylases and tryptophan deaminase activities are absent. In API ZYM strips, esterase lipase (C8), leucine-, valine-, cystine-arylamidases, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α- and β-galactosidases, α- and β-glucosidases, β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase activities are present, but lipase (C14) and α-chymotrypsin activities are absent. Esterase (C4) activity is strain-dependent. In Biolog GN2 Microplates, dextrin, gentiobiose, α-lactose, α-D-lactose, lactulose, D-raffinose, trehalose, turanose, methyl pyruvate and DL-lactic acid are utilized. Utilization of α-D-glucose, methyl β-D-glucoside and α-ketobutyric acid is strain-dependent. The following compounds are not utilized: α-cyclodextrin, D-fructose, D-galactose, Tween 80, adonitol, D-arabitol, glycogen, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, L-glutamic acid, α-D-glucose 1-phosphate, *i*-erythritol, *myo*-inositol, D-psicose, xylitol, mono-methylsuccinate, acetic acid, citric acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, L-glutamic acid, α- and β-hydroxybutyric acids, *p*-hydroxyphenylacetic acid, itaconic acid, α-ketoglutaric acid, α-ketovaleric acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, succinic acid, bromosuccinic acid, succinamic acid, glucuronamide, alaninamide, D-alanine, L-alanyl glycine, L-asparagine, L-aspartic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, L-histidine, L-proline, hydroxy-L-proline, L-leucine, L-ornithine, L-threonine, L-phenylalanine, L-pyroglytamic acid, D- and L-serine, DL-carnitine, γ-aminobutyric acid, uronic acid, inosine, uridine, thymidine, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, glycerol, DL-α-glycerol phosphate and D-glucose 6-phosphate. Susceptible to chloramphenicol, erythromycin, lincomycin and oleandomycin. In addition, the type strain is susceptible to doxycycline, streptomycin and tetracycline. Resistant to ampicillin, benzylpenicillin, gentamicin, kanamycin, carbenicillin, neomycin and polymyxin B. The predominant fatty acids of strain KMM 6032^T are the straight-chain saturated, and unsaturated and branched-chain unsaturated fatty acids C_{15:0} (22.0 %), iso-C_{15:1} (13.1 %), summed feature 3 (10.7 %; comprising

Table 2. Phenotypic characteristics of *Arenibacter echinorum* sp. nov. and other *Arenibacter* species

Taxa: 1, *A. echinorum* KMM 6032^T and KMM 6047; 2, *A. certesii* KMM 3941^T; 3, *A. latericius* (five strains); 4, *A. palladensis* (three strains); 5, *A. troitsensis* KMM 3674^T. All of the strains were positive for the following characteristics: respiratory-type metabolism; oxidase, catalase, acid and alkaline phosphatases, α - and β -galactosidases, α - and β -glucosidases, *N*-acetyl- β -glucosaminidase, esterase lipase (C8), leucine-, valine- and cystine-arylamidases, trypsin, naphthol-AS-BI-phosphohydrolase, α -mannosidase and α -fucosidase activities; growth with 1–6% NaCl and at 10–32 °C; acid formation from maltose, D-cellobiose and sucrose; utilization of L-arabinose, D-glucose, D-lactose and D-mannose; susceptibility to lincomycin and resistance to kanamycin, benzylpenicillin, gentamicin, neomycin and polymyxin B. All of the strains were negative for the following characteristics: requirement for organic growth factors; arginine dihydrolase, lysine- and ornithine-decarboxylases and tryptophan deaminase activities; production of flexirubin-type pigments and indole; degradation of agar, casein, starch, alginic acids, cellulose (CM-cellulose and filter paper) and chitin; acid production from L-arabinose, L-sorbose, adonitol, dulcitol, inositol, mannitol, fumarate and citrate; utilization of adonitol, inositol and sorbitol. +, Positive; –, negative; v, variable (<60% strains demonstrated positive reaction); v⁺, >60% strains showed a positive reaction; v[–], >60% strains showed a negative reaction. The reactions of the type strains are indicated in parentheses. Data from Ivanova *et al.* (2001), Nedashkovskaya *et al.* (2003, 2004, 2006) and this study.

Characteristic	1	2	3	4	5
Gliding motility	+	–	–	+	–
Na ⁺ requirement for growth	–	+	+	–	+
Nitrate reduction	–	+	+	–	+
H ₂ S production	–	–	–	–	+
Acetoin production	+	–	–	–	–
Degradation of:					
Gelatin	–	+	–	–	+
Urea	–	+	+	+	–
Tween 40	+	–	v [–] (–)	–	+
Growth with:					
8% NaCl	+	+	+	+	–
10% NaCl	–	+	–	+	–
Growth at 42 °C	–	+	+	–	+
Acid production from:					
D-Galactose	–	+	+	–	–
D-Glucose	+	–	+	+	–
D-Lactose	+	–	+	+	+
D-Melibiose	+	–	v ⁺ (+)	–	–
D-Raffinose	v (+)	–	+	–	+
L-Rhamnose	+	–	v [–] (+)	+	–
DL-Xylose	+	–	–	+	–
Glycerol	–	+	+	+	–
N-Acetyl- β -glucosamine	–	–	v [–] (+)	+	–
Utilization of mannitol, malate, citrate	+	–	–	–	–
Production of:					
α -Chymotrypsin	–	+	+	–	+
β -Glucuronidase	+	–	–	–	+

Characteristic	1	2	3	4	5
Susceptibility to:					
Ampicillin	–	+	+	–	–
Carbenicillin	–	–	+	–	–
Oleandomycin	+	+	+	–	+
Tetracycline	v (–)	–	–	–	+
DNA G + C content (mol%)	39–40	38	37–38	40	40

iso-C_{15:0} 2-OH and/or C_{16:1}ω7c), iso-C_{17:0} 3-OH (10.5%), iso-C_{15:0} (6.4%), C_{15:1}ω6c (4.2%), iso-C_{17:1}ω9c (3.9%) and iso-C_{15:0} 3-OH (3.5%). The DNA G + C content is 39–40 mol%.

The type strain, KMM 6032^T (=KCTC 22013^T=LMG 22574^T), was isolated from a sea urchin *Strongylocentrotus intermedius* collected in Troitsa Bay, East Sea. Another reference strain is KMM 6047 (=KCTC 22014=LMG 22582).

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