Gaetbulibacter aestuarii sp. nov., isolated from shallow coastal seawater, and emended description of the genus *Gaetbulibacter*

Seong Chan Park,¹ Han Na Choe,¹ Keun Sik Baik,¹ Kang Hyun Lee² and Chi Nam Seong¹

Correspondence
Chi Nam Seong
scnu@scnu.ac.kr

¹Department of Biology, College of Life Science and Natural Resources, Sunchon National University, Suncheon 540-742, Republic of Korea

²Biological Resource Center, Korea Research Institute of Bioscience and Biotechnology, 52 Oundong, Yuseong, Daejeon 305-333, Republic of Korea

A rod-shaped, yellow and strictly aerobic marine bacterium, designated KYW382^T, was isolated from seawater collected from the South Sea, Republic of Korea. Cells were Gram-negative and catalase- and oxidase-positive. The major fatty acids were iso-C_{15:1} G, iso-C_{15:0}, iso-C_{17:0} 3-OH, iso-C_{15:0} 3-OH and anteiso-C_{15:0}. The DNA G+C content was 32.4 mol%. A phylogenetic tree based on 16S rRNA gene sequences showed that strain KYW382^T constituted an evolutionary lineage within the radiation enclosing the members of the genus *Gaetbulibacter*. The closest neighbour was *Gaetbulibacter saemankumensis* SMK-12^T (96.1% 16S rRNA gene sequence similarity). A number of phenotypic characteristics distinguished strain KYW382^T from the described members of the genus *Gaetbulibacter*. On the basis of the data presented in this study, strain KYW382^T represents a novel species, for which the name *Gaetbulibacter aestuarii* sp. nov. is proposed. The type strain is KYW382^T (=KCTC 23303^T =JCM 17455^T). An emended description of the genus *Gaetbulibacter* is also given.

The genus *Gaetbulibacter* was described by Jung *et al.* (2005) as a new member of the family *Flavobacteriaceae* (Bernardet *et al.*, 2002; Bernardet & Nakagawa, 2006; Reichenbach, 1989) to accommodate Gram-negative, yellow-pigmented, non-flagellated and rod-shaped cells devoid of flexirubin-type pigments (Yang & Cho, 2008). At present, the genus *Gaetbulibacter* comprises two species with validly published names: *Gaetbulibacter saemankumensis* (Jung *et al.*, 2005) isolated from tidal flat sediment and *Gaetbulibacter marinus* (Yang & Cho, 2008) isolated from seawater.

During the course of a study on the microbial diversity of seawater, a yellow-pigmented bacterium, designated KYW382^T, was isolated from seawater samples collected in the shallow coastal region of Gwangyang Bay (34° 54' N 128° 43' E), South Sea, Republic of Korea. Isolation was achieved using the standard dilution-plating technique on marine agar (MA; Becton Dickinson) (Yang *et al.*, 2006) and incubation at 25 °C for 7 days. Strain KYW382^T was routinely cultured on MA and preserved as a suspension in marine broth (MB; Becton Dickinson) containing 20% (w/v) glycerol at –80 °C.

Bacterial DNA preparation and PCR amplification and sequencing of the 16S rRNA gene were carried out as described elsewhere (Chun & Goodfellow, 1995). Identification of phylogenetic neighbours and calculation of pairwise sequence similarities were achieved using the EzTaxon server (<http://www.eztaxon.org/>; Chun *et al.*, 2007). Sequences were aligned using CLUSTAL X (Thompson *et al.*, 1997) and the alignment was refined using PHYDIT version 3.1 (<http://plaza.snu.ac.kr/~jchun/phydit/>). Phylogenetic analysis was performed using PHYLIP (Felsenstein, 1993) and PAUP* 4.0 (Swofford, 1998). Phylogenetic trees were inferred using the maximum-parsimony (Fitch, 1971), neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) algorithms. The distance matrix for the neighbour-joining method was generated according to the model of Jukes & Cantor (1969). The robustness of the topology of the neighbour-joining tree was evaluated by bootstrap analysis (Felsenstein, 1985) with 1000 resamplings.

An almost-complete 16S rRNA gene sequence (1458 nt) was obtained from strain KYW382^T. Preliminary sequence comparison with 16S rRNA gene sequences in GenBank indicated that the isolate was closely related to the genus *Gaetbulibacter*. The sequence from the isolate was then manually aligned with sequences from the genus *Gaetbulibacter*. Strain KYW382^T showed the highest 16S

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain KYW382^T is GU552681.

rRNA gene sequence similarity with *G. saemankumensis* SMK-12^T (96.1%) followed by *G. marinus* IMCC1914^T (95.7%). Sequence similarity with other members of the family *Flavobacteriaceae* was <95.5%. The neighbour-joining tree (Fig. 1) showed that strain KYW382^T formed a distinct lineage in the cluster comprising *G. saemankumensis* SMK-12^T and *G. marinus* IMCC1914^T. The topology of the phylogenetic trees generated with the other algorithms was essentially the same (not shown). On the basis of 16S rRNA gene sequence similarity and phylogenetic inference, it is clear that the isolate represents a novel genomic species within the genus *Gaetbulibacter* (Wayne *et al.*, 1987). Because <97% 16S rRNA gene sequence similarity is indicative of delineation at the species level (Stackebrandt & Goebel, 1994), no DNA–DNA hybridization tests were needed to confirm the definition of a novel species.

Growth was tested on nutrient agar (NA), tryptic soy agar (TSA), R2A agar and glucose-yeast extract agar (GYEA; all from Becton Dickinson). Cell morphology and presence of flagella were examined by light microscopy and transmission electron microscopy (CM-20; Philips) with cells of exponentially growing cultures. Cells of strain KYW382^T, *G. saemankumensis* KCTC 12379^T and *G. marinus* KCCM 42380^T grown on MA at 25 °C for 3 days were used for physiological and biochemical tests. Gliding motility was examined by observing cells grown in wet mounts with phase-contrast microscopy (DS-Fi1; Nikon). Growth at 4 °C, 10–50 °C (at 5 °C intervals), 37 and 42 °C was assessed in MB. Growth at pH 4.0–10.0 was assessed in MB with the pH adjusted using 100 mM acetate buffer,

100 mM NaH₂PO₄/Na₂HPO₄ buffer or 100 mM NaHCO₃/Na₂CO₃ buffer (Yumoto *et al.*, 2004). Growth with 0–10% NaCl (in increments of 1% NaCl; Sigma) was determined in NaCl-free artificial seawater medium (ASW) (per litre distilled water: 5.9 g MgCl₂·6H₂O, 3.24 g MgSO₄·7H₂O, 1.8 g CaCl₂·2H₂O, 0.55 g KCl, 0.16 g NaHCO₃, 0.08 g KBr, 0.034 g SrCl₂·6H₂O, 0.022 g H₃BO₃, 0.008 g Na₂H₂PO₄, 0.004 g Na₂SiO₃, 0.0024 g NaF and 0.0016 g NH₄NO₃) supplemented with 5.0 g peptone and 1.0 g yeast extract (Yang & Cho, 2008). Growth in an anaerobic chamber (CO₂/H₂/N₂, 10:10:80; Sheldon Manufacturing) was assessed on MA for 1 week. Acid production from glucose and maltose was tested as described by Yamaguchi & Yokoe (2000). The presence of flexirubin-type pigments was determined by the bathochromic shift test using 20% (w/v) KOH (Bernardet *et al.*, 2002; McCammon & Bowman, 2000). Catalase and oxidase activities were determined using 3% (v/v) hydrogen peroxide and Kovács' reagent (Kovács, 1956), respectively. Nitrate reduction was tested in MB containing 0.2% KNO₃ (Skerman, 1967). H₂S production was determined on Kligler iron agar (Becton Dickinson) with 2% (w/v) NaCl. Degradation of the following macromolecules was tested on MA at 25 °C for 10 days (w/v; Sigma): casein (1% skimmed milk), chitin (1%), CM-cellulose (1%) and xylan (1%). Degradation was revealed by the formation of clear zones around colonies either directly or after flooding with appropriate stains (Teather & Wood, 1982). Hydrolysis of the following macromolecules was tested as described by Smibert & Krieg (1994), on MA at 25 °C for 10 days (w/v; Sigma): egg yolk (10%), starch (0.2%), Tween 20 (1%) and Tween 80 (1%). Hydrolysis of L-tyrosine (0.5%, w/v; Sigma) was tested on MA (Barrow &

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences (1458 unambiguously aligned nucleotides) showing the phylogenetic position of strain KYW382^T and representatives of the family *Flavobacteriaceae*. Bootstrap values (>70%) based on 1000 resamplings are shown at branch nodes. Filled circles indicate that the corresponding nodes were also recovered in trees generated with the maximum-parsimony and maximum-likelihood algorithms. Bar, 0.01 substitutions per nucleotide position.

Feltham, 1993). DNase activity was determined on DNase test agar (Becton Dickinson) supplemented with 2 % (w/v) NaCl. Other biochemical tests and enzyme activity tests were performed using API 20 NE and API ZYM strips (bioMérieux) and the GN2 MicroPlate kit (Biolog) according to the manufacturers' instructions except that cells were suspended in distilled water supplemented with 2 % (w/v) NaCl. Antibiotic resistance was determined by the disc-diffusion method using commercial antibiotic-impregnated discs (BBL Becton Dickinson). After 7 days of incubation at 25 °C on MA, the results were interpreted according to the guidelines set forth by the CLSI (2003).

Cells of strain KYW382^T were strictly aerobic, Gram-negative, non-motile rods. Detailed results of the physiological and biochemical analyses are given in Table 1 and the species description. Several phenotypic characteristics readily distinguished strain KYW382^T from its closest phylogenetic relatives.

For cellular fatty acid analysis, strain KYW382^T, *G. saemankumensis* KCTC 12379^T and *G. marinus* KCCM 42380^T were grown on MA for 3 days at 25 °C. The fatty acid methyl esters were analysed by GLC and a MIDI aerobe method (Chem Station, version 4.02) according to the instructions of the Microbial Identification System (MIDI, 1999). For G+C content analysis, DNA of strain KYW382^T was prepared in duplicate and analysed by the thermal denaturation method of Marmur & Doty (1962). The fatty acid profile of strain KYW382^T was essentially similar to those of the two *Gaetbulibacter* type strains, although the proportions of iso-C_{15:1} G, iso-C_{15:0} and summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH) were lower in *G. saemankumensis* KCTC 12379^T (Table 2). The DNA G+C content of strain KYW382^T was 32.4±0.4 mol%, a value lower than those reported for the reference strains (Table 1).

The phylogenetic, phenotypic and chemotaxonomic data clearly indicate that strain KYW382^T represents a novel species within the genus *Gaetbulibacter*, for which the name *Gaetbulibacter aestuarii* sp. nov. is proposed. The description of the genus *Gaetbulibacter* is also emended.

Emended description of the genus

Gaetbulibacter Jung *et al.* 2005 emend. Yang and Cho 2008

The description of the genus is as given by Jung *et al.* (2005) and emended by Yang & Cho (2008) with the following modification. The DNA G+C content is 32.4–38.1 mol%.

Description of *Gaetbulibacter aestuarii* sp. nov.

Gaetbulibacter aestuarii (aes.tu.a'ri.i. L. gen. n. *aestuarii* of the shallow coast, from where the type strain was isolated).

Table 1. Differential characteristics of strain KYW382^T and the type strains of species of the genus *Gaetbulibacter*

Strains: 1, *Gaetbulibacter aestuarii* sp. nov. KYW382^T; 2, *G. marinus* KCCM 42380^T; 3, *G. saemankumensis* KCTC 12379^T. All data were obtained in this study unless indicated otherwise. All strains were positive for hydrolysis of aesculin and L-tyrosine and activities of acid phosphatase, alkaline phosphatase, catalase, leucine arylamidase and valine arylamidase. All strains were negative for flexirubin-type pigments, indole production, hydrolysis of casein, chitin, CM-cellulose, DNA, Tween 80, urea and xylan and activities of cystine arylamidase, esterase (C4), esterase lipase (C8), α-fucosidase, α-galactosidase, β-glucuronidase, lipase (C14), α-mannosidase and trypsin.

Characteristic	1	2	3
Facultatively anaerobic	–	–	+
Reduction of nitrate	–	–	+
Acidification of glucose	–	+	+
Gliding motility	–	–	+
Oxidase activity	+	–	+
Hydrolysis of:			
Gelatin	+	–	+
Starch	+	–	+
Tween 20	+	–	+
Enzyme activity (API ZYM)			
N-Acetyl-β-glucosaminidase	+	–	+
α-Chymotrypsin	+	–*	–
β-Galactosidase	+	–	–
α-Glucosidase	–	+	+
β-Glucosidase	+	–*	–
Naphthol-AS-BI-phosphohydrolase	–	+	–
DNA G+C content (mol%)	32.4	38.1†	34.8†

*Results differ from those reported by Yang & Cho (2008) and Jung *et al.* (2005).

†Data from Yang & Cho (2008) and Jung *et al.* (2005).

Cells are Gram-negative, strictly aerobic, non-motile, slender rods (0.3 µm wide and 1.5–4.0 µm long). Colonies on MA are circular with entire margins, convex, smooth, opaque, yellow and approximately 1.0–2.0 mm in diameter after 3 days at 30 °C (pH 7). Growth occurs on MA, but not on GYEA, NA, R2A agar or TSA. Growth occurs with 1–9 % (w/v) NaCl (optimum 2–3 %), at pH 5–8 (optimum pH 7) and at 10–40 °C (optimum 25–35 °C). Oxidase- and catalase-positive. Flexirubin-type pigments are absent (KOH test-negative). Negative for H₂S and indole production, nitrate reduction and acid production from D-glucose and maltose. Aesculin, gelatin, starch, Tween 20 and L-tyrosine are hydrolysed, but CM-cellulose, casein, chitin, DNA, egg yolk, Tween 80 and xylan are not. With API ZYM, N-acetyl-β-glucosaminidase, acid phosphatase, alkaline phosphatase, α-chymotrypsin, β-galactosidase, β-glucosidase, leucine arylamidase and valine arylamidase are present, but cystine arylamidase, esterase (C4), esterase lipase (C8), α-fucosidase, α-galactosidase,

Table 2. Fatty acid compositions of strain KYW382^T and the type strains of species of the genus *Gaetbulibacter*

Strains: 1, *G. aestuarii* sp. nov. KYW382^T; 2, *G. marinus* KCCM 42380^T; 3, *G. saemankumensis* KCTC 12379^T. Data were obtained in this study using cells grown on MA at 25 °C for 3 days. Values are percentages of total fatty acids; fatty acids amounting to <1 % of the total in all strains are not shown. ECL, Equivalent chain-length; tr, trace (<1 %); –, not detected.

Fatty acid	1	2	3
Straight-chain			
C _{15:0}	2.0	3.1	1.8
C _{16:0}	2.2	1.3	2.3
C _{18:0}	1.7	tr	1.1
Branched			
anteiso-C _{15:0}	6.0	3.6	7.9
anteiso-C _{15:1} A	1.8	1.8	1.5
anteiso-C _{17:1} ω9c	–	–	1.2
iso-C _{13:0}	1.2	tr	1.6
iso-C _{14:0}	tr	1.2	tr
iso-C _{15:0}	21.5	22.2	15.3
iso-C _{16:0}	tr	1.6	tr
iso-C _{15:1} G	23.0	26.8	8.0
iso-C _{17:1} ω9c	tr	tr	1.5
Hydroxy			
C _{15:0} 2-OH	tr	tr	1.8
C _{17:0} 2-OH	1.8	1.2	4.1
C _{16:0} 3-OH	tr	tr	1.2
iso-C _{16:0} 3-OH	4.8	7.7	3.6
iso-C _{17:0} 3-OH	12.7	11.2	19.6
iso-C _{15:0} 3-OH	6.8	6.5	6.9
Unknown			
ECL 13.565	3.0	1.8	2.4
ECL 16.582	tr	tr	1.0
Summed feature 3*	3.0	2.9	11.3

*Summed features represent groups of two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 3 consisted of C_{16:1} ω7c and/or iso-C_{15:0} 2-OH.

α-glucosidase, β-glucuronidase, lipase (C14), α-mannosidase, naphthol-AS-BI-phosphohydrolase and trypsin activities are absent. With the Biolog GN2 MicroPlate, acetic acid, L-asparagine, L-aspartic acid, cellobiose, dextrin, D-fructose, D-galactose, gentiobiose, α-D-glucose, α-D-glucose 1-phosphate, glucose 6-phosphate, glutamic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, hydroxy-L-proline, lactose, lactulose, maltose, D-mannose, melibiose, methyl β-D-glucoside, L-ornithine, L-proline, raffinose, L-rhamnose, sucrose, trehalose, turanose and L-threonine are utilized; all other substrates are not utilized. Sensitive to (μg per disc, unless otherwise indicated): ampicillin (10), chloramphenicol (30), erythromycin (15) and penicillin (10 IU), but resistant to amikacin (30), gentamicin (10), kanamycin (30), nalidixic acid (30), polymyxin B (300 IU), streptomycin (10), tetracycline (30) and vancomycin (30). The major fatty acids (>5 % of the total) are iso-C_{15:1} G, iso-C_{15:0},

iso-C_{17:0} 3-OH, iso-C_{15:0} 3-OH and anteiso-C_{15:0}. The DNA G + C content of the type strain is 32.4 mol%.

The type strain is KYW382^T (=KCTC 23303^T =JCM 17455^T), isolated from seawater collected from the South Sea, Republic of Korea.

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