Maribacter arenosus sp. nov., isolated from marine sediment

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A Gram-staining-negative, non-spore-forming, non-gliding, aerobic bacterial strain, designated CAU 1321^T was isolated from marine sediment in Incheon, South Korea. The strain's taxonomic position was investigated using a polyphasic approach. CAU 1321^T grew optimally at 30 °C and at pH 8 in the presence of 1 % (w/v) NaCl. Phylogenetic analysis based on the 16S rRNA gene sequence revealed that CAU 1321^T represented a member of the genus *Maribacter*, closely related to *Maribacter polysiphoniae* KCTC 22021^T (similarity 98.0 %). The strain contained MK-6 as the predominant menaquinone and iso-$C_{15:0}$, iso-$C_{17:0}$ 3-OH, and iso-$C_{15:1}$ G as the major fatty acids. The polar lipids of CAU 1321^T consisted of phosphatidylethanolamine, one unidentified aminoglycolipid, five unidentified aminolipids, two unidentified aminophospholipids, two unidentified glycolipids and three unidentified lipids. The DNA G+C content was 40.6 mol% and the level of DNA–DNA relatedness between CAU 1321^T and the most closely related strain, *M. polysiphoniae* KCTC 22021^T was 25.2 %. On the basis of phenotypic and chemotaxonomic properties, and phylogenetic inference, CAU 1321^T is considered to represent a novel species of the genus *Maribacter*, for which the name *Maribacter arenosus* sp. nov. is proposed. The type strain is CAU 1321^T (=KCTC 52191^T=NBRC 111990^T).

The genus *Maribacter*, of the family *Flavobacteriaceae* was first described by Nedashkovskaya *et al.* (2004). At the time of writing, the genus *Maribacter* comprises 19 species with validly published names, *M. sedimenticola*, *M. aquivivus*, *M. orientalis*, *M. ulvicola* (Nedashkovskaya *et al.*, 2004), *M. dokdonensis* (Yoon *et al.*, 2005), *M. polysiphoniae* (Nedashkovskaya *et al.*, 2007), *M. forsetii* (Barbeyron *et al.*, 2008), *M. arcticus* (Cho *et al.*, 2008), *M. antarcticus* (Zhang *et al.*, 2009), *M. stanieri* (Nedashkovskaya *et al.*, 2010), *M. chungangensis* (Weerawongwiwat *et al.*, 2013), *M. aestuarii* (Lo *et al.*, 2013), *M. caenipelagi* (Jung *et al.*, 2014), *M. thermophilus* (Hu *et al.*, 2015), *M. spongiicola*, *M. vaceletii* (Jackson *et al.*, 2015), *M. confluentis* (Park *et al.*, 2015), *M. flavus* (Tang *et al.*, 2015) and *M. lutimaris* (Kim *et al.*, 2016). All members of this genus have been isolated from diverse marine environments. In the course of the screening of bacteria from marine territory, strain CAU 1321^T was isolated from a marine sediment sample collected from Eurwangri beach (37° 26′ 55.8″ N 126° 22′ 15.7″ E), Incheon in the Republic of Korea. The purpose of the study was to establish the taxonomic position of this bacterial strain by using a polyphasic approach that included the determination of phenotypic (including chemotaxonomic) properties, a detailed phylogenetic investigation based on 16S rRNA gene sequences and genetic analysis.

Selective isolation of strain CAU 1321^T was done according to the protocol of Gordon & Mihm (1962) by the standard dilution plate technique. The appropriate dilutions were spread on marine agar 2216 (MA; Difco) plates and incubated under aerobic conditions at 30 °C for 7 days. A single colony of CAU 1321^T was subcultured routinely on MA at 30 °C for 5 days. The strain was maintained at −80 °C in marine broth 2216 (MB; Difco) supplemented with 25 % (v/v) glycerol. *Maribacter polysiphoniae* KCTC 22021^T and *Maribacter sedimenticola* KCTC 12966^T were obtained from the Korean Collection for Type Cultures (KCTC; Jeongeup, Korea) and both strains were used as reference strains in fatty acid analysis and biochemical characterization while *M. polysiphoniae* KCTC 22021^T was used for DNA–DNA hybridization.

DNA of CAU 1321^T was isolated according to 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 an automatic DNA sequencer (model 3730; Applied Biosystems). Multiple alignments with sequences of the type strains of the 19 species of the genus *Maribacter* and calculation of sequence similarity levels were performed by using EzTaxon-e–EzBioCloud.net (http://www.ezbiocloud.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CAU 1321^T is KU719511.

Two supplementary figures are available with the online Supplementary Material.

net/eztaxon) and CLUSTAL-X 2.1 (Larkin *et al.*, 2007). Evolutionary distance matrices were calculated by the neighbour-joining method described by Jukes & Cantor (1969). Phylogenetic trees were reconstructed using the neighbour-joining (Saitou & Nei, 1987), least-squares (Fitch & Margoliash, 1967), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) algorithms in the PHYLIP package (Felsenstein, 1989). Tree topology was evaluated by the bootstrap resampling method (Felsenstein, 1985) with 1000 replicates of the neighbor-joining dataset with the SEQBOOT and CONSENSE programs from the PHYLIP package.

The almost complete 16S rRNA gene sequence of CAU 1321^T comprised 1441 bp. The sequence was compared with available reference sequences in the GenBank database (access January 2015). The neighbour-joining phylogenetic tree showed that CAU 1321^T falls within the genus *Maribacter* and is closely related to *M. polysiphoniae* KCTC 22021^T with bootstrap values of 100 % (Fig. 1). The sequence similarity of CAU 1321^T with *M. polysiphoniae* KCTC 22021^T was 98.0 % and the range of similarity values between CAU 1321^T and other species the genus *Maribacter* were 94.6–96.0 %. Moreover, the phylogenetic relatedness was found to be similar the neighbor joining trees generated using the least-squares, maximum-likelihood and maximum-parsimony algorithms (data not shown).

The DNA mol% G+C content was evaluated by the method of Tamaoka & Komagata (1984). The G+C content of the DNA of CAU 1321^T was 40.6 mol%. The extent of DNA–DNA relatedness between CAU 1321^T and its most closely related phylogenetic neighbour, *M. polysiphoniae* KCTC 22021^T was examined using the fluorometric microplate method (Ezaki *et al.*, 1989), as modified by Goris *et al.* (1998). The DNA–DNA relatedness between CAU 1321^T and the most closely related strain, *M. polysiphoniae* KCTC 22021^T was 25.2 %. The value is well below the 70 % cut-off point recommended by Wayne *et al.* (1987) for the determination of genomic species, supporting the proposal that CAU 1321^T represents a separate species.

CAU 1321^T, *M. polysiphoniae* KCTC 22021^T, and *M. sedimenticola* KCTC 12966^T were cultivated on MA at 30 °C to determine their morphological, physiological and biochemical characteristics. Cell morphology was examined by light (model DM 1000; Leica) and transmission electron (TEM; JEM 1010, JEOL) microscopy using cells from an exponential-phase culture. For TEM examination, the cells were negatively stained with 1 % (w/v) phosphotungstic acid and the grids were examined after being air-dried (Fig. S1, available in the online Supplementary Material). Gram-staining was done using the Gram-staining kit (bioMérieux). Gliding motility was tested by using the hanging-drop method and the presence of flexirubin-type pigments was investigated as described by Bernardet *et al.* (2002). Growth at 4, 10, 20, 25, 30, 35, 37, 40 and 45 °C were assessed on MA medium. Growth under aerobic and anaerobic conditions were evaluated by growth on MA medium after 5 days incubation in an aerobic incubator (model MIR-253; Sanyo) and in an anaerobic chamber (model Bactron; Sheldon) at 30 °C. To determine the pH range for growth, CAU 1321^T was cultured in MB adjusted to pH 4.5–11.5 (at intervals of 0.5 pH units) by using sodium acetate/acetic acid and Na_2CO_3 buffers for pH 4.5–8.0 and pH 8.0–11.5, respectively. Growth in the presence of 0–15.0 % (w/v) NaCl (at 1 % intervals), was investigated at 30 °C in MB medium prepared according to the formular of the Difco medium except that NaCl was excluded. Oxidase and catalase activities were tested according to the methods of Cappuccino & Sherman (2002). Hydrolysis of gelatin, casein, starch, urea and citrate were determined according to the protocol of Smibert & Krieg (1994). Biochemical features were tested using the API 20E, API 20NE, API ZYM and API 50CH systems (BioMérieux) according to the manufacturer's instruction. API 20E, API 20NE and API ZYM strips were read after 24 h, and API 50CH strips after 48 h. Antibiotic susceptibility tests were performed by the disc diffusion method, using Sensi-Disc susceptibility test discs (BD BBL) on MA and incubated at 30 °C. The following antibiotics were : amoxicillin (20 μg), ampicillin (10 μg), carbenicillin (100 μg), cefoxitin (30 μg), cephalothin (30 μg), chloramphenicol (30 μg), erythromycin (15 μg), kanamycin (30 μg), penicillin (10 U), polymyxin B (300 U), rifampin (5 μg), streptomycin (10 μg), tobramycin (10 μg), nalidixic acid (30 μg) and trimethoprim/sulfamethoxazole (1.25/23.75 μg). Inhibition zones >10 mm in diameter indicated susceptibility and the absence of inhibition zones indicated resistance.

The morphological, cultural, physiological and biochemical characteristics of CAU 1321^T are given in Table 1 and in the species description. Overall, the results obtained in this study are similar to those for the type species of the genus, *M. sedimenticola* KCTC 12966^T (Nedashkovskaya *et al.*, 2004). However, the strain differed from the close relative, *M. polysiphoniae* KCTC 22021^T and *M. sedimenticola* KCTC 12966^T by not hydrolysing gelatin and starch, and being non-gliding.

For fatty acid analysis, cell masses of CAU 1321^T, *M. polysiphoniae* KCTC 22021^T and *M. sedimenticola* KCTC 12966^T were harvested from MA after cultivation for 5 days at 30 °C. The physiological age of the biomass harvested for fatty acid analysis was standardized by observing growth development during incubation of the cultures and choosing the harvesting point according to the standard MIDI protocol (Sherlock Microbial Identification System version 6.1). Cellular fatty acid methyl esters (FAMEs) were obtained by using the method of Minnikin *et al.* (1980) and separated by a 6890N automated gas chromatography system (Agilent Technologies). Peaks were identified by using the MOORE library version 5.0 (MIDI database TSBA6). The fatty acid profile of CAU 1321^T is shown in Table 2. The fatty acid profile of CAU 1321^T was similar to those of the type strains of species of the genus *Maribacter,* although there were differences in the proportions of some fatty acids, particularly iso-$C_{15:0}$, iso-$C_{16:0}$ 3-OH and iso-$C_{17:0}$ 3-OH among strain CAU 1321^T, *M. polysiphoniae* KCTC

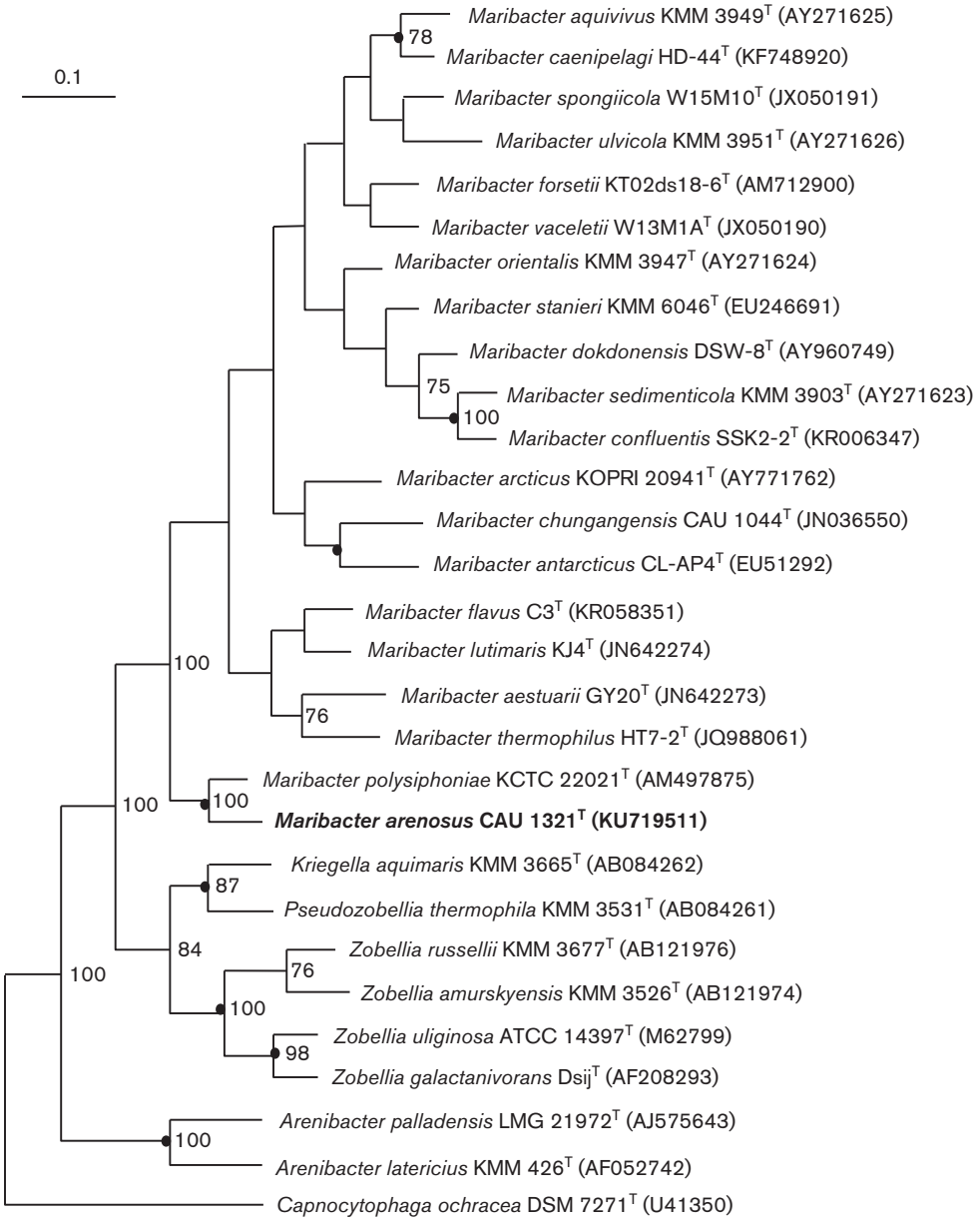

Fig. 1. Neighbour-joining phylogenetic tree based on nearly complete 16S rRNA gene sequences showing the relationships between CAU 1321^T, the type strains of species of the genus *Maribacter* with validly published names and other representatives within the family *Flavobacteriaceae*. Dots indicate that the corresponding nodes were also recovered in the trees generated with the maximum-likelihood; least-squares algorithms, and maximum-parsimony. The numbers at the nodes indicate levels of bootstrap support base on a neighbor-joining analysis of 1000 resamplings; only values of 70 % or more are shown. Bar, 0.1 substitutions per nucleotide position. *Capnocytophaga ochracea* DSM 7271^T (U41350) is used as an outgroup.

22021^T and *M. sedimenticola* KCTC 12966^T. The major fatty acids (>10 % of the total) were iso-$C_{15:0}$, iso-$C_{17:0}$ 3-OH and iso-$C_{15:1}$ G.

The menaquinones of strain CAU 1321^T were purified by the method of Komagata & Suzuki (1987) and separated by HPLC (Waters) using an isocratic solvent system [methanol/isopropyl ether (3 : 1, v/v)] and a flow rate of 1 ml^{-1}. The menaquinone of strain CAU 1321^T was MK-6. This is in agreement with results for members of the genus *Maribacter* (Nedashkovaskaya *et al.*, 2004, 2007, 2010; Yoon *et al.*, 2005; Barbeyron *et al.*, 2008; Cho *et al.*, 2008; Zhang *et al.*, 2009; Lo *et al.*, 2013; Weerawongwiwat *et al.*, 2013; Jung *et al.*, 2014; Hu *et al.*, 2015; Jackson *et al.*, 2015; Park *et al.*, 2015; Tang *et al.*, 2015; Kim *et al.*, 2016).

Table 1. Differential properties of CAU 1321^T and the type strains of the most closely related species of the genus *Maribacter*

Strains: 1, CAU 1321^T; 2, *M. polysiphoniae* KCTC 22021^T; 3, *M sedimenticola* KCTC 12966^T. Data were obtained this study. All strains were positive for oxidase and catalase, hydrolysis of aesculin, alkaline phosphatase, esterase, esterase lipase, leucine arylamidase, valine arylamidase, β-glucosidase, and *N*-acetyl-β-glucosaminidase. All strains were negative for hydrolysis of casein and citrate, nitrate reduction, flexirubin-type pigments, indole and H_2S production, arginine dihydrolase, assimilation of D-mannose, D-mannitol and D-maltose, trypsin, α-chymotrypsin, and α-galactosidase. All strains did not produce acid from glycerol, xylose, adonitol, L-rhamnose, dulcitol, inositol, D-mannitol, salicin, D-lactose, D-melibiose, sucrose, trehalose, raffinose, and fucose. +, ositive; −, negative; W, weakly positive.

Characteristic	1	2	3
Cell size (μm)	0.2–0.5×1.0–2.0	0.2–0.6×2.0–10	0.5–0.7×2–10
Gliding motility	−	+	+
Optimum temperature (°C)	30	30	22–24
NaCl for growth (%) (optimum)	0–2 (1)	0–3 (2)	1–6 (1.5–2.0)
pH for growth (optimum)	6.5–9.5 (8.0)	6.0–10.0 (8.5)	6.0–10.0 (8.0)
Hydrolysis of:			
Gelatin	−	+	−
Starch	−	+	+
Acetoin production	−	W	−
Urease activity	W	W	−
Assimilation of:			
D-Glucose	+	−	−
L-Arabinose	W	−	−
N-acetyl-glucosamine	−	+	−
Malate	−	+	−
Potassium gluconate	−	+	−
Enzyme activity (API ZYM)			
Cystine arylamidase	−	+	−
Acid phospatase	+	W	+
Napthol-AS-BI-phosphohydrolase	W	W	+
β-Galactosidase	−	−	+
α-Glucosidase	−	+	−
α-Mannosidase	+	+	−
α-Fucosidase	−	W	−
Acid production from:			
L-Arabinose	−	+	−
D-Galactose	−	+	−
D-Glucose	−	+	−
D-Fructose	−	+	−
D-Cellobiose	−	+	−
D-Maltose	−	+	−
Potassium 5-ketogluconate	+	W	+
DNA G+C content (mol%)	40.6	39.5	37.5

The major polar lipids of CAU 1321^T were separated using a TLC method (two-dimensional thin-layer chromatography) according to the protocol of Minnikin *et al.* (1984). The plate was sprayed with 10 % ethanolic molybdatophosphoric acid (for the total lipids), molybdenum blue (for phospholipids), ninhydrin (for aminolipids), and α-naphthol/sulphuric acid reagent (for glycolipids) (Sigma-Aldrich). The major polar lipids of CAU 1321^T were phosphatidylethanolamine, one unidentified aminoglycolipid, five unidentified aminolipids, two unidentified aminophospholipids, two unidentified glycolipids and three unidentified lipids (Fig. S2). This polar lipids pattern were similar to those of the species of the genus *Maribacter* (Yoon *et al.*, 2005; Lo *et al.*, 2013; Jung *et al.*, 2014; Hu *et al.*, 2015; Tang *et al.*, 2015; Kim *et al.*, 2016) but this strain is different from other species due to it having one aminoglycolipid.

From the results of the 16S rRNA gene sequence comparison and biochemical characterization, CAU 1321^T could be separated from other related strains. On the basis of the genotypic, chemotaxonomic, phenotypic and phylogenetic results, strain CAU 1321^T represents a novel species of the

Table 2. Fatty acid compositions (%) of strain CAU 1321^T

Strains: 1, CAU 1321^T; 2, *Maribacter polysiphoniae* KCTC 22021^T; 3, *Maribacter sedimenticola* KCTC 12966^T. All data for all strains are from this study. Fatty acids amounting to >1.0 % of the total fatty acids in all strains are shown. –, Not detected; TR, trace (<0.5 %).

Fatty acids	1	2	3
Saturated			
$C_{16:0}$	2.27	1.33	2.41
Unsaturated			
$C_{15:1}\omega6c$	2.16	2.86	2.28
$C_{17:1}\omega6c$	1.87	1.05	1.97
$C_{17:1}\omega8c$	2.42	1.28	–
Branched-chain			
anteiso-$C_{15:0}$	2.27	6.37	3.98
iso-$C_{15:0}$	20.05	14.39	22.81
iso-$C_{15:0}$ 3-OH	5.90	7.04	6.34
iso-$C_{16:0}$ 3-OH	5.87	15.74	4.12
iso-$C_{17:0}$ 3-OH	19.15	11.43	23.38
iso-$C_{15:1}$G	12.95	9.03	13.42
Hydroxyl			
$C_{15:0}$ 3-OH	2.29	2.46	2.20
$C_{16:0}$ 3-OH	2.42	TR	2.22
$C_{17:0}$ 2-OH	TR	2.49	TR
$C_{17:0}$ 3-OH	1.58	TR	TR
Summed feature*			
3	9.81	9.36	9.62
9	4.14	4.66	4.45

*Summed features are groups of two or three fatty acids that are treated together for the purpose of evaluation in the MIDI system and include both peaks with discrete equivalent chain lengths (ECLs) as well as those where the ECLs are not reported separately. Summed feature 3 was listed as iso-$C_{15:0}$ 2-OH/$C_{16:1}\omega7c$ and annotated here as $C_{16:1}\omega7c$ and summed feature was listed as iso-$C_{17:1}\omega9c$ and/or 10-methyl $C_{16:0}$

genus *Maribacter*, for which the name *Maribacter arenosus* sp. nov. is proposed.

Description of *Maribacter arenosus* sp. nov.

Maribacter arenosus (a.re.no′sus. L. masc. adj. *arenosus* sandy).

Cells are Gram-stain-negative, non-gliding, non-spore-forming, strictly aerobic and rod-shaped (0.2–0.5×1.0–2.0 μm). Colonies on MA are yellow, circular, convex, smooth and glistening after 5 days at 30 °C. Optimum growth occurs at 30 °C; growth occurs at 20–30 °C, but not at 35–45 °C. Optimum pH for growth is pH 8.0; growth occurs at between pH 6.5 and 9.5. Growth occurs in the presence of 0–2 % (w/v) NaCl (optimum, 1 %). Oxidase and catalase are positive. Casein, gelatin, urea and starch are not hydrolysed. Flexirubin-type pigments are not produced. Citrate is not utilized. H_2S, indole and acetoin (Voges Proskauer) are not produced. Nitrate is not reduced. Assimilation of D-glucose, PNPG (4-nitrophenyl β-D-galactopyranoside) and arabinose (weakly) are positive, but arginine dihydrolase, D-mannose, D-mannitol, *N*-acetylglucosamine, D-maltose and potassium gluconate are negative. Acid production from aesculin ferric citrate and potassium 5-ketogluconate are positive but glycerol, arabinose, xylose, D-ribose, D-galactose, D-glucose, methyl β-D-xylopyranoside, D-fructose, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, *N*-acetylglucosamine, arbutin, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-saccharose, D-trehalose, inulin, D-raffinose, fucose and potassium 2-ketogluconate are negative. In the assay with the API ZYM system, activity of alkaline phosphatase, esterase, esterase lipase, leucine arylamidase, valine arylamidase, acid phosphatase, β-glucosidase, *N*-acetyl-β-glucosaminidase and α-mannosidase are present and activity of naphtol-AS-BI-phosphohydrolase is weakly present. The results from the antibiotic susceptibility test show that this strain is susceptible to amoxicillin, carbenicillin, cefoxitin, chloramphenicol, erythromycin, kanamycin, rifampin and trimethoprim/sulfamethoxazole and resistant to ampicillin, cephalothin, penicillin, polymyxin B, streptomycin, tobramycin and nalidixic acid. The major cellular fatty acids are iso-$C_{15:0}$, iso-$C_{17:0}$ 3-OH, and iso-$C_{15:1}$G. The major polar lipids are phosphatidylethanolamine, one unidentified aminoglycolipid, five unidentified aminolipids, two unidentified aminophospholipids, two unidentified glycolipids and three unidentified lipids. The menaquinone is MK-6.

The type strain, CAU 1321^T(=KCTC 52191^T=NBRC 111990^T), was isolated from sea sand collected from Incheon, Republic of Korea. The DNA G+C content of the type strain is 40.6 mol%.

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