Halostagnicola kamekurae sp. nov., an extremely halophilic archaeon from solar salt

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A novel extremely halophilic archaeon, strain 194-10^T, was isolated from a solar salt sample imported into Japan from the Philippines. Strain 194-10^T was pleomorphic, neutrophilic and mesophilic and required at least 10 % (w/v) NaCl but no MgSO₄·7H₂O for growth; it exhibited optimal growth at 15 % (w/v) NaCl and 60 mM MgSO₄·7H₂O. Strain 194-10^T grew at 20–45 °C (optimum, 30 °C) and pH 6.0–9.0 (optimum, pH 6.5–7.0). The G + C content of its DNA was 59.8 mol%. 16S rRNA gene sequence analysis revealed closest proximity to *Halostagnicola larsenii* XH-48^T (98.5 % similarity), the sole representative of the genus *Halostagnicola*. Polar lipid analysis revealed that strain 194-10^T contained phosphatidylglycerol and phosphatidylglycerol phosphate methyl ester (the latter derived from both C₂₀C₂₀ and C₂₀C₂₅ archaeol) and several unidentified glycolipids. The results of DNA–DNA hybridization (20.7 % relatedness between *Hst. larsenii* JCM 13463^T and strain 194-10^T) and physiological and biochemical characteristics allowed differentiation of strain 194-10^T from *Hst. larsenii* XH-48^T. Therefore, strain 194-10^T represents a novel species of the genus *Halostagnicola*, for which the name *Halostagnicola kamekurae* sp. nov. is proposed, with the type strain 194-10^T (=DSM 22427^T =JCM 16110^T =CECT 7536^T).

The haloarchaea constitute a large group of extremely halophilic, aerobic archaea that are placed in the order *Halobacteriales*, family *Halobacteriaceae* (Grant *et al.*, 2001). At the time of writing (December 2009), the family *Halobacteriaceae* contains 27 genera comprising 104 species (Savage *et al.*, 2008; Oren *et al.*, 2009; <http://www.the-icsp.org/taxa/halobacterlist.htm>; <http://www.bacterio.cict.fr/>) that display a wide variety of physiological characteristics, including ranges of salinity, temperature and pH for growth. The genus *Halostagnicola*, belonging to the family *Halobacteriaceae*, currently contains only one species and one strain, *Halostagnicola larsenii* XH-48^T, isolated from sediment of Lake Xilinhot, a saline lake in Inner Mongolia, China (Castillo *et al.*, 2006). The strain is pleomorphic, neutrophilic and requires at least 15 % (w/v) NaCl. The highest 16S rRNA gene sequence similarities are obtained with *Natrialba aegyptiaca* 40^T and *Natrialba asiatica* 172P1^T. Polar lipids are phosphatidylglycerol and phos-

phatidylglycerol phosphate methyl ester, the latter derived from both C₂₀C₂₀ and C₂₀C₂₅ archaeol, and two unidentified glycolipids. We isolated a strain closely related to *Hst. larsenii* XH-48^T from an imported solar salt sample. In this report, we characterize strain 194-10^T and describe its identification. *Hst. larsenii* XH-48^T was obtained from the Japan Collection of Microorganisms as JCM 13463^T and used as a reference strain.

Strain 194-10^T was isolated from a sample of solar salt imported into Japan from the Philippines. The solar salt sample has been on the market from a Japanese firm as 'Mangrove' (<http://www.77ochiai.com/page/syouhin06-783gift.htm>) as a 200 g package. To 1 g of the salt sample, 4 ml sterile distilled water was added gradually, and three drops of the solution were spread on agar plates of JCM medium no. 168 containing the following (l⁻¹): 5.0 g yeast extract, 5.0 g Casamino acids, 1.0 g sodium glutamate, 2.0 g KCl, 3.0 g sodium citrate, 20.0 g MgSO₄·7H₂O, 200.0 g NaCl, 36.0 mg FeCl₂·4H₂O and 0.36 mg MnCl₂·4H₂O. The medium was adjusted to pH 7.0 with 40 % (w/v) KOH. About 30 colonies appeared on an agar plate, and six colonies of different colours were selected and purified by repeated serial dilution; partial 16S rRNA gene sequences were then determined. Sequences of five of the strains were closely related (more than 99.2 %

Abbreviation: PGP-Me, phosphatidylglycerolphosphate methyl ester.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 194-10^T is AB489220.

Thin-layer chromatograms of polar lipids of strain 194-10^T and related haloarchaea and a 16S rRNA gene sequence-based maximum-likelihood tree are available as supplementary figures with the online version of this paper.

similarity) to those of haloarchaea of the genera *Haloarcula* and *Halorubrum*. A pink colony designated strain 194-10^T showed 98.5 % partial 16S rRNA gene sequence similarity to *Hst. larsenii* XH-48^T. Colony morphology was observed on agar medium under optimal growth conditions after incubation at 37 °C. Gram staining was performed according to Dussault (1955). Cell morphology and motility were examined using phase-contrast microscopy (Zeiss Axiovert 135). Colonies of strain 194-10^T on agar plates were circular, smooth, opaque and white and turned pink gradually after 2–3 weeks. The cells were motile and pleomorphic, exhibiting rod-shaped or teardrop-shaped morphology, and approximately 0.8–1.0 × 2.0–2.5 µm. Cells lysed in distilled water. There were no gas vesicles inside the cells.

Growth ranges and optima for NaCl and Mg²⁺ were determined by using the growth medium containing various concentrations of NaCl (0–30 %, w/v) and MgSO₄·7H₂O (0–100 mM), at intervals of 5 % (w/v) and 10 mM, respectively. The pH range for growth was assayed in liquid medium at pH 6.0–10.0 at intervals of 0.5 pH units. The temperature range for growth of strain 194-10^T was determined at 4, 10, 15, 20, 25, 30, 35, 37, 40, 45 and 50 °C at pH 7.0 with optimal NaCl and Mg²⁺ concentrations. Strain 194-10^T was capable of growing over a wide range of NaCl concentration [10–30 % (w/v)]. Mg²⁺ was not required for growth. The strain grew optimally in the presence of 15 % (w/v) NaCl and 60 mM MgSO₄·7H₂O. Strain 194-10^T grew at 20–45 °C (optimum, 30 °C) and pH 6.0–9.0 (optimum, pH 6.5–7.0).

Total DNA was extracted by the method of Cline *et al.* (1989). The DNA G+C content was determined by the HPLC method of Tamaoka (1994). The DNA G+C content of strain 194-10^T was 59.8 mol%, which was similar to that reported for *Hst. larsenii* XH-48^T (61.0 mol%; Castillo *et al.* 2006). The 16S rRNA gene of strain 194-10^T was amplified by PCR with the forward and reverse primers 5'-ATTCCGGTTGATCCTGCCGG and 5'-AGGAGGTGATCCAGCCGAG, as described previously (Fukushima *et al.*, 2007). Amplification products were cloned into the pCR2.1 T vector (Invitrogen) and five clones were sequenced using the Big Dye sequencing kit version 3.1 (Applied Biosystems) using an ABI 310 DNA sequencer. 16S rRNA gene sequences retrieved from the

DDBJ (Miyazaki *et al.*, 2003) were aligned. A phylogenetic tree was reconstructed by the neighbour-joining method (Saitou & Nei, 1987) and was evaluated by bootstrap sampling with 1000 replicates (Felsenstein, 1985). Maximum-likelihood analysis was performed with RAxML 7.0.4 using the GTR + Γ model (Stamatakis *et al.*, 2005), and support for the maximum-likelihood tree was obtained by bootstrapping (1000 replicates) using CONSENSE in PHYLIP. The five 16S rRNA gene clones from strain 194-10^T gave exactly the same sequence, which was the closest to that of *Hst. larsenii* JCM 13463^T (DDBJ accession no. AB301489), with 98.5 % similarity; similarities to sequences of other species of the *Halobacteriaceae* with validly published names were less than 95.0 %, the highest being 94.9 % with *Nab. hulunbeirensis* AS 1.1986^T and 94.6 % with *Nab. aegyptiaca* 40^T. The sequence represented by DDBJ accession number AB301489 (1433 nt) was deposited by one of the authors of this paper (H. M.) in April 2007; this sequence was the same as that represented by AM117571 (1372 nt), deposited by A. Castillo, except for five nucleotides at positions 659–663 of AM117571. The neighbour-joining (Fig. 1) and maximum-likelihood (Supplementary Fig. S1, available in IJSEM Online) trees also supported the conclusion that the strain 194-10^T was most closely related to *Hst. larsenii* XH-48^T. DNA–DNA hybridization was performed by the fluorometric method of Ezaki *et al.* (1989); the relatedness between strain 194-10^T and *Hst. larsenii* JCM 13463^T was 20.7 % (*n*=3). This value was well below the threshold value of 70 % generally accepted for the definition of distinct species (Wayne *et al.*, 1987; Stackebrandt & Ebers, 2006).

Polar lipids were extracted with chloroform/methanol as described previously (Kamekura, 1993). TLC was performed using HPTLC silica gel 60 plates (20 × 10 cm; Merck) with the solvent system chloroform/methanol/acetic acid/water (85:22.5:10:4, by vol.). Phospholipids were detected as blue spots by spraying with Dittmer–Lester reagent (Minnikin *et al.*, 1984). Glycolipids were detected as purple spots by spraying with 0.5 % (w/v) α-naphthol in methanol/water (1:1) and then with sulfuric acid/ethanol (1:1), followed by brief heating at 160 °C. Polar lipids were detected as brown spots after prolonged heating. TLC of the polar lipids (Supplementary Fig. S2) suggested that strain 194-10^T contained phosphatidylgly-

Fig. 1. Phylogenetic tree reconstructed by the neighbour-joining method derived from 16S rRNA gene sequences showing the position of strain 194-10^T among haloarchaea. Bootstrap values >600 (from 1000 replicates) are shown. Bar, 1 % sequence divergence.

cerol and phosphatidylglycerolphosphate methyl ester (PGP-Me) derived from both C₂₀C₂₀ and C₂₀C₂₅ archaeol, as shown by the two spots for PGP-Me. Phosphatidylglycerol sulfate was not detected. Several unidentified glycolipid spots were detected. Two major spots detected above PGP-Me were not observed in *Hst. larsenii* JCM 13463^T in this study or by Castillo *et al.* (2006). Minor glycolipids similar to those of *Hst. larsenii* JCM 13463^T were also detected.

Phenotypic tests were performed according to the proposed minimal standards for the description of new taxa in the order *Halobacteriales* (Oren *et al.*, 1997). All tests were done in our laboratory on both strain 194-10^T and *Hst. larsenii* JCM 13463^T. Tests for catalase and oxidase activities and for the hydrolysis of starch, gelatin, casein and Tween 80 were performed as described by Gonzalez *et al.* (1978). Reduction of nitrate was detected by using the sulfanilic acid and α -naphthylamine reagent (Smibert & Krieg, 1981). H₂S formation was determined by monitoring the production of a black sulfide precipitate in JCM medium no. 168 containing 0.5% (w/v) sodium thiosulfate. Indole production from tryptophan and the utilization of sugars and organic acids were assessed as described by Oren *et al.* (1997). Strain 194-10^T was oxidase- and catalase-negative. Nitrate reduction and indole production from tryptophan were positive. Tween 80 was hydrolysed, but starch, gelatin and casein were not hydrolysed. Strain 194-10^T was urease-positive. Other results are included in the species description. Antibiotic sensitivity tests were performed by spreading a cell suspension on culture plates and applying discs impregnated with the following antibiotics (Becton Dickinson; amounts in mg unless indicated): ampicillin (10), bacitracin (10 U), chloramphenicol (30), erythromycin (15), gentamicin (120), kanamycin (30), nalidixic acid (30), neomycin (30), novobiocin (30), penicillin G (10 U), rifampicin (5), streptomycin (300), tetracycline (30) and vancomycin (30). Discs containing anisomycin (50 mg) and pravastatin (50 mg) were prepared in our laboratory and applied in the same way. The formation of acid from sugars and organic acids was assessed in a modified JCM medium no. 168 (Casamino acids, sodium glutamate and sodium citrate were omitted) with 0.5 g yeast extract l⁻¹, supplemented with 1% (w/v) test substrate. After incubation for 2 weeks, acid formation was determined by using bromocresol green pH test paper. The results are included in the species description and differences between strain 194-10^T and *Hst. larsenii* JCM 13463^T are highlighted in Table 1. There were no discrepancies in phenotypic test results for *Hst. larsenii* JCM 13463^T between those reported by Castillo *et al.* (2006) and those determined in our laboratory, showing that the differences between strain 194-10^T and *Hst. larsenii* JCM 13463^T highlighted in Table 1 are reliable.

The phylogenetic and phenotypic characteristics indicated strongly that strain 194-10^T represents a novel species of the genus *Halostagnicola*, for which the name *Halostagnicola kamekurae* sp. nov. is proposed.

Table 1. Characteristics that distinguish strain 194-10^T from *Hst. larsenii* JCM 13463^T

Data for *Hst. larsenii* JCM 13463^T were taken from Castillo *et al.* (2006) and this study.

Characteristic	194-10 ^T	<i>Hst. larsenii</i> JCM 13463 ^T
Cell size (µm)	0.8–1.0 × 2.0–2.5	0.5–1.0 × 1.0–3.0
NaCl optimum (% w/v)	15	20
NaCl range (% w/v)	10–30	15–30
pH optimum	6.5–7.0	7.0–8.0
Temperature optimum (°C)	30	37
Indole formation	+	–
β-Galactosidase	–	+
Hydrolysis of:		
Starch	–	+
Tween 80	+	–
Assimilation of:		
Cellobiose	+	+*
D-Fructose	–	+
Maltose	–	+
D-Mannitol	–	+*
D-Mannose	+	+*
Raffinose	+	–
Ribose	–	+
D-Xylose	–	+
Citrate	–	+*
Malate	+	–
Acid production from:		
L-Arabinose	+	–
Mannose	+	–
Sucrose	–	+
Sensitivity to:		
Anisomycin (50 mg)	+	+*
Kanamycin (30 mg)	–	–*
Pravastatin (50 mg)	+	+*
Rifampicin (5 mg)	+	–
Vancomycin (30 mg)	–	–*
Pigmentation	White†	Pink
DNA G + C content (mol%)	59.8	61.0

*Determined in this study (not reported by Castillo *et al.*, 2006).

†Colonies gradually turn pink over 2–3 weeks.

Description of *Halostagnicola kamekurae* sp. nov.

Halostagnicola kamekurae (ka.me.ku'rae. N.L. gen. n. *kamekurae* of Kamekura, named after the Japanese microbiologist Masahiro Kamekura, who contributed to the study of haloarchaea).

Gram-negative. Cells are motile and pleomorphic, rod- or teardrop-shaped. Cells are approximately 0.8–1.0 × 2.0–2.5 µm. Colonies on agar medium are circular, white and 2–3 mm in diameter, and gradually turn pink upon incubation for 2–3 weeks. Growth occurs at 10–30% (w/

v) NaCl (optimum, 15 %, w/v). Cells lyse in water. Mg^{2+} is not required for growth (optimal $MgSO_4 \cdot 7H_2O$ concentration is 60 mM). Grows at 20–45 °C (optimum, 30 °C) and at pH 6.0–9.0 (optimum, pH 6.5–7.0). H_2S is not produced from sodium sulfite. Indole is produced from tryptophan. Nitrate is reduced to nitrite, but nitrite is not reduced further and no gas is formed. Anaerobic growth does not occur with DMSO, nitrate or arginine. Oxygen is used as the terminal electron acceptor. Tests for oxidase and catalase activities are negative. Tween 80 is hydrolysed. Casein, starch and gelatin are not hydrolysed. Acid is produced from L-arabinose and mannose. Arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase are not produced. Tests for urease and phosphatase activities are positive, while β -galactosidase activity tests negative. The following substrates are utilized for growth: L-arabinose, D-galactose, D-glucose, glycerol, lactose, maltose, D-mannose, ribitol, raffinose, trehalose, acetate, glutamate, malate and propionate. No growth occurs on cellobiose, D-fructose, D-mannitol, ribose, D-sorbitol, sucrose, D-xylose, starch, citrate, fumarate or succinate. Sensitive to (mg per disc unless indicated) anisomycin (50), bacitracin (10 U), novobiocin (30) and rifampicin (5) and resistant to ampicillin (10), chloramphenicol (30), erythromycin (15), gentamicin (120), kanamycin (30), nalidixic acid (30), neomycin (30), penicillin G (10 U), pravastatin (50), streptomycin (300), tetracycline (30) and vancomycin (30). Polar lipids are phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, two major unknown glycolipids and several unknown glycolipids. The DNA G+C content of the type strain is 59.8 mol% (HPLC).

The type strain is strain 194-10^T (=DSM 22427^T =JCM 16110^T =CECT 7536^T), isolated from solar salt imported into Japan from the Philippines.

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