Roseovarius aquimarinus sp. nov., a slightly halophilic bacterium isolated from seawater

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A Gram-stain-negative, non-spore-forming, rod-shaped, motile, facultatively anaerobic bacterium, designated CAU 1059^T, was isolated from a seawater sample from Jeju Island, Republic of Korea. The bacterium grew optimally at 37 °C, at pH 7.0 and in the presence of 2 % (w/v) NaCl. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain CAU 1059^T belonged to the genus *Roseovarius*. It exhibited only 91.5–96.9 % sequence similarity to the type strains of recognized *Roseovarius* species. Similar to other species of the genus *Roseovarius* , strain CAU 1059^T had ubiquinone-10 (Q-10) as the predominant ubiquinone and $C_{16:0}$ and summed feature 8 ($C_{18:1}\omega7c/\omega6c$) as the major fatty acids. The polar lipid pattern consisted of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and phosphatidylcholine; three unidentified phospholipids, two aminolipids, an aminophospholipid and nine other lipids were also found. The G+C content of the genomic DNA was 61.9 mol%. On the basis of the data provided, strain CAU 1059^T should be classified as representing a novel species of the genus *Roseovarius*, for which the name *Roseovarius aquimarinus* sp. nov. is proposed. The type strain is CAU 1059^T (= KCTC 32014^T=CCUG 64792^T).

The genus *Roseovarius*, a member of the family *Rhodobacteraceae*, was proposed by Labrenz *et al.* (1999) with the description of *Roseovarius tolerans* as the type species of the genus within the family *Alphaproteobacteria*. At the time of writing, this genus consists of 18 recognized species (Parte, 2014): *R. tolerans* (Labrenz *et al.*, 1999), *R. nubinhibens* (González *et al.*, 2003), *R. crassostreae* (Boettcher *et al.*, 2005), *R. mucosus* (Biebl *et al.*, 2005), *R. aestuarii* (Yoon *et al.*, 2008), *R. pacificus* (Wang *et al.*, 2009), *R. halotolerans* (Oh *et al.*, 2009), *R. nanhaiticus* (Wang *et al.*, 2010), *R. marinus* (Jung *et al.*, 2011), *R. indicus* (Lai *et al.*, 2011), *R. halocynthiae* (Kim *et al.*, 2012), *R. litoreus* (Jung *et al.*, 2012), *R. sediminilitoris* (Park & Yoon, 2013), *R. lutimaris* (Choi *et al.*, 2013), *R. marisflavi* (Li *et al.*, 2013), *R. azorensis* (Rajasabapathy *et al.*, 2014), *R. albus* (Lucena *et al.*, 2014) and *R. gaetbuli* (Park *et al.*, 2014). Members of the genus *Roseovarius* have been isolated from various marine habitats such as hypersaline seawater, marine sediment, tidal flat, oysters, sea squirt and algal cells (González *et al.*, 2003; Boettcher *et al.*, 2005; Wang *et al.*, 2010; Jung *et al.*, 2011; Kim *et al.*, 2012; Choi *et al.*, 2013). Tolerance and adaptation to extreme saline environments makes them potential candidates for exploitation in biotechnology (Oren, 2010; Shivanand & Mugeraya, 2011). In the course of screening for bacteria from marine environments, a novel, slightly halophilic bacterium, designated CAU 1059^T, was isolated from a seawater sample collected from Jeju Island in the Republic of Korea (33° 229′ 47.300″ N 126° 32′ 59.320″ E). The aim of the present study was to describe the taxonomic position of this bacterial strain by using a polyphasic characterization.

Isolation was performed according to Gordon & Mihm (1962) using marine agar 2216 (MA; Difco). The seawater sample was 10-fold diluted with sterilized 0.9 % NaCl solution. Aliquots of 100 µl (diluted 10^{-2} to 10^{-7}) were spread on MA in triplicate and incubated at 30 °C for 14 days under aerobic conditions. Strain CAU 1059^T was one of the isolates and was preserved at (70 °C in marine broth 2216 (MB; Difco) supplemented with 25 % (v/v) glycerol for taxonomic studies. The type strains of the most closely related species, *R. nanhaiticus* NH52J^T (= KACC 15612^T), *R. lutimaris* 112^T (= KACC 16185^T)

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CAU 1059^T is JX233494.

Two supplementary figures are available with the online Supplementary Material.

and *R. tolerans* EL-172^T (= KACC 13027^T), were obtained from the Korean Agricultural Culture Collection (KACC) and used as reference strains.

Genomic DNA of strain CAU 1059^T was extracted according to the method of Marmur (1961). The 16S rRNA gene was amplified by PCR using the universal primers 27F and 1525R (Lane, 1991) and the conditions described by Nam *et al.* (2004). The amplified 16S rRNA gene was sequenced directly using a BigDye Terminator Cycle Sequencing kit and a 3730 automatic DNA sequencer (Applied Biosystems Life Technologies). Multiple alignments with sequences of a broad selection of *Roseovarius* and related species and calculation of sequence similarities were determined using the EzTaxon – EzBioCloud.net (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 & Cantor (1969). Phylogenetic trees were reconstructed using the neighbour-joining (Saitou & Nei, 1987), least-squares (Fitch & Margoliash, 1967) and maximum-likelihood (Felsenstein, 1981) algorithms in the PHYLIP package (Felsenstein, 1989). Tree topology was evaluated by the bootstrap resampling method (Felsenstein, 1985) with 1000 replicates of the neighbour-joining dataset with the SEQBOOT and CONSENSE programs from the PHYLIP package. The level of DNA–DNA relatedness was determined using the fluorometric microplate method (Goris *et al.*, 1998). The G+C content of the genomic DNA was determined using HPLC according to the method of Tamaoka & Komagata (1984).

The nearly complete 16S rRNA gene sequence of strain CAU 1059^T (1433 bp) was determined and compared with the corresponding sequences of other bacterial strains in the GenBank database. Phylogenetic analysis indicated that the strain fell within the genus *Roseovarius* (Fig. 1). Strain CAU 1059^T exhibited highest 16S rRNA gene sequence similarity to *R. nanhaiticus* NH52J^T (96.9 %), and 91.5–95.4 % similarity to the type strains of other *Roseovarius* species. The mean ($\pm$SD) DNA–DNA relatedness value determined between CAU 1059^T and *R. nanhaiticus* NH52J^T was 22.0$\pm$0.5 %. This value is lower than the 70 % cut-off point recommended by Wayne *et al.* (1987) for the description of genomic species, supporting the proposal that strain CAU 1059^T represents a distinct genomic species. The G+C content of the DNA of strain CAU 1059^T was 61.9 mol%, a value in the range reported for *Roseovarius* species (Labrenz *et al.*, 1999; Wang *et al.*, 2009, 2010).

Strain CAU 1059^T was cultivated routinely on MA at 37 °C to investigate all morphological, physiological and biochemical characteristics except for spore formation, which was assessed on nutrient sporulation medium (Nicholson & Setlow, 1990). Cell morphology was examined using a DM 1000 light microscope (Microsystems) and a JEM 1010 transmission electron microscope (JEOL) using cells from an exponentially growing culture. For transmission electron microscopy, 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 the bioMérieux Gram staining kit according to the manufacturer's instructions. Motility was assessed in an MB culture for 72 h using the hanging-drop method (Bowman, 2000). Growth in MB at 2, 4, 10, 20, 30, 37, 40, 42, 45, 50 and 55 °C was evaluated in an MIR-253 aerobic incubator (Sanyo Electric Biomedical) and in a Bactron anaerobic chamber (Sheldon Manufacturing) after 72 h of incubation. Growth at various pH was tested at 37 °C in MB adjusted to pH 4.0–10.0 (at increments of 0.5 pH units) by using sodium acetate/acetic acid and Na_2CO_3 buffers. Growth in the absence of NaCl and in the presence of 1–15.0 % (w/v) NaCl (at 1 % intervals) was investigated at 37 °C in MB prepared according to the formula of the Difco medium except that NaCl was excluded and that 0.45 % (w/v) $MgCl_2 . 6H_2O$ and 0.06 % (w/v) KCl were added. Catalase and oxidase activities were tested as described by Cappuccino & Sherman (2002). Hydrolysis of casein, starch, gelatin and aesculin was determined according to Smibert & Krieg (1994). Acid production from carbohydrate metabolism, enzyme activity and other biochemical features were tested using the API ZYM and API 20E systems (bioMérieux). API ZYM strips were read after 24 h at 37 °C, and API 20E strips after 36 h at 37 °C. Susceptibility to antibiotics was examined on MA at 37 °C using Sensi-Disc susceptibility test discs (BBL) containing the following compounds: 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), rifampicin (5 μg), streptomycin (10 μg), tobramycin (10 μg), nalidixic acid (30 μg) and trimethoprim/sulfamethoxazole (1.25/23.75 μg). Susceptibility was indicated by an inhibition zone >10 mm in diameter whereas resistance was represented by the absence of inhibition zones.

The morphological, cultural, physiological and biochemical characteristics of strain CAU 1059^T are given in Table 1 and in the species description. Overall, the results obtained in this study are in agreement with previously published data for *Roseovarius* species. Cells of CAU 1059^T were Gram-stain-negative, non-spore-forming, motile rods. CAU 1059^T was isolated from a saline environment; it required NaCl for growth (1–13 %, w/v). The isolate was motile by the hanging-drop method and transmission electron microscopy confirmed the presence of a single polar flagellum (Fig. S1, available in the online Supplementary Material). Strain CAU 1059^T did not hydrolyse casein, starch, gelatin or aesulin, but it had enzyme activities of acid phosphatase and naphthol-AS-BI-phosphohydrolase. However, strain CAU 1059^T differed from its closest relatives and from the type strain of the type species of the genus, *R. tolerans* EL-172^T (Labrenz *et al.*, 1999), by its nitrate reduction, optimum temperature for growth and tolerance of NaCl.

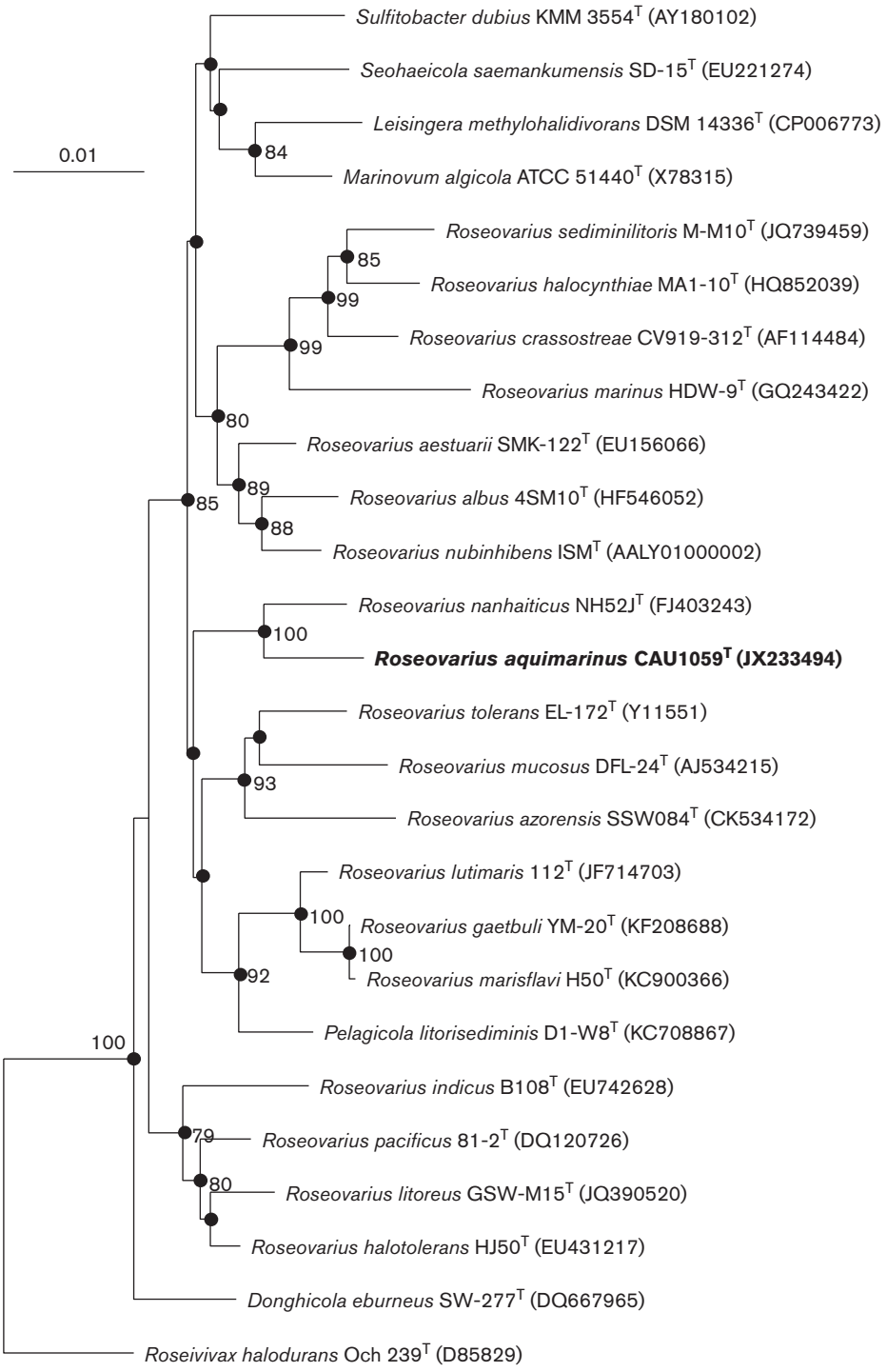

Fig. 1. Neighbour-joining phylogenetic tree based on nearly complete 16S rRNA gene sequences showing the relationships between strain CAU 1059^T, the type strains of recognized *Roseovarius* species and other representatives of the family *Rhodobacteraceae*. Filled circles indicate that the corresponding nodes were also recovered in the trees generated with the maximum-likelihood and least-squares algorithms. Numbers at nodes indicate levels of bootstrap support based on a neighbour-joining analysis of 1000 resampled datasets; only values >70 % are given. Bar, 0.01 substitutions per nucleotide position. *Roseivivax halodurans* Och 239^T (D85829) was used as an outgroup organism.

Table 1. Differential properties between strain CAU 1059^T and the type strains of the most closely related *Roseovarius* species

Strains: 1, CAU 1059^T; 2, *R. nanhaiticus* NH52J^T; 3, *R. lutimaris* 112^T; 4, *R. tolerans* EL-172^T (type species). All strains are motile. Data for the reference strains were taken from Choi *et al.* (2013). +, Positive; −, negative; W, weakly positive; NA, no data available.

Characteristic	1	2	3	4
Nitrate reduction	+	−	+	−
Ranges for growth				
Temperature (°C)	4–40	4–45	15–40	<3–43.5
NaCl (%, w/v)	1–13	0.5–10	1–6	<1–10
pH	5.5–9.0	3.0–10.0	5.0–9.0	5.3 to >9
Enzyme activities:				
Cystine arylamidase	W	W	−	+
Trypsin	W	W	−	W
α-Chymotrypsin	W	W	−	−
Acid phosphate	+	+	−	+
α-Galactosidse	−	W	−	−
Susceptibility to:				
Chloramphenicol	+	NA	+	+
Polymyxin B	−	−	−	−
Streptomycin	+	+	+	+
Kanamycin	+	+	−	NA
Acid production from:				
D-Glucose	−	−	W	−
D-Sorbitol	−	W	W	−
D-Mannitol	−	−	−	−
DNA G+C content (mol%)	61.9	62.0*[a]	58.2	63.3–63.4[b]

*Data taken from: *a*, Wang *et al.*, 2010; *b*, Labrenz *et al.*, 1999.

For fatty acid analysis, cell mass of strain CAU 1059^T was harvested from MA after cultivation for 3 days at 37 °C. The physiological age of the biomasses harvested for fatty acid analysis was standardized by observing growth development during incubation of the three different cultures and choosing the point of harvesting according to the standard MIDI protocol (Sherlock Microbial Identification System version 6.1). Cellular fatty acid methyl esters were obtained according to Minnikin *et al.* (1980) and separated using a 6890N automated gas chromatography system (Agilent Technologies). Peaks were identified by using the Microbial Identification software package (MOORE library ver. 5.0; MIDI database TSBA6). For polar lipid analysis, cells of strain CAU 1059^T were harvested from MA after cultivation for 3 days at 37 °C. The polar lipids of strain CAU 1059^T were identified using two-dimensional TLC (Silica gel 60 F 254, 20 × 20 cm; Merck) according to the method of Minnikin *et al.* (1984). The plates were sprayed with 10 % ethanolic molybdatophosphoric acid (for total lipids), molybdenum blue (for phospholipids), ninhydrin (for aminolipids), α-naphthol/sulphuric acid reagent (for glycolipids) and Dragendorff reagent (for phosphatidylcholine) (Sigma-Aldrich). Isoprenoid quinones were separated by HPLC using an isocratic solvent system (Komagata & Suzuki, 1987).

The chemotaxonomic properties support the attribution of strain CAU 1059^T to the genus *Roseovarius* (Labrenz *et al.*, 1999). The predominant isoprenoid quinone detected in strain CAU 1059^T was ubiquinone-10 (Q-10), which is consistent with data for the genus *Roseovarius*, including the type species of the genus, *R. tolerans* (Labrenz *et al.*, 1999). The fatty acid profile of strain CAU 1059^T is shown in Table 2, together with those of its closest phylogenetic relatives in the genus *Roseovarius* and the type species of the genus, *R. tolerans* (Labrenz *et al.*, 1999; Wang *et al.*, 2010; Choi *et al.*, 2013). The predominant fatty acids were summed feature 8 ($C_{18:1}\omega7c/\omega6c$) (67.3 %) and $C_{16:0}$ (10.7 %). $C_{18:1}\omega7c$ 11-methyl (6.6 %), $C_{12:0}$ (3.9 %), $C_{19:0}\omega8c$ cyclo (2.5 %), $C_{12:0}$ 3-OH (2.5 %), $C_{18:0}$ (1.4 %), summed feature 3 ($C_{16:1}\omega7c/\omega6c$) (0.8 %), $C_{14:0}$ (3.9 %), summed feature 7 ($C_{19:1}\omega6c$) (0.7 %) and $C_{10:0}$ (0.6 %) were also present. This fatty acid profile is typical for most *Alphaproteobacteria* but strain CAU 1059^T could be distinguished from closely related *Roseovarius* species by the presence/absence of minor fatty acids, namely $C_{14:0}$, $C_{17:0}$ cyclo, $C_{17:1}\omega8c$, $C_{12:0}$ 2-OH, $C_{12:1}$ 3-OH, $C_{16:0}$ 2-OH and $C_{18:1}$ 2-OH, which vary in these species. Strain CAU 1059^T contained diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and phosphatidylcholine, which were identified previously in *R. tolerans* (Labrenz *et al.*, 1999), *R. mucosus* (Biebl *et al.*, 2005), *R. halotolerans* (Oh *et al.*, 2009), *R. indicus* (Lai *et al.*, 2011), *R. halocynthiae* (Kim *et al.*, 2012), *R. sediminilitoris* (Park & Yoon, 2013) and *R. azorensis*

Table 2. Cellular fatty acid compositions (%) of strain CAU 1059^T and the type strains of the most closely related *Roseovarius* species

Strains: 1, CAU 1059^T; 2, *R. nanhaiticus* NH52J^T; 3, *R. lutimaris* 112^T; 4, *R. tolerans* EL-172^T (type species). All data were from this study. Fatty acids amounting to <0.5 % of the total fatty acids in all strains are not shown. TR, Trace amount (<0.5 %); −, not detected.

Fatty acid	1	2	3	4
Saturated				
$C_{10:0}$	0.57	0.54	−	−
$C_{12:0}$	3.86	3.64	3.35	−
$C_{14:0}$	0.76	TR	−	−
$C_{16:0}$	10.72	14.71	15.48	10.52
$C_{17:0}$	TR	−	TR	4.93
$C_{18:0}$	1.43	1.31	0.67	0.92
Unsaturated				
$C_{17:0}$ cyclo	−	−	9.27	−
$C_{17:1}\omega 8c$	−	−	−	1.21
$C_{18:1}\omega 7c$ 11-methyl	6.59	14.11	4.59	7.17
$C_{19:0}$ 10-methyl	TR	−	−	−
$C_{19:0}$ cyclo $\omega 8c$	2.53	−	5.27	−
Branched-chain				
iso-$C_{13:0}$ 3-OH	TR	−	0.50	TR
iso-$C_{15:1}$ F	TR	TR	0.67	−
Hydroxy				
$C_{12:0}$ 2-OH	−	−	−	0.63
$C_{12:0}$ 3-OH	2.51	2.97	−	−
$C_{12:1}$ 3-OH	−	−	−	3.05
$C_{15:0}$ 2-OH	−	−	−	TR
$C_{16:0}$ 2-OH	−	−	−	0.51
$C_{18:1}$ 2-OH	−	−	2.08	−
Summed features*				
3	0.80	0.76	3.96	0.77
7	0.74	TR	−	−
8	67.28	60.63	50.07	69.76

*Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 3, $C_{16:1}\omega 7c/\omega 6c$; summed feature 7, $C_{19:1}\omega 6c$; summed feature 8, $C_{18:1}\omega 7c/\omega 6c$.

(Rajasabapathy *et al.*, 2014), but this profile differed from those of the closely related species *R. gaetbuli* (Park *et al.*, 2014) and *R. lutimaris* (Choi *et al.*, 2013), which lack diphosphatidylglycerol. Similarly, *R. mucosus* (Biebl *et al.*, 2005) and *R. marisflavi* (Li *et al.*, 2013) lack the characteristic unidentified aminolipid present in CAU 1059^T and other related *Roseovarius* species. In addition, CAU 1059^T contained three unidentified phospholipids, including two aminolipids, an aminophospholipid and nine other lipids (Fig. S2). The polar lipid composition of other species of the genus *Roseovarius* clearly differs from this unidentified lipid composition. These data provide sufficient evidence to recognize strain CAU 1059^T as representing a novel species of the genus *Roseovarius*, for which the name *Roseovarius aquimarinus* sp. nov. is proposed.

Description of *Roseovarius aquimarinus* sp. nov.

Roseovarius aquimarinus (a.qui.ma.ri′nus. L. fem. n. *aqua* water; L. adj. *marinus* of the sea; N.L. masc. adj. *aquimarinus* pertaining to seawater).

Cells are Gram-stain-negative, facultatively anaerobic rods, approximately 0.5–0.7 μm in diameter and 0.7–2.7 μm in length. Cells are motile by means of a single flagellum. Endospores are not observed. Colonies on MA are cream-coloured, circular and convex with entire margins after 3 days of incubation at 37 °C. Growth occurs at 4–40 °C (optimum, 37 °C) and at pH 5.5–9.0 (optimum, pH 7.0). Growth occurs with 1–13 % (w/v) NaCl (optimum, 2.0 %). Catalase and oxidase are positive. Casein, starch, gelatin and aesculin are not hydrolysed. Nitrate is reduced to nitrite. API 20E strips show weakly positive results for citrate utilization and hydrolysis of arginine, but negative results for ONPG, lysine decarboxylase, urease and tryptophan deaminase activities, H_2S, indole, Voges–Proskauer and gelatinase production, and fermentation/oxidation of D-glucose, D-mannitol, inositol, D-sorbitol, L-rhamnose, sucrose, melibiose, amygdalin and L-arabinose. In the API ZYM system, weakly positive for alkaline phosphatase, leucine arylamidase, valine arylamidase, acid phosphatase and naphtho-AS-BI-phosphohydrolase and positive for esterase (C4), esterase lipase (C8), crystine arylamidase, trypsin, α-chymotrypsin, *N*-acetyl-β-glucosaminidase and α-fucosidase activities. Lipase (C14), α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase and α-mannosidase activities are absent. Acid production occurs from potassium 5-ketogluconate. Most carbon sources are not utilized except citrate. Susceptible to amoxicillin, ampicillin, carbenicillin, cefoxitin, cephalothin, chloramphenicol, erythromycin, kanamycin, penicillin, rifampicin, streptomycin and tobramycin, but resistant to nalidixic acid, polymyxin B and trimethoprim/sulfamethoxazole. The predominant isoprenoid quinone is Q-10. The major fatty acids (>10 % of the total fatty acids) are summed feature 8 ($C_{18:1}\omega 7c/\omega 6c$) and $C_{16:0}$. The polar lipid profile consists of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine, three unidentified phospholipids, two unidentified aminolipids, an unidentified aminophospholipid and nine unidentified lipids.

The type strain is CAU 1059^T (= KCTC 32014^T=CCUG 64792^T), isolated from seawater collected from Jeju Island in the Republic of Korea. The DNA G+C content of the type strain is 61.9 mol%.

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