Aestuariicola saemankumensis gen. nov., sp. nov., a member of the family *Flavobacteriaceae*, isolated from tidal flat sediment

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A Gram-negative, non-motile, pleomorphic bacterial strain, designated SMK-142^T, was isolated from a tidal flat of the Yellow Sea, Korea, and was subjected to a polyphasic taxonomic study. Strain SMK-142^T grew optimally at pH 7.0–8.0, 25 °C and in the presence of 2 % (w/v) NaCl. Phylogenetic analyses based on 16S rRNA gene sequences showed that strain SMK-142^T clustered with *Lutibacter litoralis* with which it exhibited a 16S rRNA gene sequence similarity value of 91.2 %. This cluster joined the clade comprising the genera *Tenacibaculum* and *Polaribacter* at a high bootstrap resampling value. Strain SMK-142^T contained MK-6 as the predominant menaquinone and iso-C_{15:0}, iso-C_{15:1} and iso-C_{17:0} 3-OH as the major fatty acids. The DNA G+C content was 37.2 mol%. Strain SMK-142^T was differentiated from three phylogenetically related genera, *Lutibacter*, *Tenacibaculum* and *Polaribacter*, on the basis of low 16S rRNA gene sequence similarity values and differences in fatty acid profiles and in some phenotypic properties. On the basis of phenotypic, chemotaxonomic and phylogenetic data, strain SMK-142^T represents a novel genus and species for which the name *Aestuariicola saemankumensis* gen. nov., sp. nov. is proposed (phylum *Bacteroidetes*, family *Flavobacteriaceae*). The type strain of the type species, *Aestuariicola saemankumensis* sp. nov., is SMK-142^T (=KCTC 22171^T=CCUG 55329^T).

A tidal flat is a part of a coast that is covered with seawater during a flood-tide and left covered with mud or slime at ebb-tide. Tidal flats are recognized as a good environment from which to screen novel and useful micro-organisms (Yoon *et al.*, 2003, 2004; Kim *et al.*, 2005; Lim *et al.*, 2006; Nam *et al.*, 2007). In recent years, bacterial taxa belonging to the phylum *Bacteroidetes* have been isolated from a variety of tidal flats on the west coast of the Korean peninsula (Yi & Chun, 2004; Jung *et al.*, 2005, 2006; Choi & Cho, 2006; Choi *et al.*, 2006; Yoon *et al.*, 2006a). In this study, we describe a bacterial strain, designated SMK-142^T, which was isolated from a tidal flat sediment at Saemankum on the west coast of Korea. Comparative 16S rRNA gene sequence analyses indicated that the strain was phylogenetically affiliated to the family *Flavobacteriaceae* (Bernardet & Nakagawa, 2006; Bowman, 2006), phylum *Bacteroidetes*. The aim of the present work was to determine the exact taxonomic position of strain SMK-142^T by using a polyphasic approach that included the determination of phenotypic properties and a detailed phylogenetic investigation based on 16S rRNA gene sequences.

Strain SMK-142^T was isolated by using the dilution plating technique at 25 °C on marine agar 2216 (MA; Difco). The morphological, physiological and biochemical characteristics of strain SMK-142^T were investigated using routine cultivation on MA at 25 °C. Cell morphology was examined using light microscopy (E600; Nikon) and transmission electron microscopy (CM-20; Philips). Flagellation was investigated by using transmission electron microscopy on cells from an exponentially growing culture that were negatively stained with 1 % (w/v) phosphotungstic acid. Grids were examined after being air-dried. Gliding motility was investigated as described by Bowman (2000). The Gram reaction was determined by using the bioMérieux Gram stain kit according to the manufacturer's instructions. Growth under anaerobic conditions was determined after incubation in a Forma anaerobic chamber on MA and on MA supplemented with 0.1 % (w/v) potassium nitrate, both of which had been prepared under a nitrogen atmosphere. Growth in the absence of NaCl was investigated using trypticase soy broth prepared according to the formula of the Difco medium except that NaCl was excluded. Growth with various NaCl concentrations (0.5 %, w/v; 1.0–7.0 %, w/v, in increments of 1.0 %) was investigated in marine broth 2216 (MB; Difco) and trypticase soy broth (Difco). Growth at various tempera-

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain SMK-142^T is EU239499.

Table 1. Differential phenotypic characteristics of strain SMK-142^T and related genera of the family *Flavobacteriaceae*

Taxa: 1, strain SMK-142^T; 2, *Lutibacter* (data from Choi & Cho, 2006); 3, *Tenacibaculum* (Choi & Cho, 2006; Jung *et al.*, 2006; Sheu *et al.*, 2007); 4, *Polaribacter* (Gosink *et al.*, 1998; Nedashkovskaya *et al.*, 2005; Choi & Cho, 2006; Yoon *et al.*, 2006b). +, Positive reaction; –, negative reaction; v, variable reaction; NA, data not available.

Characteristic	1	2	3	4
Cell morphology	Rods, ovals or cocci	Rods	Rods	Rods or filaments
Gliding motility	–	–	v*	–
Colony				
Colour	Yellow	Yellow	Yellow or pale yellow	Orange, salmon pink
Shape	Entire	Entire	Uneven edge	Entire
Requirement for sea salt	+	+	v	v
Growth at:				
pH 6	+	–	+	NA
25 °C	+	+	+	–
37 °C	–	–	v	–
Oxidase	+	–	+	v
Nitrate reduction	–	–	v	v†
Hydrolysis of:				
Aesculin	–	+	–	v
Gelatin	–	+	v	v
Starch	+	+	v	+
Utilization of:				
Sucrose	–	–	v‡	v
L-Glutamate	–	–	v	v§
L-Leucine	–	+	v§	–§
L-Proline	–	–	v	–§
Citrate	–	+	v	–
Pyruvate	–	+	–	–§
Succinate	–	+	–	–§
Major fatty acids	iso-C _{15:0} , iso-C _{15:1} , iso-C _{17:0} 3-OH	iso-C _{15:0} 3-OH, iso-C _{15:0} , anteiso-C _{15:0} , iso-C _{16:0} 3-OH	C _{16:1} ω7c and/or iso-C _{15:0} 2-OH, iso-C _{15:0} , iso-C _{17:0} 3-OH	iso-C _{15:0} 3-OH, iso-C _{15:0} , iso-C _{15:1} or iso-C _{13:0}
DNA G + C content (mol%)	37.2	33.9	30.0–35.2	30.0–33.2

*All species except *Tenacibaculum skagerrakense* are positive for this characteristic.

†All species except *Polaribacter glomeratus* are negative for this characteristic.

‡Only *T. skagerrakense* is positive for sucrose.

§Only *T. skagerrakense* is weakly positive for L-leucine.

||Data not available for *Tenacibaculum lutimaris* and *Tenacibaculum aestuarii*.

§Data not available for *Polaribacter butkevichii*.

tures (4, 10, 15 and 20 °C and from 25 to 37 °C in 1 °C increments) was measured on MA. The pH range for growth was determined in MB adjusted to various pH values (pH 4.5–9.5, in increments of 0.5 pH units) by the addition of HCl or Na₂CO₃. Catalase and oxidase activities were determined as described by Cowan & Steel (1965). Hydrolysis of casein, starch, Tweens 20, 40, 60 and 80, hypoxanthine, tyrosine and xanthine was tested on MA, using the substrate concentrations described by Cowan & Steel (1965). Hydrolysis of aesculin, gelatin and urea and reduction of nitrate were investigated as described previously (Lányi, 1987) with the modification that artificial seawater was used for the preparation of media. The artificial seawater contained the following (l⁻¹ distilled

water): 23.6 g NaCl, 0.64 g KCl, 4.53 g MgCl₂·6H₂O, 5.94 g MgSO₄·7H₂O and 1.3 g CaCl₂·2H₂O (Bruns *et al.*, 2001). H₂S production was tested as described previously (Bruns *et al.*, 2001). The presence of flexirubin-type pigments was investigated as described by Reichenbach (1992). For analysis of the pigment-absorption spectrum, strain SMK-142^T was cultivated aerobically in the dark at 30 °C in MB. The culture was centrifuged, washed twice using a MOPS buffer (MOPS/NaOH, 0.01 M; KCl, 0.1 M; MgCl₂, 0.001 M; pH 7.5) and disrupted by sonication with a Branson 450 sonifier. After removal of cell debris by centrifugation, the absorption spectrum of the supernatant was examined on a Beckman Coulter DU800 spectrophotometer. Pigments were also extracted using acetone/

methanol (1:1, v/v). Susceptibility to antibiotics was investigated on MA plates by using antibiotic discs containing the following: 100 U polymyxin B, 50 µg streptomycin, 20 U penicillin G, 100 µg chloramphenicol, 10 µg ampicillin, 30 µg cephalothin, 30 µg gentamicin, 5 µg novobiocin, 30 µg tetracycline, 30 µg kanamycin, 15 µg lincomycin, 15 µg oleandomycin, 30 µg neomycin and 100 µg carbenicillin. Growth on various substrates was tested in a basal medium containing 0.2 g NaNO₃, 0.2 g NH₄Cl and 0.05 g yeast extract in 1 l artificial seawater (Bruns *et al.*, 2001) as described by Suzuki *et al.* (2001). Enzyme activities were determined, after incubation for 8 h at 25 °C, by using the API ZYM system (bioMérieux).

Cell biomass for DNA extraction and for isoprenoid quinone analysis was obtained from a culture grown for 3 days in MB at 25 °C. Chromosomal DNA was isolated and purified according to the method described by Yoon *et al.* (1996), with the modification that RNase T1 was used in combination with RNase A to minimize contamination with RNA. The 16S rRNA gene was amplified by using a PCR with two universal primers as described previously (Yoon *et al.*, 1998). Sequencing of the amplified 16S rRNA gene and phylogenetic analysis were performed as described by Yoon *et al.* (2003). Isoprenoid quinones were analysed as described by Komagata & Suzuki (1987), using reversed-phase HPLC. For cellular fatty acid analysis, cell

mass of strain SMK-142^T was harvested after cultivation for 7 days at 25 °C on MA. The fatty acids were extracted and fatty acid methyl esters were prepared according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990). The DNA G+C content was determined using the method of Tamaoka & Komagata (1984) with the modification that DNA was hydrolysed with nuclease P1 (Sigma); the resultant nucleotides were analysed with reversed-phase HPLC.

The morphological, cultural, physiological and biochemical characteristics of strain SMK-142^T are given in the species description (see below) and in Table 1. The sonicated cell extract of strain SMK-142^T showed no absorption maximum, but the acetone/methanol extract had absorption peak maxima at 451 and 477 nm, indicating the presence of carotenoids. The almost-complete 16S rRNA gene sequence of strain SMK-142^T comprised 1482 nt. In the phylogenetic tree constructed using the neighbour-joining algorithm, strain SMK-142^T joined the lineage of *Lutibacter litoralis* with a bootstrap confidence value of 69.6% and this cluster joined the clade comprising the genera *Tenacibaculum* and *Polaribacter* with a bootstrap confidence value of 99.7% (Fig. 1). The same topology was also found in the trees constructed using the maximum-likelihood and maximum-parsimony algorithms (Fig. 1). Strain SMK-142^T exhibited 91.2% 16S rRNA gene

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the positions of strain SMK-142^T and other members of the family *Flavobacteriaceae*. Bootstrap percentages (based on 1000 replications) >50% are shown at branching points. Filled circles indicate that the corresponding nodes were also recovered in the trees generated with the maximum-likelihood and maximum-parsimony algorithms. *Bacteroides fragilis* ATCC 25285^T (GenBank accession no. X83935) was used as an outgroup. Bar, 0.05 substitutions per nucleotide position.

sequence similarity with respect to the type strain of *L. littoralis* and showed less than 91.2 % similarity with respect to the other species used in the phylogenetic analysis. The predominant isoprenoid quinone found in strain SMK-142^T was MK-6, at a peak area ratio of approximately 96 %. The cellular fatty acid profile of strain SMK-142^T is shown in Table 2 together with that of *L. littoralis* CL-TF09^T analysed previously. The major fatty acids (constituting >10 % of total fatty acids) detected in strain SMK-142^T were iso-C_{15:0}, iso-C_{15:1} and iso-C_{17:0} 3-OH. The DNA G + C content of strain SMK-142^T was 37.2 mol%.

The predominant menaquinone (MK-6) detected in strain SMK-142^T was the same as that found in phylogenetically related genera (*Lutibacter*, *Tenacibaculum* and *Polaribacter*) and in all other members of the family *Flavobacteriaceae* of the phylum *Bacteroidetes* (Suzuki *et al.*, 2001; Nedashkovskaya *et al.*, 2005; Bernardet & Nakagawa, 2006; Bowman, 2006; Choi & Cho, 2006; Yoon *et al.*, 2006b; Jung *et al.*, 2006). Phylogenetic analyses based on three different algorithms revealed that strain SMK-142^T clusters with *L. littoralis* (Fig. 1). However, the value for 16S rRNA gene sequence similarity between strain SMK-142^T and *L. littoralis* CL-TF09^T was too low (91.2 %) to allow this novel strain to be assigned to the genus *Lutibacter*. The nature and proportion of the major fatty acids and the differences in several phenotypic properties served to differentiate strain SMK-142^T from members of the genera *Lutibacter*, *Tenacibaculum* and *Polaribacter*, although these differences may have resulted partly from different culture conditions (Tables 1 and 2). On the basis of its phylogenetic distinctiveness and differential chemotaxonomic and phenotypic properties, strain SMK-142^T represents a novel genus and species within the family *Flavobacteriaceae*, phylum *Bacteroidetes*, for which the name *Aestuariicola saemankumensis* gen. nov., sp. nov. is proposed.

Description of *Aestuariicola* gen. nov.

Aestuariicola [Aes.tu.a.ri.i.co'la. L. n. *aestuarium* part of the coast that overflows with seawater during a flood-tide and is left covered with mud or slime at ebb-tide, a tidal flat; L. suff. *-cola* (from L. n. *incola*) a dweller, inhabitant; N.L. masc. n. *Aestuariicola* a dweller in a tidal flat].

Cells are Gram-negative, non-flagellated, non-gliding and pleomorphic. Catalase- and oxidase-positive. Strictly aerobic. The predominant menaquinone is MK-6. The major fatty acids are iso-C_{15:0}, iso-C_{15:1} and iso-C_{17:0} 3-OH. The type species is *Aestuariicola saemankumensis*.

Description of *Aestuariicola saemankumensis* sp. nov.

Aestuariicola saemankumensis (sae.man.kum.en'sis. N.L. masc. adj. *saemankumensis* of Saemankum, from where the organism was isolated).

Table 2. Cellular fatty acid contents (%) of strain SMK-142^T and *L. littoralis* CL-TF09^T

Data for *L. littoralis* CL-TF09^T are from Choi & Cho (2006). Fatty acids that represented <1.0 % in both strains were omitted. Both strains were grown on MA. Strain SMK-142^T was grown for 7 days at 25 °C, whereas strain CL-TF09^T was grown for 1 day at 30 °C. –, Not detected or <1.0 %.

Fatty acid	SMK-142 ^T	CL-TF09 ^T
Straight-chain fatty acids		
C _{15:0}	4.0	1.7
C _{18:0}	–	1.3
Unsaturated fatty acids		
C _{15:1} ω6c	1.9	1.5
C _{17:1} ω6c	2.6	1.0
Branched fatty acids		
iso-C _{13:0}	6.5	–
anteiso-C _{13:0}	0.4	1.4
iso-C _{14:0}	0.7	2.2
iso-C _{15:1} F*	1.2	–
iso-C _{15:1} G*	13.6	4.2
anteiso-C _{15:1} A*	0.7	1.6
iso-C _{15:0}	21.3	16.7
anteiso-C _{15:0}	1.9	15.1
iso-C _{16:1} H*	1.2	1.3
iso-C _{16:0}	1.4	–
iso-C _{17:1} ω9c	3.7	–
Hydroxy fatty acids		
iso-C _{15:0} 3-OH	7.4	17.4
C _{15:0} 2-OH	1.2	2.1
C _{15:0} 3-OH	–	1.5
iso-C _{16:0} 3-OH	3.3	13.4
iso-C _{17:0} 3-OH	12.5	3.9
C _{17:0} 2-OH	0.7	3.6
Summed features†		
1	–	1.1
3	7.6	1.0
4	1.3	–
Unknown fatty acids‡		
ECL 11.543	0.6	2.3
ECL 13.565	1.9	–

*The double-bond position, indicated by a capital letter, is unknown.

†Summed features represent groups of two or three fatty acids which could not be separated by GLC with the MIDI system. Summed feature 1 contained iso-C_{15:1} and/or C_{13:0} 3-OH; summed feature 3 contained C_{16:1}ω7c and/or iso-C_{15:0} 2-OH; summed feature 4 contained iso-C_{17:1} and/or anteiso-C_{17:1}.

‡ECL, Equivalent chain length.

Displays the following properties in addition to those given in the genus description. Cells are rod-shaped, oval or coccoid and 0.3–0.5 µm in diameter and 0.5–7.0 µm in length; cells greater than 10.0 µm in length occur in older cultures. Colonies on MA are circular with entire edges, convex, smooth, glistening, yellow and 1.0–2.0 mm in diameter after 7 days incubation at 25 °C. Optimal growth

occurs at 25 °C. Growth occurs at 4 and 33 °C, but not at 34 °C. The optimal pH for growth is 7.0–8.0; growth occurs at pH 5.5, but not at pH 5.0. Optimal growth occurs in the presence of 2 % (w/v) NaCl; growth occurs in the presence of 0.5–4 % (w/v) NaCl. Carotenoid pigments are produced, but flexirubin-type pigments are not produced. Tweens 20, 40 and 60 are weakly hydrolysed, but casein, hypoxanthine, xanthine, L-tyrosine and Tween 80 are not hydrolysed. H₂S is not produced. Growth occurs on Casamino acids, peptone and tryptone, but not on L-arabinose, cellobiose, D-fructose, D-galactose, D-glucose, maltose, D-mannose, trehalose, D-xylose, acetate, benzoate, L-malate, salicin, formate, L-arginine, L-ornithine, DL-aspartate or DL-cysteine. Susceptible to carbenicillin, cephalothin, chloramphenicol, lincomycin, novobiocin, oleandomycin and tetracycline, but not to ampicillin, gentamicin, kanamycin, neomycin, penicillin G, polymyxin B or streptomycin. In the API ZYM system, alkaline phosphatase, leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase and *N*-acetyl- β -glucosaminidase activities are present, esterase (C4) and esterase lipase (C8) activities are weak and lipase (C14), trypsin, α -chymotrypsin, naphthol-AS-BI phosphohydrolase, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, α -mannosidase and α -fucosidase activities are absent. The major fatty acids (>10 % of total fatty acids) are iso-C_{15:0}, iso-C_{15:1} and iso-C_{17:0} 3-OH. The DNA G+C content is 37.2 mol% (determined by HPLC). Other phenotypic characteristics are given in Table 1.

The type strain, SMK-142^T (=KCTC 22171^T=CCUG 55329^T), was isolated from a tidal flat sediment at Saemankum on the Yellow Sea coast of Korea.

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