Halostella salina gen. nov., sp. nov., an extremely halophilic archaeon isolated from solar salt

Hye Seon Song,† In-Tae Cha,† Jin-Kyu Rheem,† Kyung June Yim, Ah Yoon Kim, Jong-Soon Choi,† Su Jeong Baek,† Myung-Ji Seo,† Soo-Je Park,† Young-Do Nam and Seong Woon Roh†

†These authors contributed equally to this work.

Correspondence
Young-Do Nam
youngdo98@kfri.re.kr
Seong Woon Roh
seong18@gmail.com

1Biological Disaster Analysis Group, Korea Basic Science Institute, Daejeon 34133, South Korea
2Department of Food Science and Engineering, Ewha Womans University, Seoul 03760, South Korea
3Division of Bioengineering, Incheon National University, Incheon 22012, South Korea
4Graduate School of Analytical Science and Technology, Chungnam National University, Daejeon 34134, South Korea
5Department of Biology, Jeju National University, Jeju 63243, South Korea
6Research Group of Gut Microbiome, Korea Food Research Institute, Sungnam 13539, South Korea

A novel halophilic archaeon designated strain CBA1114T was isolated from solar salt in the Republic of Korea. Strain CBA1114T, cells of which were coccoid and Gram-stain-negative, grew in the presence of 15–30 % (w/v) NaCl (optimum, 20 %) and at 20–50 °C (optimum, 40 °C) and pH 7.0–9.0 (optimum, pH 8.0). Strain CBA1114T required Mg2+ for growth. Strain CBA1114T had three 16S rRNA genes, rrnA, rrnB and rrnC; levels of similarity between the sequences were 99.7–99.9 %. The 16S rRNA gene sequence of strain CBA1114T showed 91.7 % similarity to that of Haloterrigena thermotolerans PR5T. In multilocus sequence analysis (MLSA), five housekeeping genes, atpB, EF-2, radA, rpoB¢ and secY, were found to be closely related to those of the members of the genera Halorientalis (89.7 % similarity of the atpB gene sequence), Halomicroarcula (91.9 %, EF-2), Haloterrigena (85.4 %, radA), Natronoarchaeum (89.2 %, rpoB¢) and Natrinema (75.7 %, secY). A phylogenetic tree generated from the results of MLSA of the five housekeeping genes showed that strain CBA1114T was closely related to species of the genus Halorientalis in the family Halobacteriaceae. The major polar lipids were identified as phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester and unidentified lipids. The G+C content of the genomic DNA of strain CBA1114T was 68.1 mol%. According to the results of phylogenetic, phenotypic and chemotaxonomic analyses, we designate strain CBA1114T (=JCM 30111T=KCTC 4206T) as the type strain of Halostella salina gen. nov., sp. nov., a novel species of a new genus within the family Halobacteriaceae.

Haloarchaea, members of the domain Archaea, inhabit extreme environments that contain salt concentrations that do not permit the growth of most microbial species (Grant et al., 2001; Ma et al., 2010). Members of the class Halobacteria have been divided into three orders: Halobacterales, Haloferacales and Natrialbales (Gupta et al., 2015).
The order *Halobacteriales* has the sole family *Halobacteriaceae* of which the type genus is *Halobacterium* (Larsen, 1989). At the time of writing, the family *Halobacteriaceae* comprised 24 genera and over 60 species, based on the List of Prokaryotic Names with Standing in Nomenclature database (Euzéby, 1997; Parte, 2014). Members of the order *Halobacteriales* are aerobic and chemo-organotrophic, and have various cell morphologies (rod, coccus and pleomorphic). Most members of the order grow at neutral pH (Gupta et al., 2015). They have been isolated from different saline environments including salt lake sediments, soda lakes and marine salterns. In the family *Halobacteriaceae*, *Halapricum salinum* (Song et al., 2014) and *Halarchaeum salinum* (Yamauchi et al., 2013) have been isolated from solar salts. In the present study, we isolated an extremely halophilic archaeon from non-purified solar salt and determined its phylogenetic, phenotypic and chemotaxonomic properties. Strain CBA1114T is proposed as representing a novel species of a new genus within the family *Halobacteriaceae*.

To isolate haloarchaeal strains, solar salt samples collected from a solar saltern in the Republic of Korea (34° 36’ 33” N 126° 17’ 15” E) were serially diluted and inoculated onto DSM medium no. 954 (M954), which contained the following ingredients (per litre): 5 g yeast extract, 5 g Casamino acids, 20 g MgCl2·6H2O, 2 g KCl, 12 g Tris, 0.2 g CaCl2·2H2O, 200 g NaCl and 20 g agar adjusted to pH 7.4 with 1 M HCl. The plates were incubated aerobically at 37°C for 2 months. Haloarchaeal colony were successively streaked at least three times on the same medium, and a single well-isolated colony was designated strain CBA1114T. For long-term preservation, strain CBA1114T was frozen at −80°C in liquid medium M954 supplemented with 5% (v/v) DMSO. The reference strain *Haloterrigena thermotolerans* DSM 11522T was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) and *Halalkalicoccus joetgali* B3T was obtained in our previous study (Roh et al., 2007). The strains were cultured using medium M954 at 37°C.

Genomic DNA was extracted from strain CBA1114T by using a G-spin Total DNA Extraction Kit (iNtRON Biotechnology) and QuickGene DNA tissue kit S (Kurabo). The 16S rRNA gene was amplified using PCR (Weisburg et al., 1991) with forward and reverse primers 0018F (5’-ATTCCGGTGTAGCCTGGC-3’) and 1518R (5’-AGGAGGTGATCCAGCGGC-3’), respectively (Cui et al., 2009). For analysis of the heterogeneous 16S rRNA gene sequences, the PCR product was cloned using an All in One PCR cloning kit (BioFact) according to the manufacturer’s protocol. Twenty clones were picked randomly and sequenced. The genes encoding the ATP synthase subunit B (*atpB*), elongation factor 2 (*EF-2*), DNA repair protein (*radA*) and the preprotein translocase subunit (*secY*) were amplified and aligned using Megalign (DNASTar), and the DNA polymerase subunit B (*rpob*) gene was amplified according to the method of Minegishi et al. (2010). The PCR products were sequenced and assembled as described previously (Roh et al., 2008) using the SeqMan program (DNASTar). The 16S rRNA gene sequence of strain CBA1114T was aligned with sequences of related species with validly published names using the SILVA Incremental Aligner (Pruesse et al., 2012). The phylogenetic neighbours and pairwise sequence similarities based on 16S rRNA genes were determined using EzTaxon-e (Kim et al., 2012). Sequences of the housekeeping genes of related taxa used for multilocus sequence analysis (MLSA) were obtained from the GenBank database (http://www.ncbi.nlm.nih.gov) and sequence similarities for each gene were calculated using the Basic Local Alignment Search Tool (Altschul et al., 1990). Phylogenetic trees were reconstructed according to the neighbour-joining (NJ) (Saitou & Nei, 1987), maximum-parsimony (MP) (Fitch, 1971) and maximum likelihood (ML) (Felsenstein, 1981) methods using MEGAS (Tamura et al., 2013) with 1000 bootstrap replications for each.

We determined a nearly complete sequence (1457 bp) of three 16S rRNA genes, *rrnA*, *rrnB* and *rrnC*, of strain CBA1114T. Levels of 16S rRNA gene sequence similarity of *rrnA*, *rrnB* and *rrnC* were 99.7%–99.9%. Phylogenetic analysis based on the 16S rRNA genes classified CBA1114T as a member of the class *Halobacteria*; however, strain CBA1114T did not cluster with those of other haloarchaeal genera (Fig. 1). Levels of 16S rRNA gene sequence similarity between strain CBA1114T (based on the *rrnA* gene) and other genera were as follows: *Haloterrigena thermotolerans* PRS5T (91.7%), *Haloterrigena saccharovorans* AB14T (91.5%), *Natrinema pellirubrum* DSM 15624T (91.4%), *Halaliger canadensis* SH-6T (91.4%), *Natronobacterium gregoryi* SP2T (91.3%) and *Halalkalicoccus joetgali* B3T (91.3%), while levels with sequences of strains of other haloarchaeal genera were 91.1% or less. The sequences of five housekeeping genes were obtained as follows: *atpB* (495 bp), *EF-2* (507 bp), *radA* (483 bp), *rpob*’ (1833 bp) and *secY* (503 bp). Each gene was closely related to those of the following taxa: *Halorubalobus regularis* JCM 16425T (89.7% similarity of the *atpB* gene sequence); *Halomicrobium pellicula* JCM 1782T (91.9%, *EF-2*); *Haloterrigena turkmenica* DSM 5511T (85.4%, *radA*); *Natronoarchaeum rubrum* GX48T (89.2%, *rpob*’); *Natrinema pellirubrum* DSM 15624T (75.7%, *secY*). Phylogenetic trees based on the three housekeeping genes of *EF-2*, *secY* and *rpob*’ showed that strain CBA1114T clustered with different haloarchaeal strains and the tree based on the *atpB* and *radA* genes showed the strain did not cluster with any haloarchaeal strains (Fig. S1, available in the online Supplementary Material). However, the phylogenetic analysis of concatenated sequences of the five housekeeping genes indicated that strain CBA1114T clustered tightly with *Halorubalobus regularis* JCM 16425T belonging to the family *Halobacteriaceae*, regardless of the tree-making algorithms (Fig. 2).

The phenotype of strain CBA1114T was determined according to the proposed minimal standards for describing extremely halophilic archaea (Oren et al., 1997) using medium M954 as the basal medium, unless indicated

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González et al. (1978), while gelatine hydrolysis was tested according to Smibert & Krieg (1994). H₂S formation was tested according to Cui et al. (2007). Phenylalanine deaminase activity was determined as described by Ederer et al. (1971) using the suggested medium supplemented with 20% (w/v) NaCl. Arginine dihydrolase was tested following Brooks & Sodeman (1974), while production of lysine decarboxylase and ornithine decarboxylase was determined as described by Oren et al. (1997). To determine growth under anaerobic conditions, cells were incubated in the basal medium containing 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) in an atmosphere containing N₂, CO₂ and H₂ (90:5:5, by vol.). To measure the growth rate and optimal growth conditions, medium M954 was modified as required. NaCl concentrations were 10–30% (w/v) at intervals of 5%. Growth was measured from 5 to 60 °C at intervals of 5 °C. The pH range was adjusted to pH 5.0–11.0 at intervals of 1.0 pH unit using otherwise. Strain CBA1114ᵀ was observed by using a phase-contrast microscope (Eclipse 80i; Nikon) and an electron microscope (SUPRA 55VP; Carl Zeiss) to determine its morphology and size. Gram staining was performed as described by Benson (2002). Tests for activity of catalase, oxidase and urease, nitrate and nitrite reduction under aerobic conditions, indole production, and the hydrolysis of casein and starch were conducted as described by Dussault (1955). Tests for activity of catalase, oxidase and urease, nitrate and nitrite reduction under aerobic conditions, indole production, and the hydrolysis of casein and starch were conducted as described by Benson (2002). Fig. 1. Phylogenetic tree generated according ML analysis of the 16S rRNA gene sequences of strain CBA1114ᵀ and closely related taxa. Numbers at nodes indicate the bootstrap values (>70 %) calculated from probabilities determined using the ML, NJ and MP algorithms. Filled circles represent nodes recovered with the NJ and MP algorithms, and open circles indicate nodes obtained with either the NJ or the MP algorithm. Methanobacterium beijingense B-2ᵀ served as the outgroup. Bar, 0.05 changes accumulated per nucleotide.
the following buffers: 10 mM MES, pH 5.0 and 6.0; 10 mM Bis-Tris propane, pH 7.0–9.0; 10 mM CAPS, pH 10.0 and 11.0. MgCl₂ was added to media at 0, 0.005, 0.01, 0.02, 0.05, 0.1, 0.2 or 0.5 M. For comparison with media containing single carbon and energy sources, cells were grown in halophile medium (HMD) containing (per litre) 20 g MgCl₂/6H₂O, 5 g K₂SO₄, 0.1 g CaCl₂·2H₂O, 0.1 g yeast extract, 0.5 g NH₄Cl, 0.05 g KH₂PO₄, 5 g Casamino acids as carbon and energy source, 200 g NaCl and 20 g agar; final pH 7.0 (Savage et al., 2007). To determine the utilization of various substrates as sole carbon and energy sources, HMD was supplemented with 10 mM Bis-Tris propane and 1% (w/v) of the following substrates instead of Casamino acids: 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, D-sorbitose, starch, succinate, sucrose and D-xylene. Incubation was for 2 weeks at 37°C. To determine acid production, the following substrates (1%, v/v) were supplemented in modified HMD instead of Casamino acids: D-fructose, D-galactose, D-glucose, D-mannose and D-sorbitose. Acid production was measured by a decrease in pH of 1.0 after incubation for 2 weeks. For testing antibiotic sensitivity, strain CBA1114T was inoculated onto M954 plates containing discs with the following antibiotics (amount per disc): novobiocin (50 µg), bacitracin (0.1 IU), erythromycin (25 µg), penicillin G (20 IU), ampicillin (20 µg), rifampicin (10 µg), chloramphenicol (50 µg), neomycin (50 µg), norfloxacin (20 µg), ciprofloxacin (10 µg) and anisomycin (20 µg).

Fig. 2. MLSA of strain CBA1114T and related haloarchaeal strains. The NJ tree was generated according to the amino acid sequence alignments of a concatenation of atpB, EF-2, radA, rpoB and secY genes. Bootstrap values were obtained from three separate analyses and are presented (when greater than 70%) in the order NJ, MP, ML and open circles indicate nodes obtained with either the MP or ML. Bar, 0.05 changes accumulated per nucleotide.
Cells of strain CBA1114T were coccoid with a diameter of 0.7–1.4 µm, similar to members of the genera *Halococcus*, *Halalkalicus* and *Natronococcus*. Strain CBA1114T was Gram-stain-negative, formed red, smooth and round colonies that lysed in distilled water, tested positive for catalase and hydrolysis of Tween 40 but tested negative for oxidase and urease activities, hydrolysis of Tween 80, starch, casein and gelatin, and indole production from tryptophan. Nitrate was reduced to nitrite, H₂S was not produced from thiosulfate. Activities of phenylalanine deaminase, lysine decarboxylase, arginine dihydrolase and ornithine decarboxylase were not observed. Strain CBA1114T failed to grow anaerobically in the presence of nitrate, L-arginine, DMSO or TMAO but grew aerobically in the presence of 15–30 % (w/v) NaCl, 0.005–0.5 M MgCl₂ and at 20–50 °C and pH 7.0–9.0, and grew optimally in the presence of 20 % NaCl, at 40 °C, at pH 8.0 and with 0.05–0.2 M MgCl₂. Mg²⁺ was required for growth. Strain CBA1114T utilized acetate, D-galactose, D-glucose, L-glutamate, glycerol, D-lactate, maltose, D-mannose, pyruvate, starch and sucrose as carbon and energy sources. Glycine, lactate, maltitol, citrate, D-fructose, fumarate, L-lysine, sorbitol, D-sorbitose, D-ornithine, D-ribose and D-xylene were not utilized. Strain CBA1114T was sensitive to anisomycin, bacitracin, chloramphenicol, ciprofloxacin, erythromycin, neomycin, norfloxacin, novobiocin and rifampicin but was resistant to ampicillin and penicillin G.

The isoprenoid quinones were extracted from freeze-dried cells using chloroform/methanol (2:1, v/v) (Collins & Jones, 1981) and were identified using an HPLC system (YL9100; Younglin). The composition of polar lipids was determined using one- and two-dimensional TLC with Merck F254 silica gel-60 plates as described previously (Minnikin et al., 1984). The plates were sprayed with 10 % ethanolic molybdoephosphoric acid to detect total lipids, molybdenum blue to detect phospholipids and α-naphthol-sulphuric acid to detect glycolipids. The G+C content of the genomic DNA was determined using a next-generation sequencing method using an Ion Torrent PGM sequencer (Life Technologies™) according to the manufacturer’s instructions (Thermo Fisher).

The major isoprenoid quinone was menaquinone-8 (MK-8). The polar lipids of strain CBA1114T detected were phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, three unidentified glycolipids, two unidentified phospholipids and an unidentified lipid (Fig. S2a). The profile of polar lipid spots of strain CBA1114T was different from that of *Haloterrigena thermotolerans* DSM 11522T, which is the closest related species based on 16S rRNA gene sequence similarity (Fig. S2b). The genomic DNA G+C content of strain CBA1114T was 68.1 mol%, which is higher than that of *Haloterrigena thermotolerans* PRS5 (63.3 mol%) (Montalvo-Rodriguez et al., 2000) in the family Natrialbaceae and similar to that of *Halobacterium salinarum* NG4 and *Halomicrdbacter zhouti* TNB51T (67.1–71.2 and 69.1 mol%, respectively) (Grant, 2001; Yang et al., 2006) in the family Halobacteriaceae.

Phylogenetic, phenotypic, genomic and chemotaxonomic analyses were performed to evaluate the taxonomic position of strain CBA1114T. The phylogenetic analysis based on the 16S rRNA and five housekeeping gene sequences indicates that the strain is phylogenetically distinct in the family Halobacteriaceae of the order Halobacteriales. The different phenotypic, genomic and chemotaxonomic properties also distinguished the novel strain from other members in the halocarchael genera (Table 1). Therefore, based on this

### Table 1. Differential characteristics between strain CBA1114T and related species

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<th>Characteristic</th>
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<td>Morphology</td>
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<td>20–25* (15–20†)</td>
<td>Rod</td>
<td>15–20</td>
<td>Pleomorphic</td>
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<td>8.0* (7.0–7.5†)</td>
<td>7.5</td>
<td>7.2–7.8</td>
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<td>7</td>
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<td>Hydrolysis of Tween 80</td>
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<td>Indole formation</td>
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<td>Utilization as sole carbon and energy source:</td>
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<td>+ (−†)</td>
<td>–</td>
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<td>+</td>
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<tr>
<td>D-Glucose</td>
<td>+</td>
<td>+ (−†)</td>
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<td>DNA G+C content (mol%)</td>
<td>68.1</td>
<td>63.3</td>
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<td>63.2</td>
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*Data from this study.
†Results differ from those reported by Montalvo-Rodriguez et al. (2000).
polyphasic taxonomic analysis, we propose that strain CBA1114T represents a novel species of a new genus in the family Halobacteriaceae of the order Halobacterales with the name Halostella salina. gen. nov., sp. nov.

Description of Halostella gen. nov.

Halostella (Ha.lo.stell’ia; Gr. n. hals, halos salt; L. fem. n. stella star; N.L. fem. n. Halostella salt star).

Cells are Gram-stain-negative and coccoid. Growth occurs at pH 7–9, at 20–50 °C and with 15–30 % (w/v) NaCl. Optimum growth occurs at pH 8, at 40 °C and with 20 % (w/v) NaCl. Chemo-organotrophic, growing on a wide range of substrates, including single and complex carbon sources. Does not grow under anaerobic conditions with nitrate, l-arginine, DMSO or TMAO. The major polar lipids are phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester and unidentified lipids. The major isoprenoid quinone is MK-8. Phylelogenetically related to the genera Haloterrigena, Natrinema, and Halopiger.

The type species of the genus is Halostella salina. Recommended three-letter abbreviation: Hsl.

Description of Halostella salina sp. nov.

Halostella salina (sa.li’na. L. fem. adj. salina salted, salined).

Has the following characteristics in addition to those given for the genus. Cells are 0.7–1.4 μm in diameter. Colonies are red and lyse in distilled water. Requires Mg2+ for growth with optimal growth in the presence of 0.05–0.2 M Mg2+ (range 0.005–0.5 M). Nitrate is reduced to nitrite, but nitrite is not. H2S is not produced. Cells are positive for halidrolase and hydrolysis of Tween 80, starch, casein, gelatine. Acetate, D-galactose, D-glucose l-glutamate, glycerol, D-lactate, maltose, D-mannose, pyruvate, starch and sucrose are utilized as sole carbon and energy sources. Glycine, lactose, l-malate, mannitol and succinate are weakly utilized, whereas l-alanine, l-arginine, l-aspartate, citrate, D-fructose, fumarate, l-lysine, l-ornithine, D-ribose, sorbitol, D-sorbitose and D-xylose are not utilized. Acid is produced from D-fructose, D-galactose, D-glucose, D-mannose and D-sorbitose. Polar lipid composition includes phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, three unidentified glycolipids, two unidentified phospholipids and an unidentified lipid.

The type strain is CBA1114T (=JCM 30111T=KCTC 4206T), isolated from solar salt in the Republic of Korea. The genomic DNA G+C content of the type strain is 68.1 mol%.

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