# *Marimicrobium arenosum* gen. nov., sp. nov., a moderately halophilic bacterium isolated from sea sand

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A Gram-stain-negative, non-pigmented, non-spore-forming, non-motile, strictly aerobic bacterial strain, designated CAU 1038$^T$, was isolated from a sea sand sample in Modo, Republic of Korea, and its taxonomic position was examined using a polyphasic approach. Cells of strain CAU 1038$^T$ grew optimally at 30 °C, pH 7.5 in 2 % (w/v) NaCl. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain CAU 1038$^T$ formed a distinct lineage within the class *Gammaproteobacteria* as a separate deep branch, with 95.2 % or lower sequence similarity to representatives of the genera *Haliea*, *Halioglobus* and *Chromatocurvus*, and 92.3 % or lower with *Luminiphilus*, *Pseudohaliea* and *Congregibacter*. The major cellular fatty acids of strain CAU 1038$^T$ were $C_{16:0}$, $C_{16:1}\omega7c$ and $C_{18:1}\omega7c$. The polar lipid pattern of the isolate consisted of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, an unidentified aminolipid and two unidentified lipids. The strain contained lipoquinone (Q-8) as the sole respiratory quinone. The G+C content of the genomic DNA was 65 mol%. On the basis of phenotypic and chemotaxonomic data, and phylogenetic inference, strain CAU 1038$^T$ represents a novel species of a new genus in the family *Halieaceae*, for which the name *Marimicrobium arenosum* gen. nov., sp. nov. is proposed. The type strain of the type species is CAU 1038$^T$ (=KCTC 42300$^T$=NBRC 110727$^T$).

*Gammaproteobacteria* are a class of numerous medically and ecologically essential groups of bacteria, such as *Enterobacteriaceae*, *Pseudomonadaceae*, *Vibrionaceae*, *Alteromonadaceae* and *Oceanospirillaceae*. The family *Halieaceae* (Spring *et al.*, 2015) belongs to the order *Cellvibrionales* in the class *Gammaproteobacteria*. This family was found in various habitats, such as tidal flat sediment, seawater, marine and marine coastal sediments. Members of the family *Halieaceae* are Gram-negative, rod-shaped and non-spore-forming bacteria. Q-8 is the predominant isoprenoid quinone and the major fatty acid is unsaturated fatty acid ($C_{16:1}$ or $C_{18:1}$). The G+C content of the genomic DNA ranges from 52 to 66 mol% (Spring *et al.*, 2015). At the time of writing, the family *Halieaceae* comprises six genera with validly published names: *Chromatocurvus* (Csotonyi *et al.*, 2011), *Congregibacter* (Spring *et al.*, 2009), *Haliea* (Urios *et al.*, 2008), *Halioglobus* (Park *et al.*, 2012), *Luminiphilus* (Spring *et al.*, 2013) and *Pseudohaliea* (Spring *et al.*, 2013).

Modo is a small island located in Ongjingun, Incheon, in the west sea of the Republic of Korea (37° 32′ 05″ N 126° 24′ 33″ E). This area has an abundance of sediment that is interesting for the isolation of a wide variety of micro-organisms. Strain CAU 1038$^T$ was isolated from Modo Island and belongs to the class *Gammaproteobacteria*, order *Cellvibrionales* and family *Halieaceae*. The objective of this study was to evaluate the taxonomic position of this novel bacterial strain using a polyphasic approach involving the determination of phenotypic, genotypic and chemotaxonomic properties, and 16S rRNA gene sequence analysis.

A sea sand sample was serially diluted, plated on marine agar 2216 (MA; Difco), supplemented with cycloheximide (50 mg $l^{-1}$) and nalidixic acid (20 mg $l^{-1}$) to inhibit the growth of fungi, and incubated at 30 °C for 7 days under aerobic conditions as described by Gordon & Mihm (1962). Colonies were randomly selected and a single colony was purified by subculturing on MA at 30 °C for 3 days. Strain CAU 1038$^T$ was preserved at −80 °C in marine broth (MB; Difco) supplemented with 25 % (v/v) glycerol. The type strains of species of the closely related genus *Halioglobus* (*Halioglobus japonicus* KCTC 23429$^T$ and *Halioglobus pacificus* KCTC 23430$^T$), were obtained

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CAU 1038$^T$ is KP064188.

Three supplementary figures are available with the online Supplementary Material.

from the Korean Collection for Type Cultures (KCTC) and used as reference strains in most analyses.

Genomic DNA of strain CAU 1038$^T$ was extracted by the method of Marmur (1961). PCR amplification of the 16S rRNA gene of strain CAU 1038$^T$ was examined according to Lane (1991) and the conditions described by Nam *et al.* (2004). The amplified 16S rRNA gene was directly sequenced using a BigDye Terminator Cycle Sequencing kit and an automated 3730 DNA sequencer (Applied Biosystems). Multiple sequence alignments of the 16S rRNA gene sequence of strain CAU 1038$^T$ with the corresponding sequences from a broad selection of closely related strains, and calculations of the levels of sequence similarity, were made using the EzTaxon-e server (Kim *et al.*, 2012; *http://www.ezbiocloud.net/eztaxon*) and CLUSTAL X 2.1 software (Larkin *et al.* 2007). Evolutionary distance matrices were generated by the neighbour-joining method described by Jukes and 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). Branch support in the neighbour-joining tree was estimated by the bootstrap resampling method (1000 replications) (Felsenstein, 1985). The mol% G+C content of the DNA was performed using reversed-phase HPLC following the method of Tamaoka & Komagata (1984). For detection of photosynthesis genes *pufL* and *pufM* (encoding subunits of the photosynthetic reaction centre), PCR amplification was performed using primer set pufL2/pufMR2 and the following cycle conditions: initial denaturation at 98 °C for 3 min, 35 cycles of denaturation at 98 °C for 15 s, annealing at 56 °C for 25 s and extension at 72 °C for 1.5 min, followed by final extension at 72 °C for 10 min (Spring *et al.*, 2013).

For the investigation of morphological, physiological and biochemical characteristics, strain CAU 1038$^T$ was cultivated on MA at 30 °C for 3 days. Cell morphology was investigated under a DM 1000 light microscope (Leica Microsystems) and a JEM-1200EX transmission electron microscope (JEOL) using cells from an exponentially growing culture. Gram staining was carried out using the bioMérieux Gram staining kit. Motility was assessed in MB culture using the hanging-drop method (Bowman, 2000).

Growth between 4 °C and 45 °C was examined by growing the isolate on MA for 3 days in a MIR-253 aerobic incubator (Sanyo Electric Biomedical). The pH range for growth was investigated in MB that had been adjusted to pH 4.5–12.0 (at 0.5 pH unit intervals) by using $CH_3COONa$/$CH_3COOH$ and $Na_2CO_3$ buffers. The NaCl concentration for growth was determined using modified SYPHC medium as used by Spring *et al.* (2013), adjusting the concentration of NaCl from 0 to 15 % (w/v). Anaerobic growth was checked in a Bactron anaerobic chamber (Sheldon Manufacturing) under the optimal conditions. Oxidase activity was evaluated with 0.1 % (w/v) tetramethyl-*p*-phenylenediamine (Cappuccino & Sherman, 2002). Catalase activity was determined by observing bubble production in 3 % (v/v) $H_2O_2$ solution. Hydrolysis of casein, starch and urea were determined using SYPHC medium with 2 % NaCl. Acid production and assimilation from carbohydrates, enzyme activity, and other physiological and biochemical features were tested as described by Leifson (1963) using API 20NE, API 20E, API 50CHE, and API ZYM strips (bioMérieux).

For the determination of fatty acid composition, the cell biomass of strain CAU 1038$^T$, *Halioglobus japonicus* KCTC 23429$^T$ and *Halioglobus pacificus* KCTC 23430$^T$ were harvested from MA after cultivation for 3 days at 30 °C. The physiological age of the biomass used for fatty acid analysis was chosen according to the standard MIDI Sherlock Microbial Identification System (MIDI) methodology. Cellular fatty acid methyl esters were obtained and separated on a 6890N gas chromatograph fitted with a 7683 autosampler (Agilent Technologies). Peaks were identified using the Microbial Identification software package [MOORE version 5.0 (Agilent Technologies); MIDI database TSBA6]. The polar lipids of strain CAU 1038$^T$ were identified using two-dimensional thin-layer chromatography by the method of Minnikin *et al.* (1984). The plate was sprayed with 10 % ethanolic molybdatophosphoric acid (for total lipids), molybdenum blue (for phospholipids), ninhydrin (for aminolipids) and α-naphthol/sulphuric acid reagent (for glycolipids). Ubiquinones were separated by HPLC using an isocratic solvent system [methanol/isopropyl ether (3 : 1, v/v)] and flow rate of (1 ml min$^{-1}$) (Komagata & Suzuki, 1987).

The nearly complete 16S rRNA gene sequence of strain CAU 1038$^T$ (1515 bp) was determined and compared with corresponding sequences of related bacterial strains in the GenBank database. Phylogenetic analysis showed that strain CAU 1038$^T$ was distinct from a clade represented by the members of the genera *Microbulbifer*, *Saccharophagus*, *Spongiibacter*, *Dasania*, *Marinimicrobium*, *Teredinibacter*, *Haliea*, *Luminiphilus*, *Chromatocurvus*, *Pseudohaliea*, *Congregibacter* and *Halioglobus*. In pairwise analyses, *Haliea salexigens* DSM 19537$^T$ (95.2 % 16S rRNA gene sequence similarity), *Halioglobus japonicus* S1-36$^T$ (94.6 %), *Halioglobus pacificus* S1-72$^T$ (93.7 %), *Chromatocurvus halotolerans* EG19$^T$ (93.6 %), *Haliea mediterranea* 7SM29$^T$ (93.2 %), *Luminiphilus syltensis* NOR5-1B$^T$ (92.3 %), *Pseudohaliea rubra* CM41_15a$^T$ (92.2 %) and *Congregibacter litoralis* KT71$^T$ (90.5 %) were the recognised species that appeared most closely related to strain CAU 1038$^T$. The trees generated using the least-squares, maximum-likelihood, and maximum-parsimony algorithms (Fig. S1, available in the online Supplementary Material) show similar topologies to neighbour-joining tree (Fig. 1). The G+C content of the DNA of strain CAU 1038$^T$ was 65 mol%. The *pufL* and *pufM* genes were not detected in strains CAU1038$^T$ based on PCR amplification of these photosynthesis genes.

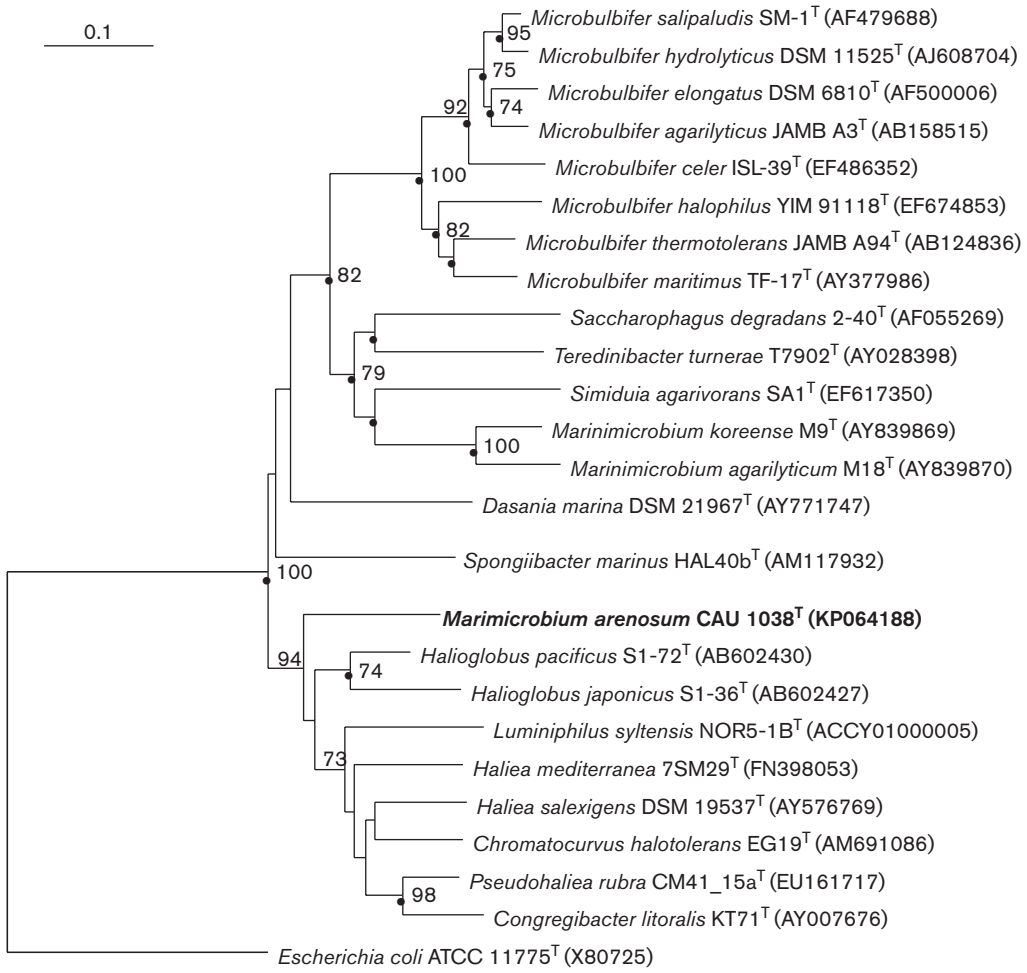


**Fig. 1.** Neighbour-joining phylogenetic tree based on nearly complete 16S rRNA gene sequences showing the position of strain CAU 1038$^T$. Filled circles indicate that the corresponding nodes were recovered in the trees generated with the maximum-likelihood and least-squares algorithms. Bootstrap values >70 %, based on a neighbour-joining analysis of 1000 resampled datasets, are shown at nodes. *Escherichia coli* ATCC 11775$^T$ (GenBank accession no. X80725) was used as an outgroup organism. Bar, 0.1 substitutions per nucleotide position.

The differential morphological, cultural, physiological and biochemical characteristics of strain CAU 1038$^T$ are presented in Table 1. Strain CAU 1038$^T$ is strictly aerobic, non-pigmented and can grow at temperatures from 20–37 °C (optimum 30 °C) and at pH 6.5–8.5 (optimum, pH 7.5). Cells were short, rod-shaped, approximately 0.3–0.5 μm in diameter and 1.0–1.1 μm in length, non-motile and did not have flagella (Fig. S2). As strain CAU 1038$^T$ was isolated from a saline environment, it required NaCl for growth around 0–3 % (w/v) (optimum 2 %). The oxidase and catalase tests were positive but urease was negative, and nitrate is reduced to nitrite but not to nitrogen. Esterase enzyme is produced. Strain CAU 1038$^T$ could utilize glucose and ribose, but was unable to utilize arabinose or maltose. The growth temperature range distinguished strain CAU 1038$^T$ (20–37 °C) from the genera *Haliea* (10–37 °C), *Halioglobus* (15–30 °C), *Chromatocurvus* (7–40 °C), *Congregibacter* (9–33 °C), *Luminiphilus* (12–32 °C) and *Pseudohaliea* (15–44 °C). In addition, the salt tolerance range distinguished strain CAU 1038$^T$ (0–3.0 %) from the genera *Haliea* (0.7–7.0 %), *Chromatocurvus* (0–18.0 %) and *Congregibacter* (1.0–7.0 %). Strain CAU 1038$^T$ differed from the close relative, *Haliea salexigens* DSM 19537$^T$ (Urios *et al.*, 2008) by the ability to utilize glucose, and from members of the genus *Halioglobus* by the inability to utilize fructose and sucrose (Table 1).

The pattern of polar lipids of strain CAU 1038$^T$ was phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylglycerol, an unidentified aminolipid and two unidentified lipids (Fig. S3). The single isoprenoid quinone detected was Q-8. The fatty acid profile of strain CAU 1038$^T$ is shown in Table 2. The strain contained

**Table 1.** Differential characteristics of strain CAU 1038$^T$ and type strains of closely related species of the family *Halieaceae*

Strain: 1, CAU 1038$^T$ (data from this study); 2, *Haliea salexigens* 3X/A02/235$^T$ (Urios *et al.* 2008); 3, *Halioglobus japonicus* KCTC 23429$^T$ (this study); 4, *Halioglobus pacificus* KCTC 23430$^T$ (this study); 5, *Chromatocurvus halotolerans* EG19$^T$ (Spring *et al.*, 2013); 6, *Congregibacter litoralis* KT71$^T$ (Spring *et al.*, 2013); 7, *Luminiphilus syltensis* NOR5-1B$^T$ (Spring *et al.*, 2013); 8, *Pseudohaliea rubra* CM41_15a$^T$ (Spring *et al.*, 2013). +, Positive; −, negative; DPG, Diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PL, phospholipid.

| Characteristic | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| Shape | Short rod | Short rod | Coccus | Coccus | Pleomorphic | Pleomorphic | Straight-to-bent | Straight rods |
| Motility | − | + | − | − | + | + | − | − |
| Growth temperature | 20–37 | 10–37 | 15–30 | 15–30 | 7–40 | 9–33 | 12–32 | 15–44 |
| (°C) range optimum | 30 | 30 | 20–25 | 20–25 | 37 | 28 | 28 | 30 |
| pH range optimum | 6.5–8.5 | 5.0–9.0 | 6.0–9.0 | 6.0–10.0 | 7.0-12.0 | 6.5–9.0 | 7.0–9.0 | 5.0–9.0 |
| | 7.5 | 8.0 | 7.0–8.0 | 7.0–8.0 | 7.0 | 7.5–8.0 | 8.0 | 8.0 |
| NaCl tolerance (%) | 0–3.0 | 0.7–7.0 | 1.0–4.0 | 1.0–5.0 | 0–18.0 | 1.0–7.0 | 1.0–9.0 | 0.7–4.2 |
| range optimum | 2.0 | 4.0 | 2.0 | 2.0 | 4.0 | 2.0 | 3.0 | 3.5 |
| Catalase | + | + | − | − | + | + | + | + |
| Oxidase | + | + | + | + | + | + | + | + |
| Utilization of: | | | | | | | | |
| Glucose | + | − | + | + | − | − | − | + |
| Fructose | − | − | + | + | − | − | − | − |
| Sucrose | − | − | + | + | − | + | − | − |
| Photosynthetic pigment | − | − | − | − | + | + | + | + |
| Polar lipids | DPG, PE, PG | DPG,PG | DPG, PE, PG, PL | DPG, PG, PL | PE, PG | PE, PG | PE, PG | PE, PG |
| Major cellular fatty acids | $C_{16:0}$, $C_{16:1}\omega7c$, $C_{18:1}\omega7c$ | $C_{16:1}\omega7c$, $C_{17:1}\omega8c$, $C_{18:1}\omega7c$ | $C_{16:0}$, $C_{16:1}\omega7c$, $C_{18:1}\omega7c$ | $C_{16:1}\omega7c$, $C_{17:1}\omega8c$, $C_{18:1}\omega7c$ | $C_{16:1}\omega7c$, $C_{17:1}\omega8c$, $C_{18:1}\omega7c$ | $C_{16:1}\omega7c$, $C_{18:1}\omega7c$ | $C_{16:0}$, $C_{16:1}\omega7c$ | $C_{16:0}$, $C_{16:1}\omega7c$, $C_{18:1}\omega7c$ |
| DNA G+C content (mol%) | 65 | 61 | 60 | 59 | 63 | 58 | 57 | 65 |

**Table 2.** Cellular fatty acids (%) of strain CAU 1038$^T$ and type strains of closely related species of the genus *Halioglobus*

Strains: 1, CAU 1038$^T$; 2, *Halioglobus japonicus* KCTC 23429$^T$; 3, *Halioglobus pacificus* KCTC 23430$^T$. All data are from this study with all strains cultured under identical conditions (marine agar for 3 days at 30 °C) and analysed using the Microbial Identification System. Fatty acids amounting to >0.5 % of the total fatty acids in all strains are shown. −, Not detected; TR, trace (<0.5 %).

| Fatty acid | 1 | 2 | 3 |
|---|---|---|---|
| Hydroxy | | | |
| $C_{10:0}$ 3-OH | − | 2.8 | 2.7 |
| $C_{11:0}$ 3-OH | − | 0.6 | 1.5 |
| $C_{12:0}$ 3-OH | 2.4 | TR | 1.5 |
| $C_{12:0}$ 2-OH | 1.7 | − | − |
| $C_{12:1}$ 3-OH | − | − | − |
| $C_{13:0}$ 3-OH | − | − | − |
| Saturated | | | |
| $C_{10:0}$ | 5.9 | 1.7 | 1.8 |
| $C_{11:0}$ | − | 0.8 | 0.6 |
| $C_{12:0}$ | − | 0.8 | 0.8 |
| $C_{13:0}$ | − | − | 0.8 |
| $C_{14:0}$ | 1.1 | 2.0 | 1.4 |
| $C_{15:0}$ | − | − | − |
| iso-$C_{15:0}$ | − | TR | − |
| $C_{16:0}$ | 27.0 | 9.4 | 3.4 |
| $C_{17:0}$ | − | 2.4 | 2.2 |
| $C_{18:0}$ | − | 0.6 | TR |
| Unsaturated | | | |
| $C_{15:1}\omega6c$ | − | 0.5 | 1.2 |
| $C_{15:1}\omega8c$ | − | 0.9 | 1.6 |
| $C_{16:1}\omega6c$ | − | TR | TR |
| $C_{16:1}\omega7c$ | 42.3 | 35.2 | 31.4 |
| $C_{17:1}\omega6c$ | − | TR | TR |
| $C_{17:1}\omega8c$ | − | 4.2 | 13.1 |
| $C_{18:1}\omega7c$ | 14.7 | 34.7 | 28.0 |
| $C_{19:1}\omega6c$ | − | − | − |

hydroxyl, saturated and unsaturated fatty acids. The major cellular fatty acids (>10 %) were $C_{16:0}$ (27.0 %), $C_{16:1}\omega7c$ (42.3 %), and $C_{18:1}\omega7c$ (14.7 %). These fatty acids were present in higher amounts than those recorded for the type strains of the type species of the genera *Haliea*, *Halioglobus*, *Chromatocurvus*, *Luminiphilus*, *Pseudohaliea* and *Congregibacter*. The fatty acids $C_{12:0}$ 3-OH, $C_{12:0}$ 2-OH, $C_{10:0}$ and $C_{14:0}$ were present in strain CAU 1038$^T$ at >1 %.

In conclusion, it is evident from the phenotypic, chemotaxonomic and genotypic data that strain CAU 1038$^T$ positioned within the family *Halieaceae*, which encompasses the genera *Haliea* and *Halioglobus* (Fig. 1). Strain CAU 1038$^T$ showed very low 16s rRNA gene sequence similarity (≤95.2 %) with respect to members of the family *Halieaceae*, which supports the placement of the novel isolate within a separate genus. In addition, differences in chemotaxonomic and phenotypic characteristics with closely related taxa were observed (Table 1). The novel isolate could be distinguished from members of the genera of the family *Halieaceae* by its phylogenetic relationship, and phenotypic and chemotaxonomic characteristics. Therefore, we propose that strain CAU 1038$^T$ be classified as a representative of a novel genus and species, *Marimicrobium arenosum* gen. nov., sp. nov.

## Description of *Marimicrobium* gen. nov.

*Marimicrobium* (Ma.ri.mi.cro′bi.um. L. n. *mare* the sea; N.L. neut. n. *microbium* microbe; N.L. neut. n. *Marimicrobium* small, marine microbe).

Cells are Gram-stain-negative, non-pigmented, strictly aerobic, oxidase- and catalase-positive, rod-shaped, non-spore-forming and non-motile. The temperature range for growth is 20–37 °C. Growth occurs in the presence of 0–3 % (w/v) NaCl. The pH range for growth is 6.5–8.5. The respiratory lipoquinone is Q-8. The polar lipids consist of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, an unidentified aminolipid and two unidentified lipids. The major fatty acids are $C_{16:0}$, $C_{16:1}\omega7c$ and $C_{18:1}\omega7c$. The type species is *Marimicrobium arenosum*.

## Description of *Marimicrobium arenosum* sp. nov.

*Marimicrobium arenosum* (a.re.no′sum. L. neut. adj. *arenosum* sandy, dwelling in marine sediment sand).

Cells are approximately 0.3–0.5 μm in diameter and 1.0–1.1 μm in length. Colonies on MA are cream and circular, and cells are small, straight, regular rods after incubation for 3 days at 30 °C. Optimum growth occurs at 30 °C, at pH 7.5 and with 2 % (w/v) NaCl. Gelatin, casein, and starch are not hydrolysed. Nitrate is reduced to nitrite but not to nitrogen. Assimilates D-mannitol and trisodium citrate, but not L-arabinose, D-mannose, maltose, *N*-acetylglucosamine, potassium gluconate, capric acid, adipic acid, malic acid or phenylacetic acid. Uses D-ribose, D-galactose and D-glucose as sole carbon sources, but not erythritol, L-arabinose, L-xylose, L-sorbose, L-rhamnose, L-fucose, L-arabitol, D-arabinose, D-xylose, D-adonitol, D-fructose, D-mannose, dulcitol, inositol, D-mannitol, D-sorbitol, cellobiose, sucrose, melezitose, turanose, D-lyxose, D-tagarose, D-fucose, D-arabitol, methyl α-D-glucopyranoside, methyl β-D-xylopyranoside, *N*-acetylglucosamine, aesculin ferric citrate, starch, glycogen, xylitol, potassium gluconate, potassium 2-ketogluconate or potassium 5-ketogluconate. In the API ZYM system, alkaline phosphatase, esterase (C4), esterase lipase (C8), α-chymotrypsin, acid phosphatase, and naphthol-AS-BI-phosphohydrolase activities are positive, but lipase (C14), leucine arylamidase, valine arylamidase, crystine arylamidase, trypsin, α-galactosidase, β-glucuronidase, β-galactosidase, β-glucosidase, α-glucosidase, α-mannosidase, α-fucosidase and *N*-acetyl-β-glucosaminidase

activities are negative. The fatty acids comprise $C_{16:0}$, $C_{16:1}\omega7c$, $C_{18:1}\omega7c$, $C_{12:0}$ 3-OH, $C_{12:0}$ 2-OH, $C_{10:0}$ and $C_{14:0}$.

The type strain CAU 1038$^T$ (=KCTC 42300$^T$=NBRC 110727$^T$), was isolated from a sea sand sample collected in Modo in the Republic of Korea. The DNA G+C content of the type strain is 65 mol%.

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