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ORIGINAL PAPER

# *Halococcus sediminicola* sp. nov., an extremely halophilic archaeon isolated from a marine sediment

**Kyung June Yim · In-Tae Cha · Tae Woong Whon · Hae-Won Lee · Hye Seon Song · Kil-Nam Kim · Young-Do Nam · Sung-Jae Lee · Jin-Woo Bae · Sung-Keun Rhee · Jong-Soon Choi · Myung-Ji Seo · Seong Woon Roh · Daekyung Kim**

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**Abstract** A novel, red-pigmented and coccoid haloarchaeon, designated strain CBA1101$^T$, was isolated from a marine sediment. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain CBA1101$^T$ is most closely related to the genus *Halococcus* in the family *Halobacteriaceae*. Strain CBA1101$^T$ had a highest 16S rRNA gene sequence similarity of 98.4 % with *Halococcus dombrowskii* DSM 14522$^T$, followed by 93.7–98.3 % with sequences of other type strains in the genus *Halococcus*. The RNA polymerase subunit B′ gene sequence similarity of strain CBA1101$^T$ with that of *Halococcus qingdaonensis* JCM 13587$^T$ is 89.5 % and lower with those of other members of the genus *Halococcus*. Strain CBA1101$^T$ was observed to grow at 25–40 °C, pH 6.0–9.0 and in the presence of 15–30 % (w/v) NaCl, with optimal growth at 35–40 °C, pH 7.0 and with 20 % NaCl. The cells of strain CBA1101$^T$ are Gram-negative and did not lyse in distilled water. The major polar lipids were identified as phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, sulfated diglycosyl diether, unidentified phospholipids and unidentified glycolipids. The genomic DNA G+C content was determined 66.0 mol%. The DNA–DNA hybridization experiment showed that there was less than 40 % relatedness between strain CBA1101$^T$ and the reference species in the genus *Halococcus*. Based on this polyphasic taxonomic analysis, strain

Kyung June Yim and In-Tae Cha contributed equally to this work.

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K. J. Yim · H.-W. Lee · H. S. Song · K.-N. Kim · J.-S. Choi · S. W. Roh (✉) · D. Kim (✉)
Jeju Center and Division of Life Science, Korea Basic Science Institute, Cheju 690-140, Republic of Korea
e-mail: seong18@gmail.com

D. Kim
e-mail: dkim@kbsi.re.kr

K. J. Yim · H. S. Song
Department of Biology, Jeju National University, Cheju 690-756, Republic of Korea

I.-T. Cha · S.-K. Rhee
Department of Microbiology, Chungbuk National University, Ch'ongju 361-763, Republic of Korea

T. W. Whon · S.-J. Lee · J.-W. Bae
Department of Life and Nanopharmaceutical Sciences and Department of Biology, Kyung Hee University, Seoul 130-701, Republic of Korea

Y.-D. Nam
Fermentation and Functionality Research Group, Korea Food Research Institute, Sungnam 463-746, Republic of Korea

M.-J. Seo
Division of Bioengineering, Incheon National University, Inchon 406-772, Republic of Korea

CBA1101$^T$ is considered to represent a new species in the genus *Halococcus*, for which the name *Halococcus sediminicola* sp. nov. is proposed. The type strain is CBA1101$^T$ (=JCM 18965$^T$ = CECT 8275$^T$).

**Keywords** Haloarchaea · *Halococcus sediminicola* · Marine sediment · Polyphasic taxonomy

## Introduction

Extremely halophilic archaea (haloarchaea) are adapted to hypersaline environments, although high salinity is toxic to most cells (Grant 2004). They have usually red or pink pigmented colonies and can grow optimally in the presence of 20–30 % (w/v) NaCl (Colwell et al. 1979). All of the haloarchaea are classified within the family *Halobacteriaceae* in the phylum *Euryarchaeota*. Currently, this family comprises 40 recognized genera based on the list of Prokaryotic names with standing in nomenclature (http://www.bacterio.net/archaea.html). Among the haloarchaea, the genus *Halococcus* was proposed by Schoop (1935) and it currently includes seven validly named species: *Halococcus dombrowskii*, *Hcc. hamelinensis*, *Hcc. morrhuae*, *Hcc. qingdaonensis*, *Hcc. saccharolyticus*, *Hcc. salifodinae* and *Hcc. thailandensis*. The present study assessed the taxonomic position of a new haloarchaeon, designated CBA1101$^T$, to propose a novel species in the genus *Halococcus* using a polyphasic taxonomy approach, according to the proposed minimal standards for the description of new taxa in the order *Halobacteriales* (Oren et al. 1997).

## Materials and methods

### Archaeal strain and culture conditions

Strain CBA1101$^T$ was isolated from a marine sediment (34°88′37″ N, 127°92′13″ E) collected from the bay of Gangjin in the Republic of Korea. A dilution-plating technique was used where marine agar 2216 (MA; BD) was supplemented with 20 % (w/v) NaCl and incubated at 37 °C. A red colony was subcultured repeatedly to obtain a pure culture. To facilitate its long-term preservation, strain CBA1101$^T$ was frozen at −80 °C in marine broth 2216 (MB; BD) supplemented with 5 % (v/v) dimethyl sulfoxide (DMSO). For the comparative taxonomic analyses, *Hcc. dombrowskii* DSM 14522$^T$, *Hcc. morrhuae* DSM 1307$^T$, *Hcc. qingdaonensis* JCM 13587$^T$, *Hcc. thailandensis* JCM 13552$^T$ were selected as reference strains. Reference strains were obtained from DSMZ and JCM culture collections.

### Determination of the 16S rRNA and RNA polymerase subunit B (*rpoB′*) gene sequence and phylogenetic analysis

Genomic DNA of strain CBA1101$^T$ was extracted using a genomic DNA extraction kit (G-spin$^{TM}$; iNtRON Biotechnology). The 16S rRNA gene of strain CBA1101$^T$ was amplified and sequenced using a PCR pre-Mix (iNtRON Biotechnology) and the primer set Arch21F and 1492R (DeLong 1992), as described previously (Roh et al. 2008). PCR-mediated amplification and sequencing of the *rpoB′* genes were carried out according to Minegishi et al. (2010). The nearly full-length 16S rRNA and *rpoB′* gene sequences were assembled using SeqMan software (DNASTAR). EzTaxon-e (Kim et al. 2012) was used to identify closely related species with validly published names and to calculate the pairwise 16S rRNA gene sequence similarities of strain CBA1101$^T$ and its phylogenetic neighbours. The 16S rRNA gene sequences of strain CBA1101$^T$ and related taxa were aligned using the SILVA Incremental Aligner (Pruesse et al. 2012). To analyze the phylogenetic tree of the *rpoB′* gene, the gene sequences of related taxa were obtained from GenBank (http://www.ncbi.nlm.nih.gov). Multiple sequence alignments were carried out using the Clustal_W program. Phylogenetic trees were constructed with the neighbour-joining (NJ) (Saitou and Nei 1987), minimum-evolution (ME) (Nei et al. 1998) and maximum-likelihood (ML) algorithms (Felsenstein 1981) based on Kimura's two-parameter model (Kimura 1980) with 1,000 randomly selected bootstrap replicates using MEGA5 (Tamura et al. 2011).

### Morphological, physiological, and biochemical characterization

All of the phenotypic tests were performed using a complex medium (DSM medium no. 954), which was adjusted to pH 7.0 and contained the following ($l^{-1}$):

5 g casamino acids (BD), 5 g yeast extract (BD), 20 g $MgCl_2 \cdot 6H_2O$, 2 g KCl, 12 g Tris, 0.2 g $CaCl_2 \cdot 2H_2O$ and 200 g NaCl; or a halophilic medium (HMD) that contained the following ($l^{-1}$): 20 g $MgCl_2 \cdot 6H2O$, 5 g $K_2SO_4$, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.1 g yeast extract, 0.5 g $NH_4Cl$, 0.05 g $KH_2PO_4$, 0.5 g casamino acids as the carbon source and 180 g NaCl (Savage et al. 2007), unless indicated otherwise. Reference strains were cultured under identical conditions for comparative tests. The cell morphology and size were examined using a scanning electron microscope (SUPRA 55 VP; Carl Zeiss), as described previously (Lee et al. 2012). Gram staining was performed according to the method for haloarchaea (Dussault 1955). Cell lysis in distilled water was detected by microscopic examination after cells had been suspended in distilled water for 1 week previously. Growth with various NaCl concentrations was tested using medium 954, which was supplemented with 0–30 % (w/v) NaCl at intervals of 5 %. Growth at different temperatures (5–60 °C, at intervals of 5 °C) was assayed using medium 954. The pH range that supported growth was tested in the pH range of 5.0–11.0 at intervals of 1.0 using HMD, which was adjusted by adding the following buffers: 10 mM MES for pH 5.0 and 6.0; 10 mM bis–Tris propane for pH 7.0–9.0; and 10 mM CAPS for pH 10.0 and 11.0. The requirement of $Mg^{2+}$ for growth was determined using HMD without $MgCl_2 \cdot 6H_2O$, which was supplemented with 10 mM bis–Tris propane. Tests for catalase and oxidase activities, nitrate reduction, indole production, urease activity and the hydrolysis of casein and starch were conducted as described by Benson (2002) using medium 954 as the basal medium. Hydrolysis of Tween 20, Tween 40 and Tween 80 was tested as described by Gonzalez et al. (1978), while gelatin hydrolysis was tested according to Smibert & Krieg (1994) using medium 954 as the basal medium. Growth in anaerobic conditions was determined using medium 954 supplemented with either 30 mM nitrate, 5 g L-arginine, 5 g DMSO or 5 g trimethylamine *N*-oxide (TMAO) at 37 °C in an anaerobic chamber (Coy Laboratory Products), where the atmosphere comprised $N_2/CO_2/H_2$ (90:5:5, by vol.). The capacity to utilize various substrates as sole carbon and energy sources was tested using HMD supplemented with 10 mM bis–Tris propane and 1 % (w/v) of the following substrates: acetate, L-alanine, L-arginine, L-aspartate, citrate, D-fructose, fumarate, D-galactose, D-glucose, L-glutamate, glycerol, glycine, DL-lactate, lactose, L-lysine, L-malate, maltose, mannitol, D-mannose, L-ornithine, pyruvate, D-ribose, sorbitol, L-sorbose, starch, succinate, sucrose or D-xylose. $H_2S$ formation was tested by growing strains in medium 954 supplemented with 0.5 % (w/v) $Na_2S_2O_3$ (Cui et al. 2007). For testing antibiotic sensitivity, the strain was inoculated on agar medium plates using antibiotic discs with the following amounts (μg per disc, unless indicated): ampicillin (20), bacitracin (0.1 IU), chloramphenicol (50), ciprofloxacin (10), erythromycin (25), neomycin (50), norfloxacin (20), novobiocin (50), penicillin G (20 IU) and rifampin (10). The strain was incubated for 2 weeks at 37 °C.

### Determination of the DNA G + C content, DNA–DNA hybridization (DDH) and polar lipid analysis

The genomic DNA G + C content of strain CBA1101$^T$ was determined using high-performance liquid chromatography, as described by Mesbah and Whitman (1989). DDH was used to determine the genetic relatedness of strain CBA1101$^T$ and four type strains of *Halococcus* species that shared 16S rRNA gene sequence similarities of >97 % with the isolate. DDH was performed using the fluorometric method with photobiotin-labeled DNA probes and a microwell plate (MaxiSorp, FluoroNunc), as described by Ezaki et al. (1989). Values were determined from five replicates. The polar lipids were extracted and analyzed using thin-layer chromatography (TLC) on a silica gel 60 F254 plate (Merck), according to method of Minnikin et al. (1984). The separated polar lipid spots were detected by spraying each plate with specific detection reagents as follows: sulfuric acid–ethanol (1:2, by vol.) for total lipids, ninhydrin for amino-containing lipids, molybdenum blue for phospholipids and α-naphthol–sulfuric acid for glycolipids. The designations of the polar lipid spots were compared with those of the four reference strains of *Hcc. dombrowskii* DSM 14522$^T$, *Hcc. morrhuae* DSM 1307$^T$, *Hcc. qingdaonensis* JCM 13587$^T$ and *Hcc. thailandensis* JCM 13552$^T$.

## Results and discussion

The nearly full-length 16S rRNA and *rpoB′* gene sequences of strain CBA1101$^T$ were obtained (1,406

and 1,836 bp, respectively; GenBank accession numbers JX989265 and KF700331, respectively). The phylogenetic analyses based on the 16S rRNA gene sequences showed that strain CBA1101$^T$ is closely related to the following members of the genus *Halococcus* in the family *Halobacteriaceae*: *Hcc. dombrowskii* DSM 14522$^T$ (98.4 % 16S rRNA gene sequence similarity), *Hcc. morrhuae* DSM 1307$^T$ (98.3 %), *Hcc. qingdaonensis* JCM 13587$^T$ (98.2 %), *Hcc. thailandensis* JCM 13552$^T$ (97.5 %), *Hcc. hamelinensis* 100A6$^T$ (94.2 %), *Hcc. salifodinae* Blp$^T$ (94.0 %) and *Hcc. saccharolyticus* P-423$^T$ (93.7 %). Strain CBA1101$^T$ clustered with *Hcc. dombrowskii* DSM 14522$^T$, *Hcc. morrhuae* DSM 1307$^T$, *Hcc. qingdaonensis* JCM 13587$^T$ and *Hcc. thailandensis* JCM 13552$^T$ in the phylogenetic trees, with high bootstrap values (99, 99 and 100 % in the NJ, ME, and ML trees, respectively) (Fig. 1a). The phylogenetic analyses based on *rpoB′* gene sequences showed that strain CBA1101$^T$ is closely related to the following

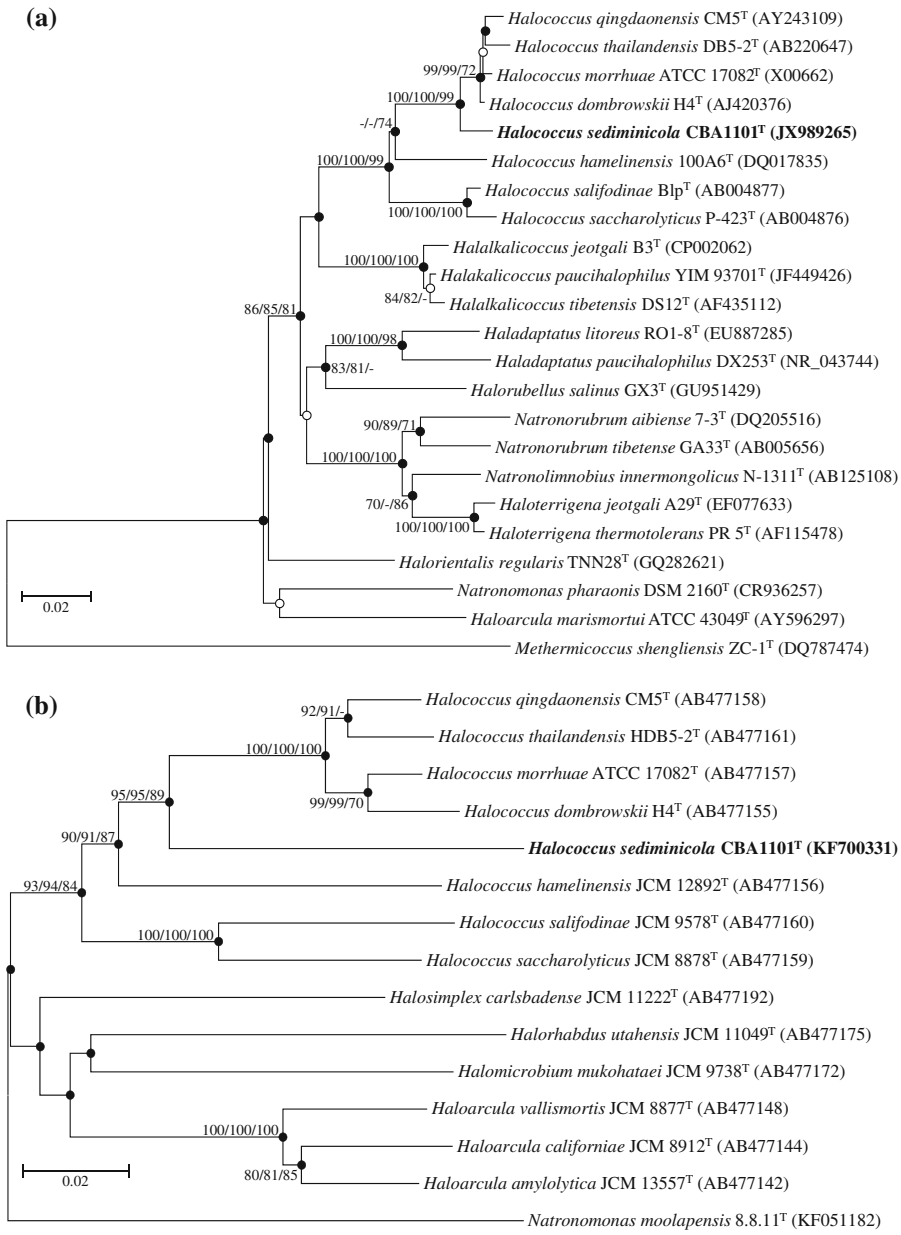


**Fig. 1** Phylogenetic tree based on the neighbour-joining (NJ) algorithm for the 16S rRNA (**a**) and *rpoB′* (**b**) gene sequences of strain CBA1101$^T$ and closely related taxa. The *numbers* on the nodes indicate the bootstrap values (>70 %) calculated using the NJ/minimum-evolution (ME)/maximum-likelihood (ML) probabilities. The *closed circles* represent nodes recovered by both the ME and ML methods, while the *open circles* indicate nodes recovered using either the ME or ML methods. *Methermicoccus shengliensis* ZC-1$^T$ and *Natronomonas moolapensis* 8.8.11$^T$ served as the outgroups in the phylogenetic trees of the 16S rRNA and *rpoB′* gene, respectively. *Bar* 0.02 accumulated changes per nucleotide

**Table 1** Differential characteristics of strain CBA1101$^T$ and closely related species in the genus *Halococcus*

| Characteristic | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| NaCl range for growth (%, w/v) | 15–30 | 15–30 | 10–25 | 15–30 | 15–30 |
| pH range for growth | 6.0–9.0 | 6.0–8.0 | 6.0–8.0 | 6.0–8.0 | 6.0–8.0 |
| Optimum pH | 7.0 | 7.0 | 6.0 | 7.0 | 7.0 |
| Temperature range for growth (°C) | 25–40 | 20–45 | 20–45 | 20–40 | 20–45 |
| Optimum temperature | 35–40 | 40 | 35 | 35 | 40 |
| Indole formation | − | + | − | + | + |
| Nitrate reduction | − | + | + | + | + |
| Utilization of | | | | | |
| L-Alanine | + | − | − | + | − |
| L-Arginine | − | − | + | − | − |
| L-Aspartate | − | + | + | + | + |
| D-Fructose | − | + | + | + | + |
| L-Glutamate | + | + | + | + | − |
| Glycerol | + | + | + | + | − |
| DL-Lactate | + | + | − | + | + |
| Lactose | − | + | + | + | − |
| L-Malate | + | + | − | + | + |
| Maltose | − | + | + | + | − |
| Mannitol | + | + | − | + | + |
| Pyruvate | + | + | − | + | + |
| Sorbitol | + | + | − | + | + |
| Succinate | + | + | − | + | − |
| D-Xylose | − | − | + | + | + |

Taxa: 1, *Halococcus sediminicola* CBA1101$^T$ sp. nov.; 2, *Hcc. dombrowskii* DSM 14522$^T$; 3, *Hcc. morrhuae* DSM 1307$^T$; 4, *Hcc. qingdaonensis* JCM 13587$^T$; 5, *Hcc. thailandensis* JCM 13552$^T$. All data from this study. All strains showed optimal growth with 20 % (w/v) NaCl and were positive for hydrolysis of gelatin, Tween 20, Tween 40 and Tween 80, and utilization of acetate, citrate, fumarate, D-galactose, D-glucose, D-mannose, starch and sucrose; negative for cell lysis in distilled water, urease activity, $H_2S$ production, hydrolysis of starch and casein, and utilization of glycine, L-lysine, L-ornithine, D-ribose and L-sorbose. +, Positive; −, negative

members of the genus *Halococcus*: *Hcc. qingdaonensis* JCM 13587$^T$ (89.5 % *rpoB′* gene sequence similarity), *Hcc. morrhuae* DSM 1307$^T$ (89.5 %), *Hcc. thailandensis* JCM 13552$^T$ (89.5 %), *Hcc. dombrowskii* DSM 14522$^T$ (89.4 %), *Hcc. hamelinensis* 100A6$^T$ (87.3 %), *Hcc. saccharolyticus* P-423$^T$ (87.2 %) and *Hcc. salifodinae* Blp$^T$ (85.8 %) (Fig. 1b).

The cells of strain CBA1101$^T$ were observed to be aerobic, Gram-negative, and coccoid (1.1–1.5 μm in diameter). Strain CBA1101$^T$ did not lyse in distilled water and found to grow in the presence of 15–30 % (w/v) NaCl, at 25–40 °C and at pH 6.0–9.0, with optimum growth in the presence of 20 % NaCl, at 35–40 °C and at pH 7.0. $Mg^{2+}$ was not found to be required for the growth of strain CBA1101$^T$, although the growth without $Mg^{2+}$ is weak. Strain CBA1101$^T$ was determined to be positive for catalase and oxidase, and for the hydrolysis of gelatin, Tween 20, Tween 40 and Tween 80, but negative for indole formation, nitrate reduction, urease activity and the hydrolysis of starch and casein. The detailed phenotypic data for strain CBA1101$^T$ are presented in the species description. Table 1 compares the characteristics of strain CBA1101$^T$ with the closely related type strains in the genus *Halococcus*, which shows that strain CBA1101$^T$ can be distinguished from other members of the genus *Halococcus*. Strain CBA1101$^T$ was found to be sensitive to erythromycin and neomycin, but resistant to ampicillin, bacitracin, chloramphenicol, ciprofloxacin, norfloxacin, novobiocin, penicillin G and rifampin.

The DNA G + C content of strain CBA1101$^T$ was determined to be 66.0 mol%, which is higher than those of the closely related strains *Hcc. dombrowskii* DSM 14522$^T$ and *Hcc. qingdaonensis* JCM 13587$^T$ (61.3 and 61.2 mol%, respectively) (Stan-Lotter et al. 2002; Wang et al. 2007). The DDH values of strain CBA1101$^T$ with the type strains of *Hcc. dombrowskii* DSM 14522$^T$, *Hcc. morrhuae* DSM 1307$^T$, *Hcc. qingdaonensis* JCM 13587$^T$ and *Hcc. thailandensis* JCM 13552$^T$ were 33 ± 14, 32 ± 11, 28 ± 15 and 28 ± 19 %, respectively. DDH values of <70 % indicate species distinctness in current prokaryotic systematics (Stackebrandt and Goebel 1994; Wayne et al. 1987), so strain CBA1101$^T$ can be considered a distinct genospecies in the genus *Halococcus*. The polar lipids detected in strain CBA1101$^T$ comprised phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, sulfated diglycosyl diether, unidentified phospholipids and unidentified glycolipids, which is consistent with the polar lipid profile of the reference strains in the genus *Halococcus* (Supplementary Fig. S1).

In conclusion, the results of the phylogenetic, phenotypic, genomic and chemotaxonomic analyses showed that the extremely halophilic archaeal strain CBA1101$^T$ can be associated with members of the genus *Halococcus*. Strain CBA1101$^T$ can be differentiated from closely related type strains in the genus *Halococcus* based on its different phenotypic characteristics, as shown in Table 1. Thus, based on this polyphasic taxonomic analysis, strain CBA1101$^T$ represents a new species in the genus *Halococcus*, for which the name *Halococcus sediminicola* sp. nov. is proposed.

## Description of *Halococcus sediminicola* sp. nov.

*Halococcus sediminicola* (se.di.mi.ni'co.la. L. n. sedimen -inis, sediment; L. suff. -cola, inhabitant, dweller; N. L. n. *sediminicola*, sediment-dweller, isolated from a marine sediment collected at a cage-cultured ark clam farm on the South coast of Korea).

Cells are Gram-negative, coccoid and 1.1–1.5 μm in diameter. Colonies are red on agar medium. Cell lysis does not occur in distilled water. Growth occurs with 15–30 % (w/v) NaCl (optimum, 20 %), at 25–40 °C (optimum, 35–40 °C) and at pH 6.0–9.0 (optimum, pH 7.0). $Mg^{2+}$ is not required for the growth, but the growth without $Mg^{2+}$ is weak. Anaerobic growth does not occur in the presence of nitrate, L-arginine, DMSO or TMAO. Cells are positive for catalase and oxidase, and the hydrolysis of gelatin, Tween 20, Tween 40 and Tween 80, but negative for indole formation, nitrate reduction, urease activity and the hydrolysis of starch and casein. Acetate, L-alanine, citrate, fumarate, D-galactose, D-glucose, L-glutamate, glycerol, DL-lactate, L-malate, mannitol, D-mannose, pyruvate, sorbitol, L-sorbose, starch, succinate and sucrose are utilized as carbon and energy sources, whereas L-arginine, L-aspartate, D-fructose, glycine, lactose, L-lysine, maltose, L-ornithine, D-ribose and D-xylose are not. $H_2S$ is not produced from $Na_2S_2O_3$. The major polar lipids comprise phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, sulfated diglycosyl diether, unidentified phospholipids and unidentified glycolipids. The DNA G + C content of the type strain is 66.0 mol%.

The type strain, CBA1101$^T$ (= JCM 18965$^T$ = CECT 8275$^T$), was isolated from a marine sediment collected from the bay of Gangjin in the Republic of Korea. The GenBank/EMBL/DDBJ accession number for the 16S rRNA and *rpoB′* gene sequences of strain CBA1101$^T$ are JX989265 and KF700331, respectively.

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