Aestuariibacter litoralis sp. nov., isolated from a sandy sediment of the Sea of Japan

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The phenotypic and phylogenetic characteristics of an aerobic, Gram-negative, motile, non-pigmented *Alteromonas*-like bacterium (designated strain KMM 3894^T), isolated from a sandy sediment sample collected offshore of the Sea of Japan, were investigated. Comparative 16S rRNA gene sequence analysis revealed that strain KMM 3894^T belonged to the genus *Aestuariibacter* and was most closely related to *Aestuariibacter halophilus* JC2043^T (95.5% sequence similarity). Fatty acid analysis showed C_{16:1}ω7c, C_{18:1}ω7c, and C_{16:0} as the dominant components. Strain KMM 3894^T could be differentiated from recognized species of the genus *Aestuariibacter* by its ability to grow at 4 °C and at 30 °C, the optimum temperature for growth, and its inability to utilize most carbohydrates. On the basis of the phenotypic, chemotaxonomic and phylogenetic data, strain KMM 3894^T is considered to represent a novel species of the genus *Aestuariibacter*, for which the name *Aestuariibacter litoralis* sp. nov. is proposed. The type strain is KMM 3894^T (=NRIC 0754^T=JCM 15896^T).

The genus *Aestuariibacter* was described by Yi *et al.* (2004) and comprises two species, *Aestuariibacter halophilus* JC2043^T and *Aestuariibacter salexigens* JC2042^T, which were isolated from a getbol sediment sample collected from Ganghwa Island, Korea. Here we report the phenotypic and phylogenetic characterization of a Gram-negative, aerobic bacterium, designated strain KMM 3894^T, which was isolated from a sandy sediment sample collected offshore of the Sea of Japan. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain KMM 3894^T was potentially a novel member of the genus *Aestuariibacter*. Differential phenotypic properties, together with the phylogenetic distinctiveness demonstrated that strain KMM 3894^T differed from other recognized *Aestuariibacter* species. On the basis of the phenotypic and molecular data obtained, a novel *Aestuariibacter* species is described.

Strain KMM 3894^T was isolated from a sandy sediment sample collected offshore of the Sea of Japan (42°44.348' N

133°14.490' E) at a depth 3 m, as described previously (Romanenko *et al.*, 2004). Strain KMM 3894^T was grown aerobically on/in marine 2216 agar (MA; Difco) and marine broth (MB; Difco) or seawater medium (SWM). The SWM medium contained: 5.0 g peptone, 2.5 g yeast extract, 1.0 g glucose, 0.2 g K₂HPO₄, 0.05 g MgSO₄, and 15.0 g agar, 750 ml natural seawater/250 ml distilled water. The sodium ion requirement and tolerance of various NaCl concentrations (0–20%) were examined on SWM prepared with an artificial seawater base (ASW) (Lyman & Fleming, 1940), supplemented with an appropriate amount of NaCl. Sea salts requirement was tested using various concentrations of ASW, supplemented with 10.0 g Bacto peptone l⁻¹, 2.0 g yeast extract l⁻¹, 0.028 g FeSO₄ l⁻¹ and 15.0 g agar l⁻¹. Strain KMM 3894^T was stored at –80 °C in liquid MB supplemented with 30% (v/v) glycerol. Motility was observed by using the hanging drop method as described by Gerhardt *et al.* (1994). Phenotypic properties were tested according to the standard methods described by Smibert & Krieg (1994). The oxidation/fermentation medium of Leifson (1963) for marine bacteria was used to test acid production from carbohydrates, with 1% (w/v) of each compound. Growth at various temperatures and pH values and in the presence of various NaCl concentrations and antibiotic resistance were studied as described previously (Romanenko *et al.*, 2003, 2004). In addition, biochemical tests were carried out using API 20NE, API 20E and API 50 CH test kits (bioMérieux), according to the

Abbreviations: ASW, artificial seawater base; MP, maximum-parsimony; NJ, neighbour-joining; SWM, seawater medium.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain KMM 3894^T is AB473549.

A maximum-parsimony phylogenetic tree based on 16S rRNA gene sequences showing the position of strain KMM 3894^T and related species is available as supplementary material with the online version of this paper.

manufacturer's instructions, except that the culture was suspended in ASW. For comparative fatty acid analysis, strain KMM 3894^T was cultivated on MA at 28 °C for 3 days, and lipids were extracted using the chloroform-methanol extraction method of Bligh & Dyer (1959). Fatty acid methyl esters (FAMES) were obtained by using alkaline methanolysis (15 % NaOH/methanol). The resultant FAMES were extracted with hexane and analysed using a GLC-MS Hewlett Packard model 6890 gas chromatograph equipped with a HP 5 MS 5 % phenyl methyl siloxane capillary column (30 m × 250 µm × 0.25 µm) and connected to a Hewlett Packard model 5973 mass spectrometer. A 16S rRNA gene sequence of 1504 nucleotides was determined for strain KMM 3894^T, as described by Shida *et al.* (1997). The sequence obtained was compared with 16S rRNA gene sequences retrieved from the GenBank/EMBL/DBJ databases by using the FASTA program (Pearson & Lipman, 1988). Phylogenetic analysis of 16S rRNA gene sequences was performed using the software package MEGA 4 (Tamura *et al.*, 2007) after multiple alignment of data by using CLUSTAL_X (version 1.83; Thompson *et al.*, 1997). Phylogenetic trees were constructed by using the neighbour-joining (NJ) and maximum-parsimony (MP) methods and the distances were calculated according to the Kimura two-parameter model. The robustness of the phylogenetic trees was estimated by using a bootstrap analysis of 1000 replicates.

Phylogenetic trees constructed using the NJ and MP methods based on the 16S rRNA gene sequence both gave similar results, as shown, respectively, in Fig. 1 and Supplementary Fig. S1 (available in IJSEM Online). FASTA searches displayed that strain KMM 3894^T belonged to the genus *Aestuuriibacter*; *Aestuuriibacter halophilus* JC2043^T and *Aestuuriibacter salexigens* JC2042^T were most closely related to the novel isolate, with 95.5 and 94.9 % sequence similarities, respectively. Strain KMM 3894^T shared lower sequence similarities with other species, including *Alteromonas genovensis* LMG 24078^T (94.4 %), *Alteromonas hispanica* F-32^T (94.4 %), *Alteromonas stellipolaris* LMG 21861^T (93.9 %) and *Glaciecola chathamensis* S18K6^T (93.8 %). The 16S rRNA gene sequence similarities obtained for strain KMM 3894^T were significantly lower than the threshold similarity value of 97 % proposed by Stackebrandt & Goebel (1994) and re-evaluated as 98.7 % by Stackebrandt & Ebers (2006), indicating that strain KMM 3894^T could be assigned to the genus *Aestuuriibacter* as an individual species. Strain KMM 3894^T contained C_{16:1}ω7c, C_{16:0} and C_{18:1}ω7c as the dominant fatty acids (Table 1), which is in accordance with data reported previously for *Aestuuriibacter* species (Yi *et al.*, 2004). The detailed fatty acid composition of strain KMM 3894^T is given in Table 1. The differential phenotypic features of strain KMM 3894^T and related species of the genus *Aestuuriibacter* are listed in Table 2 and in the species

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences available from the GenBank/EMBL/DBJ databases (accession numbers are given in parentheses) showing the relationship of strain KMM 3894^T and recognized *Aestuuriibacter* species. Phylogenetic analysis was performed using the software package MEGA 4 (Tamura *et al.*, 2007) after multiple alignments of data by using CLUSTAL_X (version 1.83; Thompson *et al.*, 1997). Bootstrap values based on 1000 replications are given as percentages at branch points; only values > 90 % are given. Bar, 0.01 substitutions per nucleotide position.

Table 1. Cellular fatty acid compositions (%) of strain KMM 3894^T and related *Aestuariibacter* species

Strains: 1, KMM 3894^T (*Aestuariibacter litoralis* sp. nov.); 2, *Aestuariibacter salexigens* JC2042^T (data from Yi *et al.*, 2004); 3, *Aestuariibacter halophilus* JC2043^T (Yi *et al.*, 2004). –, Not detected.

Fatty acid	1	2	3
C _{10:0} 3-OH	—	0.6	4.6
C _{12:0}	2.3	1.8	3.0
C _{11:0} 3-OH	—	1.0	0.3
C _{12:1} 3-OH	1.3	2.2	1.1
C _{12:0} 3-OH	1.6	1.0	0.6
C _{14:0}	3.8	3.9	3.1
C _{15:1} ω8c	—	1.0	0.5
C _{15:0}	1.2	3.8	2.4
C _{16:1} ω7c alcohol	—	2.1	0.7
C _{12:0} alde/iso-C _{16:1} I/C _{14:0} 3-OH	—	2.0	1.6
iso-C _{16:0}	—	1.1	0.1
C _{16:1} ω9c	2.0	—	—
C _{16:1} ω7c/iso-C _{15:0} 2-OH	—	27.2	32.5
C _{16:1} ω7c	29.1	—	—
C _{16:0}	17.7	15.8	23.9
iso-C _{17:1} ω5c	—	—	1.6
C _{17:1} ω8c	—	6.8	3.1
C _{17:1} ω6c	3.4	—	—
C _{17:0}	2.7	4.5	2.8
C _{18:1} ω7c	20.8	14.0	11.1
C _{18:0}	1.1	2.9	0.9
C _{19:1} ω6c/C _{19:0} ω10c cyclo/C _{19:0} ω6c	—	1.1	0.3

description. Strain KMM 3894^T could be distinguished from recognized *Aestuariibacter* species in not being able to grow in artificial sea salts-based medium containing <20 % sea salts and the ability to utilize most compounds included in the API 20NE, API 20E and API 50 CH panels and in the optimum temperature for growth. Based on the results obtained it is proposed to assign strain KMM 3894^T to the genus *Aestuariibacter* as representing a novel species, *Aestuariibacter litoralis* sp. nov.

Description of *Aestuariibacter litoralis* sp. nov.

Aestuariibacter litoralis (li.to.ra'lis. L. masc. adj. *litoralis* of the seashore).

Aerobic, Gram-negative, oxidase- and catalase-positive, motile, rod-shaped bacterium (approx. 2 µm in length). Colonies are non-pigmented, opaque, whitish and shiny, with wrinkled surface and regular edges of 2–3 mm in diameter on MA. Growth occurs at 4–40 °C, with optimum growth at 30 °C. Sodium ions are essential for growth. Growth occurs in medium containing 0.5–6 % NaCl without other sea salts added. No growth is observed on media containing 0.5–12 % (w/v) artificial sea salts, but is observed on media containing 20–100 % (w/v) artificial sea salts. pH range for growth is 5.5–10.5, with optimum growth at pH 6.5–8.0. Positive for Tween 80, starch, aesculin and DNA hydrolysis. Negative for casein and

Table 2. Differential phenotypic characteristics of strain KMM 3894^T and recognized *Aestuariibacter* species

Strains: 1, KMM 3894^T (*Aestuariibacter litoralis* sp. nov.; data from this study); 2, *Aestuariibacter salexigens* JC2042^T (Yi *et al.*, 2004); 3, *Aestuariibacter halophilus* JC2043^T (Yi *et al.*, 2004). All strains are positive for motility, nitrate reduction and hydrolysis of aesculin, starch, DNA and Tween 80. All strains are negative for β-galactosidase and hydrolysis of chitin, agar and cellulose. –, Negative; +, positive; NG, no growth; S, slow reaction; W, weakly positive.

Characteristic	1	2	3
Temperature range for growth (°C)	4–40	20–40	15–40
Temperature optimum (°C)	30	35	40
Sea salts range for growth (%)	20–100	1–10	0.5–10
Growth in NaCl (%)*	0.5–6	NG	1–8
Hydrolysis of:			
Casein	—	—	+
Gelatin	S	+	+
Utilization of:			
D-Glucose	—	—	+
N-Acetylglucosamine	—	+	+
Sucrose	—	—	+
Acetate	—	+	+
D-Ribose	—	W	+
D-Sorbitol	—	W	—

*Growth in medium containing NaCl, without the addition of sea salts.

chitin hydrolysis, and for H₂S production. Gelatin is slowly hydrolysed over 4–5 days routinely and in API 20NE, and 2 days in API 20E tests. Does not produce melanin-like pigments or clearance zone on medium containing L-tyrosine. Acid is not produced from D-glucose, D-mannitol, sucrose, D-lactose, maltose, D-galactose, D-mannose, cellobiose, D-xylose, D-sorbitol, L-arabinose or L-rhamnose. In addition, according to API 20NE tests, positive for aesculin hydrolysis and nitrate reduction, and negative for indole production, glucose acidification, arginine dihydrolase, urease production and the p-nitrophenyl α-D-glucopyranoside test, and assimilation of D-glucose, D-mannitol, maltose, L-arabinose, D-mannose, N-acetylglucosamine, D-gluconate, caprate, adipate, L-malate, citrate and phenylacetate. In API 20E tests, positive for trisodium citrate utilization, and negative for ONPG, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, H₂S production, urease, tryptophan deaminase, indole production, acetoin production, and utilization of D-glucose, D-mannitol, inositol, D-sorbitol, L-rhamnose, sucrose, melibiose, amygdalin and D-arabinose. In API 50 CH tests, weakly positive for utilization of potassium 5-ketogluconate and negative for utilization of glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, methyl β-D-xylopyranoside, D-galactose, D-glucose, D-fructose, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl α-D-

mannopyranoside, methyl α -D-glucopyranoside, N-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, D-lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, starch, glycogen, xylitol, gentiobiose, turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, L-arabitol, D-arabitol, potassium 2-ketogluconate and gluconate. Predominant fatty acids are C_{18:1} ω 7c, C_{16:0} and C_{16:1} ω 7c (detailed fatty acid composition of the type strain is given in Table 2). Susceptible to the following antibiotics (content per disc): ampicillin (10 µg), vancomycin (30 µg), gentamicin (10 µg), kanamycin (30 µg), carbenicillin (100 µg), chloramphenicol (30 µg), nalidixic acid (30 µg), oleandomycin (15 µg), ofloxacin (5 µg), polymyxin B (300 U), rifampicin (5 µg), streptomycin (30 µg), erythromycin (15 µg), tetracycline (30 µg), cephalozin (30 µg) and cephalexin (30 µg); and resistant to benzylpenicillin (10 U), lincomycin (15 µg), neomycin (30 µg) and oxacillin (10 µg).

The type strain, KMM 3894^T (=NRIC 0754^T=JCM 15896^T), was isolated from a marine sandy sample, collected offshore of the Sea of Japan, Russia at a depth of 3 m.

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