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TAXONOMIC DESCRIPTION

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Tessaracoccus arenae sp. nov., isolated from sea sand

Chutimon Thongphrom,[1] Jong-Hwa Kim,[1] Nagamani Bora[2,*] and Wonyong Kim[1,*]

Abstract

A Gram-stain positive, non-spore-forming, non-motile, facultatively anaerobic bacterial strain, designated CAU 1319^T, was isolated from sea sand and the strain's taxonomic position was investigated using a polyphasic approach. Strain CAU 1319^T grew optimally at 30 °C and at pH 7.5 in the presence of 2 % (w/v) NaCl. Phylogenetic analysis, based on the 16S rRNA gene sequence, revealed that strain CAU 1319^T belongs to the genus *Tessaracoccus*, and is closely related to *Tessaracoccus lapidicaptus* IPBSL-7^T (similarity 97.69 %), *Tessaracoccus bendigoensis* Ben 106^T (similarity 95.64 %) and *Tessaracoccus flavescens* SST-39^T (similarity 95.84 %). Strain CAU 1319^T had LL-diaminopimelic acid as the diagnostic diamino acid in the cell-wall peptidoglycan, MK-9 (H_4) as the predominant menaquinone, and anteiso-$C_{15:0}$ as the major fatty acid. The polar lipids consisted of phosphatidylglycerol, phosphatidylinositol, two unidentified aminolipids, three unidentified phospholipids and one unidentified glycolipid. Predominant polyamines were spermine and spermidine. The DNA–DNA hybridization value between strain CAU 1319^T and *T. lapidicaptus* IPBSL-7^T was 24 %±0.2. The DNA G+C content of the novel strain was 69.5 mol %. On the basis of phenotypic and chemotaxonomic properties, as well as phylogenetic relatedness, strain CAU 1319^{T}should be classified as a novel species of the genus *Tessaracoccus*, for which the name *Tessaracoccus arenae* sp. nov. is proposed. The type strain is CAU 1319^T(=KCTC 39760^T=NBRC 111973^T).

The genus *Tessaracoccus*, a member of the family *Propionibacteriaceae*, was first described by Maszenan et al. [1] with the description of a single novel species, *Tessaracoccus bendigoensis* Ben 106^T. The genus *Tessaracoccus* consists of Gram-stain-positive, facultatively anaerobic (except *Tessaracoccus lubricantis*), non-motile, non-spore-forming and oval to rod-shaped bacteria that are characterized by the presence of LL-diaminopimelic acid (LL-DAP) as the cell wall peptidoglycan, MK-9 (H_4) as the predominant menaquinone, and anteiso-$C_{15:0}$ as the predominant cellular fatty acid [1–7]. At the time of writing, the genus *Tessaracoccus* consists of 7 species with validly published names, *T. bendigoensis*, isolated from activated sludge biomass [1], *Tessaracoccus flavescens*, isolated from marine sediment [2], *T. lubricantis*, isolated from metal working fluid [3], *Tessaracoccus oleiagri*, isolated from crude oil-contaminated saline soil [4], *Tessaracoccus lapidicaptus*, isolated from a deep-subsurface drilling core [5], *Tessaracoccus flavus*, isolated from the drainage system of a lindane-producing unit [6] and *Tessaracoccus rhinocerotis*, isolated from the faeces of *Rhinoceros unicortis* [7]. The novel bacterial strain, CAU 1319^T, was isolated from a sea sand sample collected from Eurwangri beach (37° 26′ 55.8″ N 126° 22′ 15.7″ E), Incheon in the Republic of Korea. The purpose of this study was to establish the taxonomic position of this bacterial strain by using a polyphasic characterization that included the determination of phenotypic (including chemotaxonomic) properties and a detailed phylogenetic investigation, based on the 16S rRNA gene sequence.

Selective isolation of strain CAU 1319^T was performed according to Gordon and Mihm [8]. 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 strain CAU 1319^T was sub-cultured on MA at 30 °C for 3 days. The strain was maintained at −80 °C in marine broth 2216 (MB; Difco) supplemented with 25 % (v/v) glycerol. Stain CAU 1319^T has been deposited in the Korean Collection for Type Cultures (KCTC; Jeongeup, Korea) and National Institute of Technology and Evaluation (NBRC; Chiba, Japan), respectively. The type strains of the most closely related species, *T. lapidicaptus* IPBSL-7^T (=CECT 8385^T), *T. bendigoensis* Ben 106^T (=JCM 13525^T) and *T. flavescens* SST-39^T (=JCM 16025^T) were obtained from the Spanish Type Culture Collection (CECT; Valencia, Spain) and the Japan Collection of Micro-organisms (JCM; Tsukuba, Japan), and were used as reference strains for fatty acid, biochemical and genetic analyses

Genomic DNA of strain CAU 1319^T was extracted by the method of Marmur [9] and PCR amplification was carried

Author affiliations: [1]Department of Microbiology, Chung-Ang University College of Medicine, Seoul, Republic of Korea; [2]School of Biosciences, University of Nottingham, Sutton Bonington, UK.

***Correspondence:** Nagamani Bora, nagamani.bora@gmail.com; Wonyong Kim, kimwy@cau.ac.kr

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out following established procedures [10]. The amplified 16S rRNA gene was sequenced directly using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) and an automatic DNA sequencer (model 3730; Applied Biosystems). Multiple sequence alignments with sequences of a broad selection of genes of the genus *Tessaracoccus* and calculation of sequence similarity levels were carried out by using EzTaxon [11] and CLUSTAL_X 2.1 [12]. Evolutionary distance matrices were generated by the neighbour-joining method described by Jukes and Cantor [13]. Phylogenetic trees were generated using the neighbour-joining [14], least-squares [15], maximum-likelihood [16] and maximum-parsimony [17] algorithms in the PHYLIP package [18]. Tree topology was evaluated by the bootstrap resampling method [19] with 1000 replicates of the neighbour-joining dataset with the SEQBOOT and CONSENSE programs from the PHYLIP package. DNA–DNA hybridization experiments were performed with strain CAU 1319^T and *T. lapidicaptus* IPBSL-7^T, according to Ezaki et al. [20], by using the fluorometric microplate method, as modified by Goris *et al.* [21]. The mol% G+C content of the genomic DNA was determined using HPLC by the method of Tamaoka and Komagata [22].

The almost complete 16S rRNA gene sequence of strain CAU 1319^T (1436 bp) was determined and compared with the available reference sequences in the GenBank database (accessed October 2016). Phylogenetic analysis, based on the 16S rRNA gene sequence, indicated that the strain belonged to the genus *Tessaracoccus*. The neighbour-joining phylogenetic tree is presented in Fig. 1. The trees generated with other treeing methods showed a similar topology (data not shown). In the neighbour-joining tree, strain CAU 1319^T clustered with *T. lapidicaptus* IPBSL-7^T (similarity 97.69 %), *T. bendigoensis* Ben 106^T (similarity 95.64 %) and *T. flavescens* SST-39^T (similarity 95.84 %). The G+C content of the DNA of strain CAU 1319^T was 69.5 mol%. The DNA–DNA hybridization value between CAU 1319^{T}and the most closely related reference strain, *T. lapidicaptus* IPBSL-7^T, was 24 %±0.2. This is well below the 70 % cut-off point recommended by Wayne *et al.* [23] for the delineation of genomic species, supporting the proposal that strain CAU 1319^T represents a separate species.

Strain CAU 1319^T and the three reference strains were routinely cultivated on MA at 30 °C to examine all morphological, physiological and biochemical characteristics, except for spore formation, which was assessed on nutrient sporulation medium [24]. Cell morphology was examined by light microscopy (model DM 1000; Leica) and transmission electron microscopy (TEM, JEM 1010, JEOL) using cells from an exponentially growing culture. For TEM, the cells were negatively stained with 1 % (w/v) phosphotungstic acid and the grids were examined after being air-dried. Gram staining was carried out using a Gram staining kit (bioMérieux), according to the manufacturer's instructions. Gliding motility was examined on an MB culture for 72 h using the hanging-drop method [25].

Growth on MA medium at 4, 10, 20, 25, 30, 37, 40, 45 and 55 °C in an aerobic incubator (MIR-253; Sanyo) and in an anaerobic chamber (Bactron; Sheldon) was evaluated by measuring the growth on a plate after 3 days. The pH range for growth was investigated in MB that had been adjusted to pH 4.5–11.5 (at intervals of 0.5 pH unit) by using sodium acetate/acetic acid and Na_2CO_3 buffers. Growth patterns were investigated at 30 °C using a range of 0–15 % (w/v) MB medium which was a modified version of Difco medium, by excluding NaCl and adding 0.45 % (w/v) $MgCl_2.6H_2O$ and 0.06 % (w/v) KCl. Oxidase activity was evaluated from the oxidation of 0.1 % (w/v) tetramethyl-*p*-phenylenediamine [26]. Catalase activity was determined by bubble production in 3 % (v/v) H_2O_2 solution. Hydrolysis of gelatin, casein, starch and citrate were determined according to Lányi [27] and Smibert and Krieg [28]. Biochemical characterizations were tested using the API 20E, API 20NE, API 50CH and API ZYM (bioMérieux) systems. API 20E, API 20NE, API 50CH and API ZYM strips were read after 24 h, respectively. Antibiotic susceptibility was tested on MA at 30 °C by using Sensi-Disc susceptibility test discs (BBL), antibiotics tested were: amoxicillin (20 µg), ampicillin (10 µg), carbenicillin (100 µg), cefoxitin (30 µg), cephalothin (30 µg), chloramphenicol (30 µg), erythromycin (15 µg), gentamicin (10 µg), kanamycin (30 µg), penicillin (10 U µg), nalidixic acid (30 µg), polymyxin B (300 U), rifampin (5 µg), streptomycin (10 µg), tetracycline (30 µg), tobramycin (10 µg) and trimethoprim/sulfamethoxazole (1.25/23.75 µg).

Detailed phenotypic characteristics of strain CAU 1319^{T}are given in the species description and in Table 1. Overall, results obtained in this study are in accord with the type strains of closely related species of the genus *Tessaracoccus*. However, the strain differed from its closest relatives, *T. lapidicaptus* IPBSL-7^T, *T. bendigoensis* Ben 106^T and *T. flavescens* SST-39^T in terms of optimum salinity for growth, and positivity for urease, valine arylamidase, crystine arylamidase, trypsin, aesculin ferric citrate, negativity for α-galactosidase, α-glucosidase and weak positivity for acid production from D-fructose. Strain CAU 1319^T was Gram-stain positive, facultatively anaerobic, non-motile and non-spore-forming. Cells were rod-shaped, and approximately 0.2–0.5 µm in diameter and 2.1–3.0 µm in length. Colonies were pale yellow, circular, convex with diameters of 0.1–0.3 mm after 3 days of cultivation on MA at 30 °C. Flagella were not observed (Fig. S1, available in the online Supplementary Material). Growth of strain CAU 1319^T was observed at 20–37 °C (optimum 30 °C) and pH 6.5–9.0 (optimum pH 7.5) in the presence of 0–3 % (w/v) NaCl (optimum 2 %). Strain CAU 1319^T showed resistance against gentamicin, kanamycin, nalidixic acid, polymyxin B, tobramycin, and trimethoprim/sulfamethoxazole.

The chemotaxonomic properties of CAU 1319^T and the three reference strains were compared under the same culture conditions. For fatty acid analysis, all strains were harvested from marine agar (MA; Difco) after cultivation at 30 °C for 3 days. 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 moment of harvesting 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.* [29] and separated by a 6890 N automated gas chromatography system (Agilent). Peaks were identified by using the Microbial Identification software package (MOORE library ver. 5.0; MIDI database TSBA6).

The fatty acid profile of strain CAU 1319^T is shown in Table 2. The main fatty acid was anteiso-$C_{15:0}$, which is a characteristic element of the fatty acids of the type strains of the species of the genus *Tessaracoccus*. The fatty acid profile also contained: iso-$C_{14:0}$, $C_{14:0}$, iso-$C_{13:0}$ 3-OH, iso-$C_{15:1}$ F, anteison-$C_{15:1}$ A, iso-$C_{15:0}$, $C_{16:0}$ N alcohol, iso-$C_{16:0}$, $C_{16:0}$, anteiso-$C_{17:1}\omega 9c$, iso-$C_{17:0}$, anteiso-$C_{17:0}$, $C_{17:0}$, $C_{18:0}$.

The isomer of diaminopimelic acid in the cell wall was analyzed according to Staneck and Roberts [30]. The respiratory quinones of strain CAU 1319^T were extracted as described by Komagata and Suzuki [31] and analyzed by HPLC (Waters) using an isocratic solvent system [methanol/isopropyl ether (3 : 1, v/v)] and a flow rate of 1 ml min^{-1}. The polar lipids of strain CAU 1319^T were separated by using two-dimensional TLC, according to the method of Minnikin *et al.* [32]. The plate was sprayed with 10 % (v/v) ethanolic molybdatophosphoric acid (for total lipids), molybdenum

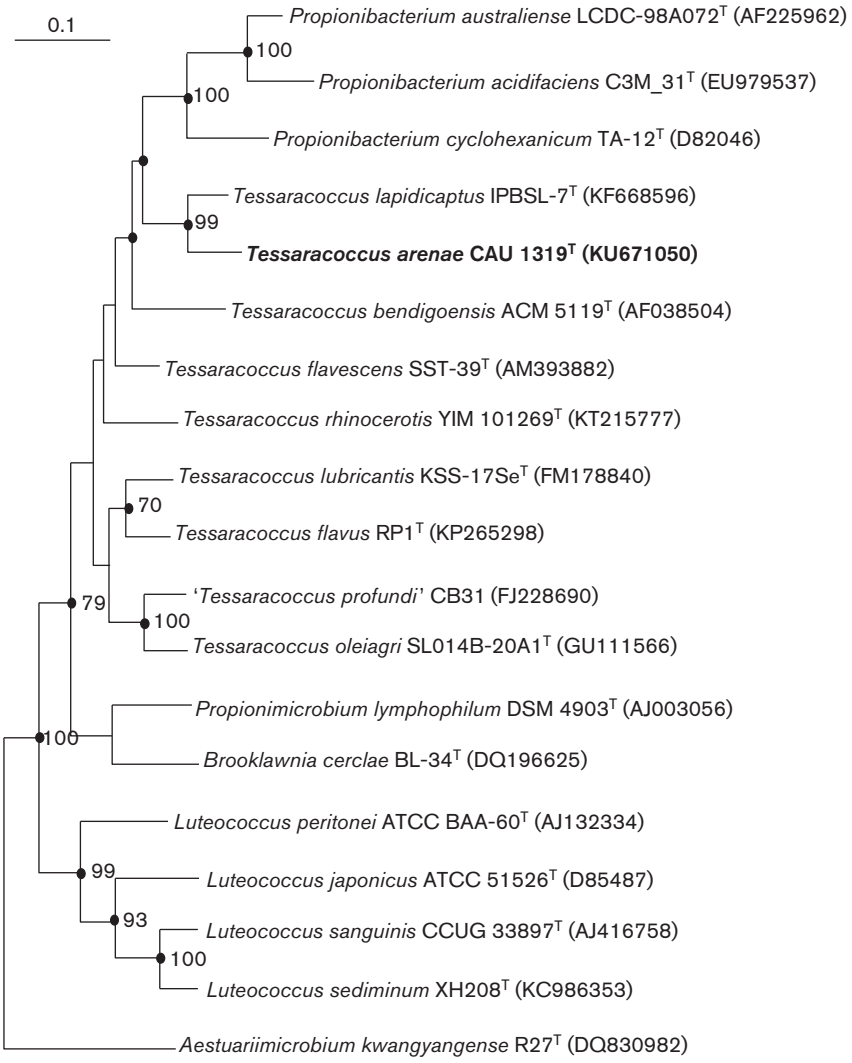

Fig. 1. Neighbour-joining phylogenetic tree, based on nearly complete 16S rRNA gene sequences, showing the relationships between strain CAU 1319^T and other members of the family *Propionibacteraceae*. Bootstrap values less than 70 % are not shown. Nodes were also recovered in the trees generated with the maximum-likelihood and least squares algorithms. *Aestuariimicrobium kwangyangense* R27^T (DQ830982) was used as an outgroup organism. Bar, 0.1 substitutions per nucleotide position.

Table 1. Differential characteristics of strain CAU 1319^T and type strains of the most closely related species of the genus *Tessaracoccus*

Strains: 1, CAU 1319^T; 2, *T. lapidicaptus* IPBSL-7^T; 3, *T. bendigoensis* Ben 106^T; 4, *T. flavescens* SST-39^T. All strains were non-motile, positive for catalase, and negative for oxidase and citrate. All strains were positive for esterase, esterase lipase, leucine arylamidase, β-galactosidase, β-glucosidase, and the hydrolysis of aesculin. All strains were negative for alkaline phosphatase, lipase, α-chymotrypsin, acid phosphatase, β-glucuronidase, indole and H_2S production, arginine dihydrolase, lysine and ornithine decarboxylase, and the hydrolysis of gelatin. Data are all from the present study. +, Positive; −, negative; W, weakly positive

Characteristic	1	2	3	4
Temperature range for growth (°C)	20–37	15–40	15–40	15–40
Optimum temperature	30	37	25	30
NaCl concn range for growth (%, w/v)	0–3	0–2	0–8	0–8
Optimum NaCl concn	2	0	4	0
pH range for growth	6.5–9.0	6.0–9.0	5.0–9.0	6.0–9.0
Optimum pH	7.5	8.0	8.0	8.5
Hydrolysis of urea	+	−	−	−
Acetoin production	−	+	−	−
Acid production (Data from API 20E)				
L-Arabinose	−	−	+	+
L-Rhamnose	−	+	−	−
D-Mannitol	−	+	+	−
Inositol	−	W	−	+
Fermentation of D-glucose	−	+	−	+
Carbon source assimilation (Data from API 20NE)				
D-Glucose	−	+	−	+
Arabinose	−	−	+	−
Mannose	−	+	−	+
Mannitol	−	+	−	−
N-Acetyl-glucosamine	−	−	−	+
Maltose	−	+	−	+
Acid production (Data from API 50CH)				
Glycerol	−	+	−	−
D-Xylose	−	−	+	+
D-Glucose	W	+	−	−
D-Fructose	W	−	−	−
D-Mannose	−	+	−	−
Aesculin ferric citrate	+	−	−	−
Cellobiose	−	+	−	−
Maltose	−	−	+	+
Lactose	−	−	+	−
Sucrose	W	−	+	W
Raffinose	−	+	−	−
API ZYM				
Valine arylamidase	+	−	−	−
Crystine arylamidase	+	−	−	−
Trypsin	+	−	−	−
Napthol-AS-BI-phosphohydrolase	−	−	W	W
α-Galactosidase	−	+	+	+

Table 2. Cellular fatty acid compositions (as a percentage of the total) of strain CAU 1319^T and type strains of the most closely related species of the genus *Tessaracoccus*

Strains: 1, CAU 1319^T; 2, *T. lapidicaptus* IPBSL-7^T; 3, *T. bendigoensis* Ben 106^T; 4, *T. flavescens* SST-39^T. Data were all taken from this study. TR, trace (<1 %).

Fatty acids	1	2	3	4
Saturated				
$C_{14:0}$	2.3	2.6	1.4	–
$C_{15:0}$	–	–	–	–
$C_{15:0}$ 2-OH	TR	–	–	–
$C_{16:0}$	2.8	4.4	2.0	TR
$C_{16:0}$ N alcohol	1.6	–	–	1.1
$C_{17:0}$	2.4	TR	–	TR
$C_{18:0}$	TR	2.3	–	TR
Branched-chain				
anteiso-$C_{15:0}$	65.1	60.6	58.6	54.7
anteiso-$C_{15:1}$ A	TR	1.3	–	1.3
anteiso-$C_{17:0}$	5.5	2.3	1.3	3.0
anteiso-$C_{17:1}\omega$9c	TR	1.4	–	–
iso-$C_{13:0}$ 3-OH	1.0	1.1	–	1.0
iso-$C_{14:0}$	3.6	1.9	4.9	6.9
iso-$C_{15:0}$	1.1	5.4	4.4	6.7
iso-$C_{15:1}$ F	1.9	TR	–	1.0
iso-$C_{16:0}$	8.0	2.3	3.4	11.4
iso-$C_{17:0}$	TR	TR	–	1.3
Summed features*				
1	TR	TR	TR	TR
2	TR	–	14.1	2.5
4	TR	–	4.4	2.3

*Summed features are groups of fatty acids that could not be separated by GC with the MIDI system. Summed features 1, 2 and 4 comprised $C_{13:0}$ 3-OH/$C_{15:1}$ i H, $C_{14:0}$ 3-OH/ iso-$C_{16:1}$ I, and anteiso-$C_{17:1}$ B/ iso I, respectively.

blue (for phospholipids), ninhydrin (for aminolipids), α-naphthol/sulphuric acid reagent (for glycolipids) (Sigma-Aldrich). Bacterial polyamine was determined by TLC after extraction, as described by Busse and Auling [33].

The cell wall peptidoglycan of strain CAU 1319^T had LL-DAP as the diagnostic diamino acid, which is a characteristic of species of the genus *Tessaracoccus*. Strain CAU 1319^T contained MK-9 (H_4) as the respiratory quinone , as in *T. lapidicaptus* IPBSL-7^T, *T. flavescens* SST-39 and *T. lubricantis* KSS-17Se^T. The polar lipid profile of strain CAU 1319^T comprised phosphatidylglycerol, phosphatidylinositol, two unidentified aminolipids, three unidentified phospholipids, and one unidentified glycolipid (Fig. S2). This polar lipid pattern was similar to those of type strains of species of the genus *Tessaracoccus* in that phosphatidylglycerol was a major polar lipid, but the novel strain differed from the most closely related strain, *T. lapidicaptus* IPBSL-7^T, due to the absence of phosphoglycolipid (Fig. S3). The polyamine pattern predominantly comprised spermine and spermidine, as in other species of the genus *Tessaracoccus* [3, 6, 34].

To conclude, data from the phenotypic, chemotaxonomic, phylogenetic and genotypic studies provide sufficient evidence to recognize strain CAU 1319^T as a representative of a novel species of the genus *Tessaracoccus*, for which the name *Tessaracoccus arenae* sp. nov. is proposed.

DESCRIPTION OF *TESSARACOCCUS ARENAE* SP.NOV.

Tessaracoccus arenae (a.re′nae. L. gen. fem. n. *arenae* of sand).

Cells are Gram-stain positive, non-motile, non-spore-forming, facultatively anaerobic, rod- shaped (0.2–0.5×2.1–3.0 µm). Colonies grown on MA after 3 days of incubation at 30 °C are pale yellow, circular and convex with entire margins. Growth occurs at 20–37 °C (optimum, 30 °C), at pH 6.5–9.0 (optimum, 7.5), and with NaCl at 0–3 % (w/v) (optimum, 2 %). Catalase positive but oxidase negative. Aesculin and urea are hydrolyzed, but casein, gelatin and starch are not hydrolyzed. Nitrate is reduced. Citrate is not utilized. The production of acetoin (VP), H_2S and indole are negative. In API 20NE, assimilation of D-glucose, maltose, mannitol, mannose and *N*-acetyl-glucosamine are negative. Acid production in API 20E was negative for D-mannitol, L-arabinose, L-rhamnose, inositol and D-glucose fermentation. The API 50 CH system showed that aesculin ferric citrate is strongly positive, D-fructose, D-glucose and D-surcrose are weakly positive, but cellobiose, lactose, maltose, D-mannose, raffinose, D-xylose and glycerol are negative. In API ZYM strips, β-galactosidase, β-glucosidase, crystine arylamidase, esterase, esterase lipase, leucine arylamidase, valine arylamidase and trypsin are positive, but acid phosphatase, alkaline phosphatase, α-chymotrpsin, α-fucosidase, α-galactosidase, α-glucosidase, α-mannosidase, β-glucoronidase, lipase, *N*-acetyl-β-glucosaminidase and Napthol-AS-BI-phosphohydrolase are negative. The major cellular fatty acid is anteiso-$C_{15:0}$. LL-diaminopimelic acid is the diagnostic diamino acid in the cell wall peptidoglycan. The predominant menaquinone is MK-9(H_4). The major polar lipids are phosphatidylglycerol, phosphatidylinositol, two unidentified aminolipids, three unidentified phospholipids, and an unidentified glycolipid. The main polyamines are spermine and spermidine.

The type strain, CAU 1319^T (=KCTC 39760^T=NBRC 111973^T), was isolated from sea sand collected from Eurwangri beach in Incheon, Republic of Korea. The G+C content of the DNA is 69.5 mol%.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
The authors have declared that no ethical issues exist.

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