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ORIGINAL PAPER

Halapricum salinum gen. nov., sp. nov., an extremely halophilic archaeon isolated from non-purified solar salt

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Abstract A halophilic archaeal strain, designated CBA1105^T, was isolated from non-purified solar salt. Strain CBA1105^T was found to have three 16S rRNA genes, *rrnA*, *rrnB* and *rrnC*; similarities between the 16S rRNA gene sequences were 99.5–99.7 %. The phylogenetic analysis of the 16S rRNA gene sequences showed that strain CBA1105^T forms a distinct clade with the strains of the closely related genera, *Halorientalis* and *Halorhabdus,* with similarities of 94.2 % and 93.9–94.2 %, respectively. Multilocus sequence analysis confirmed that strain CBA1105^T is closely related to the genus *Halorhabdus* or *Halorientalis*. Growth of the strain was observed in 15–30 % NaCl (w/v; optimum 20 %), at 30–45 °C (optimum 37 °C) and pH 7.0–8.0 (optimum pH 7.0) and with 0–0.5 M $MgCl_2 \cdot 6H_2O$ (optimum 0.05–0.2 M). The cells of the strain were observed to be Gram-stain negative and pleomorphic with coccoid or ovoid-shape. The cells lysed in distilled water. Tweens 20, 40 and 80 were found to be hydrolysed but starch, casein and gelatine were not. The cells were unable to reduce nitrate under aerobic conditions. Assays for indole formation and urease activity were negative and no growth was observed under anaerobic conditions. Cells were found to be able to utilize L-glutamate, D-glucose, L-maltose, D-mannose and sucrose as sole carbon

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sources. The polar lipids were identified as phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, unidentified glycolipids and an unidentified phospholipid. The G+C content of strain CBA1105^T was determined to be 66.0 mol%. The phenotypic, chemotaxonomic and phylogenetic properties suggest that the strain represents a novel species of a novel genus within the family *Halobacteriaceae*, for which the name *Halapricum salinum* is proposed with CBA1105^T (= KCTC 4202^T = JCM 19729^T) as the type strain.

Keywords Haloarchaeon · *Halapricum salinum* · Solar salt · Polyphasic taxonomy

Introduction

The family *Halobacteriaceae* within the order *Halobacteriales* is comprised of 40 genera containing over 150 species (Cui et al. 2011a, b, c, d; Inoue et al. 2011; Shimane et al. 2011; Amoozegar et al. 2012; Makhdoumi-Kakhki et al. 2012a, b; Mou et al. 2012). Most of these extremely halophilic archaea live and grow in hyper-saline environments where salt concentrations exceed 20 % (Oren 2006). Although organisms require water to live, haloarchaeal strains can live in environments with minimal water, such as on rock salts (Denner et al. 1994). Some halophilic archaea such as *Halarchaeum acidiphilum*, which was described by Minegishi et al. (2010), were grown and isolated from commercial solar salt. In this study, we also isolated an extremely halophilic archaeon, strain CBA1105^T, from an unpurified solar salt, and characterized it phylogenetically and phenotypically as the representative strain of a proposed novel genus and a novel species, for which the name *Halapricum salinum* is proposed.

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Materials and methods

Archaeal strain and culture conditions

An extremely halophilic archaeon, designated CBA1105^T, was isolated from a non-purified solar salt sample that was collected from a solar saltern (E 126°36′, N 35°35′) in Gomso Bay, the Republic of Korea. Five grams of the solar salt was dissolved in 100 ml of DSM medium no. 372 (M372) which contained following (l^{-1}): 5 g yeast extract, 5 g casamino acids, 1 g sodium glutamate, 3 g trisodium citrate, 2 g KCl, 20 g $MgSO_4 \cdot 7H_2O$, 36 mg $FeCl_2 \cdot 4H_2O$, 0.36 mg $MnCl_2 \cdot 4H_2O$ and 200 g NaCl, adjusted to pH 7.0–7.2. The aliquots were serially diluted in M372 medium and spread onto M372 agar plates. The inoculated plates were incubated for 2 months at 37 °C. After this initial cultivation, colonies were successively restreaked on M372 agar plates at least three times to obtain pure colonies. The reference strain *Halorientalis regularis* JCM 16425^T was obtained from the Japan Collection of Microorganisms (JCM) and grown under comparable conditions.

Determination of the 16S rRNA gene sequence and phylogenetic analysis

Genomic DNA from strain CBA1105^T was extracted and purified using a G-spin DNA extraction kit (iNtRON Biotechnology) and the 16S rRNA genes were amplified with the primer set forward primer 0018F (5′-ATTCCGGTTGATCCTGCC-3′) and reverse primer 1518R (5′-AGGAGGTGATCCAGCC GC-3′) (Cui et al. 2009). The heterogeneous 16S rRNA gene sequences were analyzed using an All-in PCR cloning kit (BioFact) according to the manufacturer's protocol, followed by picking randomly and PCR-amplifying multiple clones. The PCR products were sequenced and assembled as previously described (Roh et al. 2008) using the SeqMan program (DNAStar) to obtain most of the 16S rRNA gene sequences for strain CBA1105^T, which were subsequently aligned with those of validly named related species using the SILVA Incremental Aligner (Pruesse et al. 2012). The phylogenetic neighbours and pairwise sequence similarities were determined using EzTaxon-e (Kim et al. 2012) and the phylogenetic trees were constructed with MEGA5 (Tamura et al. 2011) using the neighbour-joining (NJ) (Saitou and Nei 1987),

maximum-parsimony (MP) (Fitch 1971) and maximum likelihood (ML) (Felsenstein 1981) algorithms with 1,000 randomly selected bootstrap replicates.

Analysis of multilocus sequence analysis (MLSA)

The three housekeeping gene fragments, ATP synthase subunit B (*atpB*), elongation factor 2 (*EF-2*) and DNA repair and recombination protein (*radA*) of strain CBA1105^T and *Hos. regularis* JCM 16425^T were amplified according to Papke et al. (2011). The RNA polymerase subunit B (*rpoB′*) gene of strain CBA1105^T and *Hos. regularis* JCM 16425^T was also amplified according to the method of Minegishi et al. (2010). The PCR products were sequenced and assembled as previously described (Roh et al. 2008). The housekeeping gene fragments of related taxa were obtained from GenBank (http://www.ncbi.nlm.nih.gov). The gene fragments were aligned and translated into amino acids. The MLSA trees were constructed using the methods as previously described by Papke et al. (2011).

Morphological, physiological and biochemical characterization

The phenotypic characterization of strain CBA1105^T was performed according to the proposed minimal standards for describing extremely halophilic archaea (Oren et al. 1997). Unless otherwise indicated, the phenotype was characterized during growth on DSM medium no. 954 (M954) that contained the following (l^{-1}): 5 g yeast extract, 5 g casamino acids, 20 g $MgCl_2{\cdot}6H_2O$, 2 g KCl, 12 g Tris, 0.2 g $CaCl_2{\cdot}2H_2O$, 200 g NaCl and 20 g agar, adjusted at pH 7.0; or on a halophile medium (HMD) that contained the following (l^{-1}): 20 g $MgCl_2{\cdot}6H_2O$, 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). Gram staining of the haloarchaea was performed as described by Dussault (1955) and the cell morphology and size were determined using a transmission electron microscope (SUPRA 55VP, Carl Zeiss).

To test the strain for survival in distilled water, the cells were suspended in distilled water and examined for lysis microscopically after 1 week. The optimal NaCl conditions for growth were determined by supplementing M954 as a basal medium with 0–30 % (w/v) NaCl at 5 % increments. The optimal pH range for growth was assayed from pH 5.0–11.0 at intervals of 1.0 using HMD as the basal medium and adjusting the pH with 10 mM 2-(*N*-morpholino) ethanesulfonic acid (pH 5.0 and 6.0), 10 mM bis–Tris propane (pH 7.0–9.0), or 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (pH 10.0 and 11.0). To determine the growth temperature range, the strain was inoculated onto M954 and cultivated at temperatures of 5, 10, 15, 20, 25, 30, 37, 40, 45, 50 and 55 °C. The requirement for magnesium ions for growth was determined by the ability to grow on M954 initially made without magnesium, which was then supplemented with $MgCl_2{\cdot}6H_2O$ which was supplemented with 10 mM bis–Tris propane.

The basal medium for the following assays was M954. The standard phenotypic tests for nitrate reduction under aerobic conditions, indole formation, catalase, oxidase and urease activity, and casein and starch hydrolysis were performed as described by Benson (2002). The hydrolysis of Tweens 20, 40 and 80 was tested as described by González et al. (1978) and the hydrolysis of gelatine was assayed as described by Smibert and Krieg (1994). The ability to grow anaerobically was examined by growth in the presence of either 30 mM nitrate, 5 g L-arginine, 5 g dimethyl sulfoxide (DMSO) or 5 g trimethylamine *N*-oxide (TMAO) at 37 °C in an anaerobic chamber (Coy Laboratory Products) with an $N_2{\cdot}CO_2{\cdot}H_2$ (90:5:5, v:v:v) atmosphere. To assess the utilization of sole carbon and energy sources, the HMD was supplemented with 10 mM bis–Tris propane and 1 % 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. For testing antibiotic sensitivity, strain CBA1105^T was inoculated on M954 with an antibiotic disc containing (μg per disc, unless otherwise indicated) ampicillin (20), bacitracin (0.1 IU), chloramphenicol (50), ciprofloxacin (10), erythromycin (25), neomycin (50), norfloxacin (20), novobiocin (50), penicillin G (20 IU) or rifampicin (10). The plates were incubated for 2 weeks at 37 °C.

DNA G+C content and polar lipid analysis

The DNA G+C content was determined as described by González and Saiz-Jimenez (2002). Polar lipids were extracted and detected using thin layer chromatography (TLC) on a Merck F254 silica gel-60 plate as described by Minnikin et al. (1984). The composition of the polar lipid spots was determined by spraying each plate with the following specific detection reagents: sulphuric acid–ethanol (1:2, v/v) for total lipids, molybdenum blue for phospholipids and α-naphthol-sulphuric acid for glycolipids.

Results and discussion

Strain CBA1105^T was found to have three 16S rRNA genes, *rrnA*, *rrnB* and *rrnC* (1,472 bp of each sequence; GenBank accession numbers KF314042, KJ184543 and KJ184544, respectively); similarities between the 16S rRNA gene sequences were 99.5–99.7 %. Strain CBA1105^T (based on the 16S rRNA *rrnA* gene sequence) shared the highest levels of sequence similarity with the validly named strains *Hos. regularis* JCM 16425^T (94.2 %), *Halorhabdus tiamatea* JCM 14471^T (94.2 %), *Halorhabdus utahensis* DSM 12940^T (93.9 %), *Halosimplex carlsbadense* 2-9-1^T (93.8 %), *Halomicrobium zhouii* TNB51^T (93.0 %), *Halomicrobium mukohataei* DSM 12286^T (92.7 %) and 92.3 % similarity or less with sequences from strains in other genera. Four housekeeping gene sequences of strain CBA1105^T and *Hos. regularis* JCM 16425^T were obtained: *atpB* (489 and 492 bp, respectively; GenBank accession numbers KJ411816 and KJ411817, respectively), *EF-2* (507 bp of each sequence; GenBank accession numbers KJ411818 and KJ411819, respectively), *radA* (498 and 495 bp, respectively; GenBank accession numbers KJ411820 and KJ411821, respectively) and *rpoB′* gene (1,827 bp of each sequence; GenBank accession

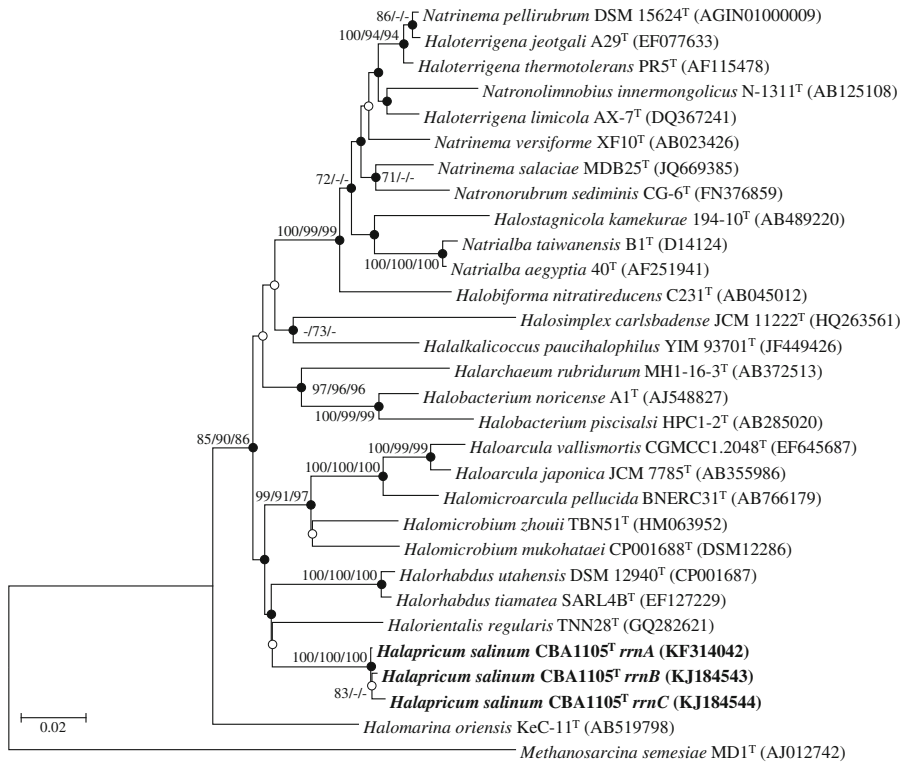

Fig. 1 Phylogenetic tree based on the neighbour-joining (NJ) algorithm for the 16S rRNA gene sequences of strain CBA1105^T and closely related taxa. The *numbers* on the *nodes* indicate the bootstrap values (>70 %) calculated using the NJ, maximum-parsimony (MP) and maximum-likelihood (ML) algorithm probabilities. *Closed circles* represent nodes recovered with both the MP and ML algorithms and *open circles* indicate nodes recovered with either the MP or ML algorithm. *Methanosarcina semensiae* MD1^T served as the outgroup. The *bar* represents 0.02 accumulated changes per nucleotide

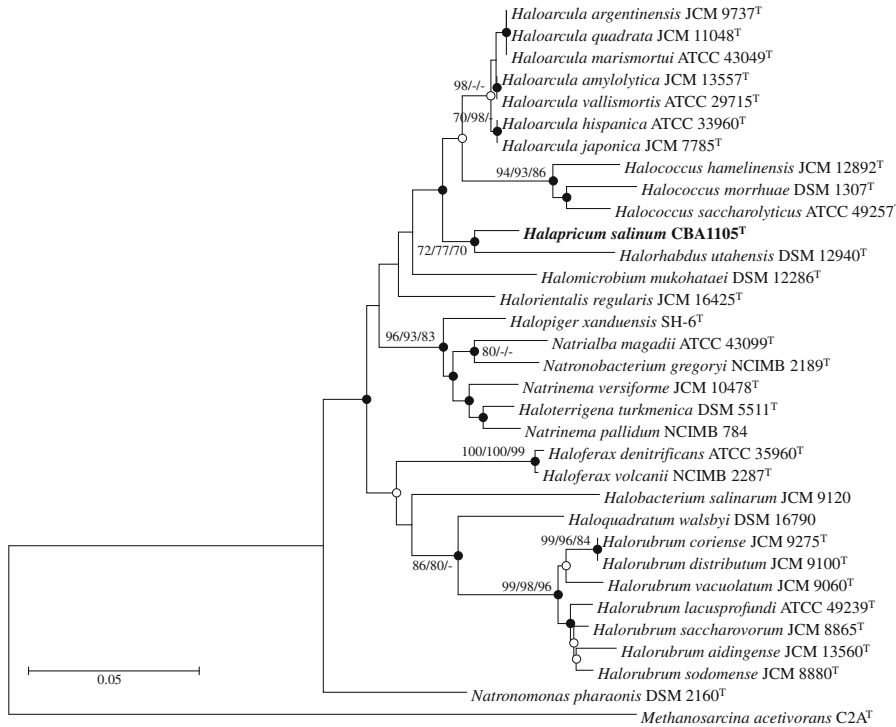

Fig. 2 Multilocus sequencing analysis of strain CBA1105^T and related *Halobacteriales* strains. NJ tree based on amino acid sequence alignments of a concatenation of the four genes *AtpB*, *EF-2*, *RadA* and *RpoB'*. Bootstrap values more than 70 % were obtained from three separate analyses and are presented in the order NJ/MP/ML

numbers KJ411818 and KJ411819, respectively). Phylogenetic trees based on the 16S rRNA gene sequences constructed with the NJ, MP and ML algorithms showed that strain CBA1105^T clusters with the genera *Halorientalis* and *Halorhabdus* (Fig. 1). Multilocus sequence analysis showed also that strain CBA1105^T is closely related to the genus *Halorhabdus* or *Halorientalis* depending on the compared gene sequences (Fig. 2 and S1).

The cells of strain CBA1105^T were observed to be Gram-stain negative and pleomorphic with coccoid or ovoid-shape with a diameter of 0.6–1.1 μm (Fig. S2). The colonies were pigmented red and had a smooth and rounded shape on solid medium. Strain CBA1105^T was found to grow in the presence of 15–30 % (w/v) NaCl, at 30–45 °C and pH 7.0–8.0 and with $MgCl_2{\cdot}6H_2O$ concentrations of 0–0.5 M. The magnesium ions were not required for growth. The optimal growth conditions were obtained in the presence of 20 % (w/v) NaCl, at 37 °C and pH 7.0 and with 0.05–0.2 M $MgCl_2{\cdot}6H_2O$. After 1 week in distilled water, cells of strain CBA1105^T were observed to have lysed. Strain CBA1105^T could not reduce nitrate under aerobic conditions. Catalase activity was found to be negative and oxidase activity was positive. The novel strain hydrolysed Tween 20, 40 and 80, but did not hydrolyse casein, gelatine or starch. Indole production and urease activity were both absent. Strain CBA1105^T was unable to grow anaerobically in the presence of nitrate, L-arginine, DMSO or TMAO. Strain CBA1105^T was found to be sensitive to bacitracin, erythromycin, neomycin, novobiocin and rifampicin, but resistant to ampicillin, chloramphenicol, ciprofloxacin, norfloxacin and penicillin G. Detailed results of the phenotypic tests and the nutritional features of strain CBA1105^T are presented in the species description. Table 1 shows the different characteristics of strain CBA1105^T in comparison to those of closely related type strains in the order *Halobacteriales*. Especially, the growth temperature range of strain CBA1105^T is relatively narrow, more like the range of *Natronolimnobius baerhuensis* (30–46 °C) (Itoh et al. 2005), than the phylogenetically related strains.

Table 1 Characteristics of strain CBA1105^T and closely related species of other genera

Characteristic	1	2	3	4
Growth range of NaCl (%, w/v)	15–30	15–30[a]	9–30	10–30
Optimum NaCl (%)	20	18–20[a]	27	27
Growth range of Mg^{2+} (M)	0– 0.5	0.005–1[a]	0.002–0.8	0–1
Growth range of temperature (°C)	30–45	20–50[a]	17–55	15–55
Optimum temperature (°C)	37	37	50	44–46
Growth range of pH	7–8	6–9	5.5–8.5	5.5–8.5
Optimum pH	7	7	6.7–7.1	6.5–7.0
Hydrolysis of				
Starch	−	−	−	w
Gelatine	−	−	−	+
Tween 80	+	−	−	−
Nitrate reduction	−	+	−	+
Catalase	−	+	+	+
Oxidase	+	+	+	−
Anaerobic growth of nitrate	−	−	+	+
Utilization of				
Acetate	−	+	−	−
L-Alanine	−	+	−	−
D-Fructose	−	−	+	+
D-Galactose	−	+	−	w
Glycerol	−	+	−	−
DL-Lactate	−	+	−	−
L-Lysine	−	+	−	−
Maltose	+	−	−	+
Starch	−	+	−	w
Sucrose	+	−	−	−
D-Xylose	−	−	+	+
DNA G+C content (mol%)	66	61.5–61.9[a]	64	61.7

Taxa: 1, *Halapricum salinum* CBA1105^T gen. nov., sp. nov.; 2, *Halorientalis regularis* JCM 16425^T; 3, *Halorhabdus utahensis* DSM 12940^T (data from Wainø et al. 2000); 4, *Hrd. tiamatea* JCM 14471^T (Antunes et al. 2008). Based on our data, strain CBA1105^T and *Hos.regularis* JCM 16425^T were positive for oxidase activity and utilization of L-glutamate, maltose, sucrose, D-glucose and D-mannose; negative for hydrolysis of starch, gelatin and casein, indole formation, urease activity, anaerobic growth under nitrate, arginine, DMSO and TMAO and utilization of L-arginine, lactose, D-fructose, D-ribose, L-sorbose and D-xylose. +, Positive; −, negative; w, weakly positive

[a] Data from Cui et al. (2011b)

The DNA G+C content of strain CBA1105^T was determined to be 66.0 mol%. The DNA G+C value of strain CBA1105^T is higher than those of phylogenetically related strains *Hos. regularis* (61.5-61.9 mol%), *Hrd. tiamatea* (61.7 mol%) and *Hrd. utahensis* (64 mol%). The polar lipids of strain CBA1105^T were identified as phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, three unidentified glycolipids and an unidentified phospholipid (Fig. S3). Based on the one-dimensional TLC analysis (Fig. S3b), strain CBA1105^T differs from *Hos. regularis* JCM 16425^T by the absence of a glycolipid that is chromatographically identical to sulfated mannosyl glucosyl diether (S-DGD-1) (Cui et al. 2011b).

In conclusion, the results of the phenotypic, phylogenetic and chemotaxonomic analyses showed that the haloarchaeal strain CBA1105^T belongs to the order *Halobacteriales.* Strain CBA1105^T can be distinguished from other taxa in the family *Halobacteriaceae* as a member of a new genus, *Halapricum*. Thus,

based on this polyphasic taxonomic study, strain CBA1105^T can be considered to represent a novel species in a novel genus *Halapricum*, for which the name *Halapricum salinum*. gen. nov., sp. nov. is proposed.

Description of *Halapricum* gen. nov.

Halapricum (Ha.la.pri.cum. Gr. n. *hals halos* salt; L, neut. adj. *apricum* sunny or loves the sun; N.L. neut. n. *Halapricum* salt and sun-loving or solar salt).

Cells are Gram-stain negative and pleomorphic with coccoid or ovoid forms. The major polar lipids are phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester and unidentified glycolipids and phospholipid. The genomic DNA G+C content of the type strain of the type species is 66.0 mol%.

The type species is *Halapricum salinum*. Recommended three-letter abbreviation: *Hpr*.

Description of *Halapricum salinum* sp. nov.

Halapricum salinum (sa.li′num. L. neut. adj. *salinum* salted, saline)

Cells are Gram-stain negative and pleomorphic with coccoid or ovoid-shape with a diameter of 0.6–1.1 μm. Colonies are red, smooth and round in shape. Lyse in distilled water. Growth occurs in the presence of 15–30 % (w/v) NaCl (optimum, 20 %), at 30–45 °C (optimum, 37 °C) and pH 7.0–8.0 (optimum, pH 7.0) and with 0–0.5 M $MgCl_2 6H_2O$ (optimum 0.05–0.2 M). Anaerobic growth does not occur in the presence of nitrate, L-arginine, DMSO or TMAO. Cells are positive for oxidase activity and hydrolyse Tweens 20, 40 and 80, but are negative for catalase, nitrate reduction under aerobic conditions, indole formation, urease activity and the hydrolysis of casein, gelatine and starch. Cells utilize L-glutamate, D-glucose, maltose, D-mannose and sucrose as sole carbon sources, but are unable to utilize acetate, L-alanine, L-arginine, L-aspartate, citrate, fumarate, glycerol, glycine, DL-lactate, lactose, L-lysine, L-malate, mannitol, pyruvate, sorbitol, starch, succinate, D-fructose, D-galactose, L-ornithine, D-ribose, L-sorbose, or D-xylose. The polar lipids are phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, three unidentified glycolipids and an unidentified phospholipid. The genomic DNA G+C content of the type strain is 66.0 mol%.

The type strain, CBA1105^T (= KCTC 4202^T = JCM 19729^T), was isolated from non-purified solar salt in the Republic of Korea. The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA *rrnA*, *rrnB* and *rrnC* gene sequences of strain CBA1105^T are KF314042, KJ184543 and KJ184544, respectively; and of the *atpB*, *EF-2*, *radA* and *rpoB′* gene sequences are KJ411816, KJ411818, KJ411820 and KJ364599, respectively.

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