Algoriphagus chungangensis sp. nov., isolated from a tidal flat sediment

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A Gram-stain-negative, non-spore-forming, non-motile, strictly aerobic, rod-shaped bacterial strain, designated CAU 1002^T, was isolated from a tidal flat sediment and its taxonomic position was investigated using a polyphasic approach. Strain CAU 1002^T grew optimally at 30 °C and pH 7.5. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain CAU 1002^T formed a distinct lineage within the genus *Algoriphagus* and was most closely related to *Algoriphagus lutimaris* KCTC 22630^T and *Algoriphagus halophilus* KCTC 12051^T (97.75 and 97.74 % 16S rRNA gene sequence similarity, respectively). The strain contained MK-7 as the major isoprenoid quinone and iso-$C_{15:0}$ and $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH (summed feature 3) as the major fatty acids. The cell-wall peptidoglycan of strain CAU 1002^T contained *meso*-diaminopimelic acids. The major whole-cell sugars were glucose, arabinose, sucrose, and ribose. The polar lipid profile was composed of phosphatidylethanolamine, five unidentified aminolipids, one unidentified aminophospholipid, one unidentified phospholipid, one unidentified aminoglycolipid, one unidentified glycolipid and twelve unidentified lipids. The DNA G+C content of strain CAU 1002^T was 38.0 mol%. On the basis of phylogenetic inference, phenotypic, chemotaxonomic and genotypic data, strain CAU 1002^T should be classified into the genus *Algoriphagus* as a member of a novel species, for which the name *Algoriphagus chungangensis* sp. nov. is proposed. The type strain is CAU 1002^T (=KCTC 23759^T=CCUG 61890^T). The description of the genus *Algoriphagus* is emended.

The genus *Algoriphagus*, a member of the phylum *Bacteroidetes*, was described by Bowman *et al.* (2003). At the time of writing, this genus comprised 19 species with validly published names (http://www.bacterio.cict.fr/). Recently, members of the genus *Algoriphagus* have been isolated from various environmental habitats such as freshwater (Liu *et al.*, 2009; Rau *et al.*, 2012), oil-contaminated soil (Young *et al.*, 2009), a tidal flat sediment (Park *et al.*, 2010) and seawater (Oh *et al.*, 2012). Members of this genus are Gram-negative, rod-shaped, non-motile, non-sporulating, and possess menaquinone-7 (MK-7) as the major respiratory quinone and iso-$C_{15:0}$ as the predominant cellular fatty acid (Yoon *et al.*, 2006). In the course of screening bacteria with biotechnological potential from marine samples, a bacterial strain, designated CAU 1002^T, was isolated from a tidal flat sediment sample collected from Sido Island (37° 32′ 13.05″ N 126° 25′ 07.24″ E) in the Yellow Sea, Republic of Korea. The purpose of the present study was to establish the taxonomic position of this bacterial strain by using a polyphasic approach that included the determination of phenotypic and chemotaxonomic properties, a detailed phylogenetic investigation based on 16S rRNA gene sequences and genetic analysis.

The procedure for isolation of the strain followed that of Gordon & Mihm (1962) using marine agar 2216 (MA; Difco), supplemented with cycloheximide (50 mg l^{-1}) and nalidixic acid (20 mg l^{-1}). The crushed sample was diluted with sterile saline solution. Serial dilutions were spread on MA plates, and incubated aerobically at 30 °C for 3 days. Pure cultures of isolates were preserved at −70 °C in marine broth (MB; Difco) supplemented with 25 % (v/v) glycerol. The type species of three closely related species were used as reference strains for phenotypic, chemotaxonomic and genotypic analysis. *Algoriphagus lutimaris* KCTC 22630^T, *Algoriphagus halophilus* KCTC 12051^T, and *Algoriphagus*

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CAU 1002^T is JN967625.

Four supplementary figures are available with the online version of this paper.

ratkowskyi DSM 22686T (type species of the genus) were obtained from the Korean Collection for Type Cultures (KCTC; Taejon, Korea) and the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany), respectively.

To investigate morphological, physiological, and biochemical characteristics, strain CAU 1002^T was cultivated routinely on MA at 30 °C except for spore-formation assay where nutrient sporulation medium (NSM) was used to induce the production of spores (Nicholson & Setlow, 1990). After 5 days of growth, spore formation was determined by staining with malachite green as described previously by Conn *et al.* (1957). Cell morphology was examined by light microscopy (DM 1000; Leica), scanning electron microscopy (SEM; JSM-5410LV; JEOL), and transmission electron microscopy (TEM; JEM 1010; JEOL) using cells from an exponentially growing culture. For TEM, cells were negatively stained with 2 % (w/v) uranyl acetate acid and the grids were examined after being air-dried. Gram staining was carried out using the bioMérieux Gram-staining kit according to the manufacturer's instructions. Motility was checked using a hanging-drop method (Schaal, 1986). Gliding motility was assessed using the hanging-drop method.

Growth in MB at 4, 10, 30, 37, 45, and 55 °C in an aerobic incubator (MIR-253; Sanyo) and in an anaerobic chamber (Bactron; Sheldon) was evaluated by measuring the turbidity of the broth after 72 h. Growth was tested at 30 °C in MB adjusted to pH 6.0–10.0 in increments of 0.5 pH unit by using sodium acetate/acetic acid and Na_2CO_3 buffers. Growth in the absence of NaCl and in the presence of 0–15.0 % (w/v) NaCl at 1 % intervals was investigated at 30 °C in MB prepared according to the formula of the Difco medium except that NaCl was excluded and that 0.45 % (w/v) $MgCl_2 . 6H_2O$ or 0.06 % (w/v) KCl was added.

Catalase activity was determined by bubble production in a 3 % (v/v) hydrogen peroxide solution. Oxidase activity was tested by means of the oxidation of 1 % (w/v) tetramethyl-*p*-phenylenediamine (Cappuccino & Sherman, 2002). Hydrolysis of casein, gelatin, starch and Tween 80, and nitrate reduction were determined according to Lányí (1987) and Smibert & Krieg (1994). Acid production from carbohydrates, enzyme activity, and other physiological and biochemical features were tested using the API 20E and API ZYM systems (bioMérieux), and the GN2 MicroPlate (Biolog) according to the manufacturers' instructions. API 20E and API ZYM strips were read after 24 h and 6 h, respectively. GN2 MicroPlates were read after 24 h and 48 h.

For determination of fatty acid composition, the physiological age of the biomasses harvested for fatty acid analysis was standardized by observing growth development during incubation of the plates and choosing the moment of harvesting according to a standard MIDI protocol (Sherlock Microbial Identification System version 6.1). The cell mass of strain CAU 1002^T, *A. ratkowskyi* DSM 22686^T, *A. lutimaris* KCTC 22630^T, and *A. halophilus* KCTC 12051^T was harvested from MA after cultivation for 72 h at 30 °C. Cellular fatty acid methyl esters (FAMEs) were obtained as previously described (Minnikin *et al.*, 1980) and separated by an automated gas chromatography system (6890N and 7683 autosampler; Agilent). Peaks were identified by using the Microbial Identification software package (MOORE library ver. 5.0; MIDI database TSBA6). The polar lipids of strain CAU 1002^T and the three reference strains were identified by using two-dimensional thin-layer chromatography (TLC) according to the method of Minnikin *et al.* (1984). The plate were sprayed with 10 % ethanolic molybdatophosphoric acid (for total lipids), molybdenum blue (for phospholipids), ninhydrin (for aminolipids), and α-naphthol/sulphuric acid reagent (for glycolipids) (all detection reagents from Sigma-Aldrich). The following three analyses were performed on strain CAU 1002^T only: menaquinones were analysed as described previously (Komagata & Suzuki, 1987) using reverse-phase HPLC; whole cell sugars were analysed by TLC according to the method of Komagata & Suzuki (1987); peptidoglycan was analysed as described by Schleifer (1985), with the modification that cellulose sheets were substituted for paper chromatography.

Genomic DNA of strain CAU 1002^T was isolated by the method of Marmur (1961). The 16S rRNA gene was amplified by PCR following established procedures (Nam *et al.*, 2004). The amplified 16S rRNA gene was sequenced directly using a BigDye Terminator Cycle Sequencing kit (Applied Biosystems) and an automatic DNA sequencer (3730; Applied Biosystems). Multiple alignments of the 16S rRNA gene sequences of strain CAU 1002^T with the 16S rRNA gene sequences of a broad selection of members of the genus *Algoriphagus* and calculation of sequence similarity levels were carried out by using the EzTaxon server (http://147.47.212.35:8080/) and CLUSTAL_X (Thompson *et al.*, 1997). Evolutionary distance matrices were generated by the neighbour-joining method described by Jukes & Cantor (1969). Phylogenetic trees were constructed using the neighbour-joining (Saitou & Nei, 1987), least-squares (Fitch & Margoliash, 1967), and maximum-likelihood (Felsenstein, 1981) algorithms in the PHYLIP software package (Felsenstein, 1989). Tree topology was evaluated by the bootstrap resampling method (Felsenstein, 1985) with 1000 replicates of the neighbour-joining dataset using the SEQBOOT and CONSENSE programs from the PHYLIP package. The extent of DNA–DNA relatedness between isolate CAU 1002^T and two closely related species, *A. lutimaris* KCTC 22630^T and *A. halophilus* KCTC 12051^T was estimated using the fluorometric microplate method (Ezaki *et al.*, 1989), as modified by Goris *et al.* (1998). The G+C content of the genomic DNA was determined using HPLC by the method of Tamaoka & Komagata (1984) with the modification that DNA was hydrolysed and the resultant nucleotides were analysed by reversed-phase HPLC.

The morphological, cultural, physiological, and biochemical characteristics of strain CAU 1002^T are given in Table 1

Table 1. Differential properties of strain CAU 1002^T and type strains of the most closely related species of the genus *Algoriphagus*

Strains: 1, CAU 1002^T; 2, *A. ratkowskyi* DSM 22686^T; 3, *A. lutimaris* KCTC 22630^T; 4, *A. halophilus* KCTC 12051^T. Data from Bowman *et al.* (2003), Nedashkovskaya *et al.* (2007), Park *et al.* (2010), Yi & Chun (2004), and this study. +, Positive; −, negative; W, weakly positive, ND, not determined.

Characteristic	1	2	3	4
Isolation source	Tidal flat sediment	Sea ice	Tidal flat sediment	Seawater and algae
Cell morphology	Rod	Rod	Rod	Rod
Colony colour	Scarlet	Pink	Reddish orange	Pink
Production of catalase	+	−	+	+
Temperature for growth (°C)				
Range	10–45	−2–25	4–40	5–40
Optimum	30	16–19	30	35
pH for growth				
Range	5.5–9.5	ND	7–8	6–13
Optimum	7.5	ND	5	7
NaCl tolerance for growth (%)				
Range	0–15	0.1–1.0	0–8.0	1–10
Optimum	2	ND	2	5
Hydrolysis of:				
Casein	−	+	+	−
Gelatin	+	−	+	+
Starch	−	W	−	+
Tween 80	−	−	+	+
Utilization of:				
Inositol	−	+	−	−
Malonate	−	+	−	−
Mannitol	−	+	−	−
Sorbitol	−	+	−	−
Acid production from:				
L-Arabinose	W	+	−	−
Cellobiose	+	+	−	−
D-Fructose	+	+	−	+
D-Galactose	−	+	+	−
Lactose	+	−	−	−
Maltose	−	+	+	+
Melibiose	W	+	−	−
Raffinose	+	+	−	−
L-Rhamnose	−	+	−	−
Sucrose	−	−	−	+
Trehalose	W	−	+	−
D-Xylose	−	+	−	+
Enzyme activity				
Alkaline phosphatase	W	W	+	+
Esterase (C4)	+	+	−	+
Valine arylamidase	+	−	−	−
Trypsin	+	−	+	+
α-Chymotrypsin	+	W	+	+
Naphthol-AS-BI phosphohydrolase	W	−	W	−
β-Galactosidase	−	−	−	+
α-Glucosidase	−	−	−	+
β-Glucosidase	−	−	−	+
N-Acetyl-β-glucosaminidase	−	−	+	−
DNA G+C content (mol%)	38.0	35–37	41.4	37

and in the species description. Overall, the results obtained in this study were in agreement with previously published data for recognised species of the genus *Algoriphagus*. However, strain CAU 1002^T differed from its closest relatives, *A. lutimaris* KCTC 22630^T, *A. halophilus* KCTC 12051^T (Table 1), and from the type species of the genus, *A.*

ratkowskyi DSM 22686^T (Bowman *et al.*, 2003), by its colony colour, production of catalase, and some physiological, and biochemical characteristics.

Colonies of strain CAU 1002^T were scarlet and convex with entire margins and a diameter of 0.6–0.8 mm after 3 days cultivation at 30 °C on MA. No colonies formed under anaerobic growth conditions. Cells of strain CAU 1002^T were Gram-stain-negative and rod-shaped, approximately 0.4–0.6 μm wide and 1.0–2.2 μm long (Fig. S1 available in IJSEM online). Strain CAU 1002^T was non-motile with cells lacking flagella (Fig. S2). The bacterium grew at temperatures between 10 °C and 45 °C (optimum 30 °C) and at pH 4.5 to pH 10.0 (optimum pH 7.5). Growth was observed at salt concentrations ranging from 0–15.0 % (w/v) NaCl, with optimal growth on media containing 2 % (w/v) NaCl.

The cellular fatty acid profile of strain CAU 1002^T contained branched-chain, unsaturated and hydroxy fatty acids (Table 2). The major (>5 %) fatty acids were iso-$C_{15:0}$, summed feature 3 ($C_{16:1}\omega7c$/iso-$C_{15:0}$ 2-OH), summed feature 9 (iso-$C_{17:1}\omega9c$/10-methyl $C_{16:0}$) and minor amount of iso-$C_{16:0}$, iso-$C_{17:0}$ 3-OH, $C_{16:1}\omega5c$, anteiso-$C_{15:0}$, summed feature 4 ($C_{17:1}$ iso I/$C_{17:1}$), iso-$C_{15:0}$ 3-OH, $C_{17:1}\omega6c$, $C_{16:0}$ 3-OH, iso-$C_{16:1}$ H, $C_{15:1}\omega6c$, and iso-$C_{17:0}$ were also present. Overall, the results obtained in this study are in agreement with previously published data for ten species of the genus *Algoriphagus* (Liu *et al.*, 2009; Nedashkovskaya *et al.*, 2004, 2007; Park *et al.*, 2010; Van Trappen *et al.*, 2004; Yoon *et al.*, 2005a, b, 2006), but differed from those of the type species of the genus, *A. ratkowskyi* DSM 22686^T (Bowman *et al.*, 2003). Phosphatidylethanolamine was the only polar lipid identified in strain CAU 1002^T. The other unidentified polar lipids were five unidentified aminolipids, one unidentified aminophospholipid, one unidentified phospholipid, one unidentified aminoglycolipid, one unidentified glycolipid and 12 unidentified lipids (Fig. S3). The peptidoglycan of strain CAU 1002^T contained *meso*-diaminopimelic acid, and menaquinone-7 (MK-7) was the major respiratory quinone. TLC analysis of whole-cell hydrolysates of strain CAU 1002^T revealed the presence of glucose, arabinose, sucrose and ribose.

The almost complete 16S rRNA gene sequence of strain CAU 1002^T (1430 bp) was determined and compared with the corresponding sequences of other bacterial strains in the GenBank database. Phylogenetic analysis indicated that the strain fell into the genus *Algoriphagus* (Fig. 1) Pairwise analysis showed that the most closely related strains were *A. lutimaris* KCTC 22630^T (97.75 % 16S rRNA gene sequence similarity), *A. halophilus* KCTC 12051^T (97.74 %), *A. antarcticus* LMG 21980^T (96.40 %), and *A. ratkowskyi* DSM 22686^T (96.12 %). The mean DNA–DNA relatedness values and standard deviations determined between strain CAU 1002^T and closely related strains, *A. lutimaris* KCTC 22630^T and *A. halophilus* KCTC 12051^T were 28.0 % ± 2.9 and 26.9 % ± 2.6, respectively. These values are below the 70 % cut-off point recommended by Wayne *et al.* (1987) for the delineation of a genomic species, supporting the proposal that strain CAU 1002^T is a separate species. The genomic DNA of strain CAU 1002^T had a G+C content of 38.0 mol%.

Table 2. Cellular fatty acid content (%) of strain CAU 1002^T, and type strains of the most closely related species of the genus *Algoriphagus*

Strains: 1, CAU 1002^T; 2, *A. ratkowskyi* DSM 22686^T; 3, *A. lutimaris* KCTC 22630^T; 4, *A. halophilus* KCTC 12051^T. All data from this study. Only those fatty acids amounting to >1.0 % in all strains are shown; –, not detected.

Fatty acid	1	2	3	4
Saturated				
$C_{16:0}$	–	1.56	–	2.67
$C_{18:0}$	–	–	–	1.89
Unsaturated				
$C_{15:1}\omega6c$	1.08	1.72	–	–
$C_{16:1}\omega5c$	3.46	7.00	4.40	3.96
$C_{17:1}\omega6c$	2.59	–	3.42	2.96
Branched-chain				
anteiso-$C_{11:0}$	–	1.54	1.86	1.92
anteiso-$C_{15:0}$	3.21	4.62	3.35	2.55
iso-$C_{14:0}$	–	1.49	–	–
iso-$C_{15:0}$	26.23	33.49	24.38	21.89
iso-$C_{16:0}$	5.83	5.56	4.72	5.16
iso-$C_{17:0}$	1.05	–	–	–
iso-$C_{16:1}$ H	1.92	2.88	1.47	1.95
iso-$C_{15:0}$ 3-OH	2.60	1.71	1.54	2.60
iso-$C_{16:0}$ 3-OH		2.73		1.54
iso-$C_{17:0}$ 3-OH	5.28	6.50	5.86	6.92
Hydroxy				
$C_{16:0}$ 3-OH	2.12	1.46	–	–
Summed feature*				
3	26.59	20.54	26.28	23.68
4	3.12	1.17	3.26	3.89
8	–	–	–	1.52
9	11.16	1.94	10.47	8.46

*Summed features represent two or more fatty acids that could not be separated by GLC with the MIDI system; summed feature 3 comprises $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH; summed feature 4 comprises $C_{17:1}$ iso I and/or $C_{17:1}$; summed feature 8 comprises 18 : 1$\omega7c$ and/or 18 : 1$\omega6c$; summed feature 9 comprises iso-$C_{17:1}\omega9c$ and/or 10-methyl $C_{16:0}$.

These data together provide sufficient evidence to support the proposal to recognize strain CAU 1002^T as a novel species of the genus *Algoriphagus*, for which the name *Algoriphagus chungangensis* sp. nov. is proposed, and the genus description is emended

Emended description of the genus *Algoriphagus* Bowman *et al.* 2003, emend. Nedashkovskaya *et al.* 2004, emend. Nedashkovskaya *et al.* 2007

This description is as given by Bowman *et al.* (2003) and Nedashkovskaya *et al.* (2004, 2007) with the following

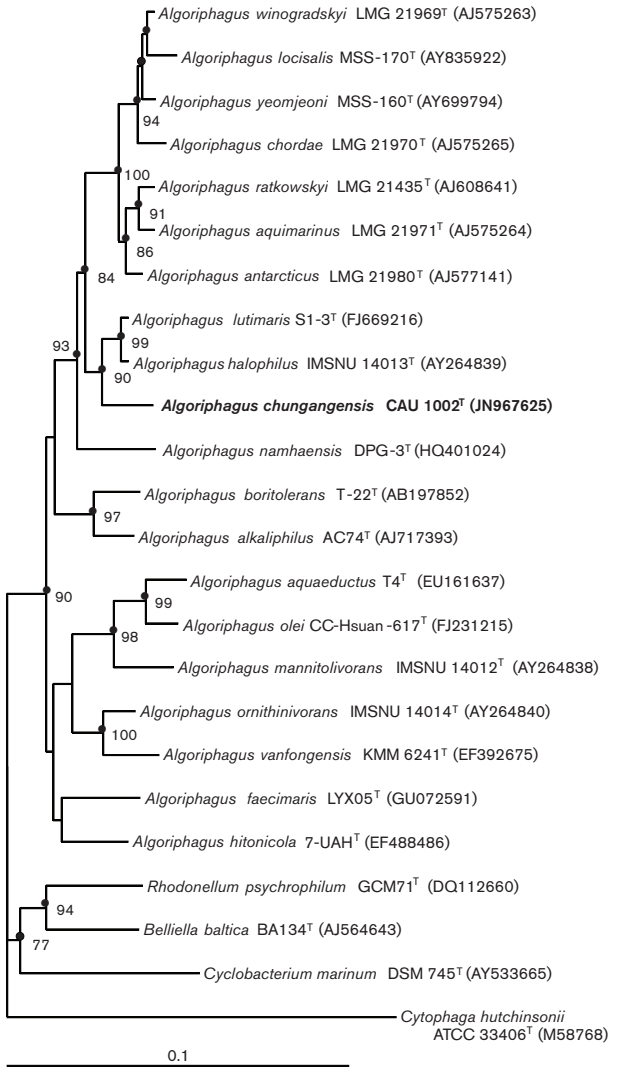

Fig. 1. Neighbour-joining phylogenetic tree based on almost complete 16S rRNA gene sequences showing the relationships between strain CAU 1002^T and recognized species of the genus *Algoriphagus*. Filled circles indicate that the corresponding nodes were also recovered in the trees generated with the maximum-likelihood and least-squares algorithms (Fig. S4). Numbers at nodes indicate levels of bootstrap support based on a neighbour-joining analysis of 1000 resampled datasets; only values >70 % are given. Bar, 0.1 substitutions per nucleotide position.

amendments. Unidentified aminolipids, aminophospholipid, phospholipid, aminoglycolipid, glycolipid and lipids may also occur. The whole-cell hydrolysate contains glucose, arabinose, sucrose and ribose.

Description of *Algoriphagus chungangensis* sp. nov.

Algoriphagus chungangensis (chung.ang.en′sis. N.L. masc. adj. *chungangensis* belonging to Chung-Ang University, where the taxonomic studies on this species were performed).

Cells are Gram-stain-negative, non-motile, strictly aerobic rods approximately 0.4–0.6 μm in diameter and 1.0–2.2 μm in length. Colonies on MA are scarlet and convex with entire margins after 3 days of incubation at 30 °C. Growth occurs at 10–45 °C (optimum, 30 °C) and at pH 4.5–10.0 (optimum, pH 7.5). NaCl is not required for growth but up to 15.0 % (w/v) NaCl is tolerated. Catalase- and oxidase-positive. Hydrolyses gelatin, but not casein, starch or Tween 80. Nitrate is reduced to nitrite. Acid is produced from α-cyclodextrin, cellobiose, D-fructose, α-D-glucose, lactose, methyl β-D-glucoside, raffinose, succinic acid monomethyl ester, succinic acid, hydroxyl-L-proline, L-ornithine, and weak for L-arabinose, α-lactose, D-mannose, melibiose, trehalose, acetic acid, α-ketoglutaric acid, succinamic acid, glycyl L-glutamic acid, L-proline, L-threonine, and putrescine. Positive for enzyme activities of esterase (C4), leucine arylamidase, valine arylamidase, trypsin, α-chymotrypsin, α-chymotrypsin, and weakly positive for alkaline phosphatase and naphthol-AS-BI-phosphohydrolase, but negative for esterase lipase (C8), cystine arylamidase, acid phosphatase, α-galactosidase, β-glucuronidase, β-glucosidase, α-glucosidase, β-galactosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase activities. The major isoprenoid quinone is MK-7. The whole-cell hydrolysate contains glucose, arabinose, sucrose and ribose. The cell-wall peptidoglycan contains *meso*-diaminopimelic acid. The polar lipid pattern consists of phosphatidylethanolamine, five unidentified aminolipids, one unidentified aminophospholipid, one unidentified phospholipid, one unidentified aminoglycolipid, one unidentified glycolipid and 12 unidentified lipids. The predominant cellular fatty acids (>20 % of the total fatty acids) were iso-$C_{15:0}$ and $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH (summed feature 3).

The type strain, CAU 1002^T (=KCTC 23759^T=CCUG 61890^T), was isolated from a tidal flat sediment sample collected from Sido in the Yellow Sea, Republic of Korea (37° 32′ 13.05″ N, 126° 25′ 07.24″ E). The DNA G+C content of the type strain was 38.0 mol%.

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