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Salarchaeum japonicum gen. nov., sp. nov., an aerobic, extremely halophilic member of the *Archaea* isolated from commercial salt

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Strain YSM-79^T was isolated from commercial salt made from seawater in Yonaguni island, Okinawa, Japan. The strain is an aerobic, Gram-negative, chemo-organotrophic and extremely halophilic archaeon. Cells are short rods that lyse in distilled water. Growth occurs at 1.5–5.3 M NaCl (optimum 2.5–3.0 M), pH 5.0–8.8 (optimum pH 5.2–6.3) and 20–55 °C (optimum 40 °C). Mg^{2+} is required for growth, with maximum growth at 200–300 mM Mg^{2+}. Polar lipid analysis revealed the presence of phosphatidylglycerol, phosphatidylglycerophosphate methyl ester, sulfated diglycosyl diether-1 and five unidentified glycolipids. The G+C content of the DNA was 64 mol%. On the basis of 16S rRNA gene sequence analysis, strain YSM-79^T was determined to be a member of the family *Halobacteriaceae*, with the closest related genus being *Halobacterium* (94 % sequence identity). In addition, the *rpoB′* gene sequence of strain YSM-79^T had <88 % sequence similarity to those of other members of the family *Halobacteriaceae*. The results of phenotypic, chemotaxonomic and phylogenetic analysis suggested that strain YSM-79^T should be placed in a new genus, *Salarchaeum* gen. nov., as a representative of *Salarchaeum japonicum* sp. nov. The type strain is YSM-79^T (=JCM 16327^T =CECT 7563^T).

Extremely halophilic and aerobic members of the *Archaea* are placed in the order *Halobacteriales*, family *Halobacteriaceae* (Grant *et al.*, 2001). The current classification of this group is based mainly on four taxonomic characters: 16S rRNA gene sequence, polar lipid composition, phenotypic features and DNA–DNA hybridization (Oren *et al.*, 1997; Grant *et al.*, 2001). The value of RNA polymerase subunit B′ (*rpoB′*) gene sequence analysis for the classification of members of the family *Halobacteriaceae* has also been reported (Walsh *et al.*, 2004; Minegishi *et al.*, 2010).

Abbreviations: PG, phosphatidylglycerol; PGP-Me, phosphatidylglycerophosphate methyl ester; S-DGD-1, sulfated diglycosyl diether-1.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain YSM-79^T is AB454051.

Three supplementary figures are available with the online version of this paper.

At the time of writing, the aerobic, extremely halophilic archaea are classified within 35 different genera and more than 100 species that display a wide variety of physiological characters, including pH range for growth. However, most of them grow well in neutral or alkaline conditions. Only *Halococcus hamelinensis* 100A6^T, *Halococcus qingdaonensis* CM5^T and *Halarchaeum acidiphilum* MH1-52-1^T have an acidic optimum pH for growth, of 6.0 (Goh *et al.*, 2006), 6.0 (Wang *et al.*, 2007) and 4.8 (Minegishi *et al.*, 2008, 2010), respectively.

Recently, we isolated a slightly acidophilic and halophilic archaeon, strain YSM-79^T, from commercially available salt made in Yonaguni island, Okinawa, Japan, in 2009. The salt sample was coarse and well-dried because it had been made from seawater by a process involving heating, concentrating and crystallization over about 10 days (the temperature was not described on the packaging). In the present study, we report the phylogenetic and phenotypic

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characterization of strain YSM-79^T. We propose that the strain represents a novel genus and species.

Strain YSM-79^T was isolated from commercial salt that was produced and sold in Japan, using 168M medium containing (l^{-1}) 200 g NaCl, 2.0 g KCl, 20 g $MgSO_4.7H_2O$, 0.5 g Casamino acids (Difco), 0.5 g yeast extract (Difco), 2.0 g locustbean gum (Sigma-Aldrich), 0.3 g sodium citrate, 0.1 g sodium glutamate, 36 mg $FeCl_2.4H_2O$ and 0.36 mg $MnCl_2.4H_2O$. The pH was adjusted to 7.2 with 1 M KOH. Solid media contained 20 g Bacto agar (Difco). The medium was based on JCM medium no. 168 (Japan Collection of Microorganisms, RIKEN BioResource Center). Salt samples suspended in sterile water were spread on 168M agar plates and then incubated in plastic bags at 37 °C. After incubation for 2–8 weeks, clear red colonies appeared and were transferred to a new plate. The strain was isolated and purified by repeated streaking. Cells of strain YSM-79^T grown under optimal conditions were Gram-negative, motile, short rods (see Supplementary Fig. S1, available in IJSEM Online), approximately 0.5–1.0 × 0.5–2.0 μm in size in liquid medium. On agar plates, this strain formed clear red microcolonies.

According to the proposed minimum standard tests for the description of a new taxon in the order *Halobacteriales* (Oren *et al.*, 1997), we carried out the following phenotypic tests on strain YSM-79^T: anaerobic growth in the presence of nitrate, DMSO or arginine, catalase and oxidase activities, and the utilization of sugars, alcohols and organic acids.

Medium A, containing (l^{-1}) 165 g NaCl, 20 g $MgCl_2.6H_2O$, 10 g K_2SO_4, 3.0 g Casamino acids, 1.0 g yeast extract, 3.0 g sodium glutamate, 18 mg $FeCl_2.4H_2O$ and 0.18 mg $MnCl_2.4H_2O$, was used for culture maintenance. Solid media contained 20 g Bacto agar. The pH was adjusted to 5.5 with 1 M KOH or HCl. The pH was elevated to 7.5 (at stationary phase) during the growth of YSM-79^T. Medium B, containing (l^{-1}) 165 g NaCl, 20 g $MgCl_2.6H_2O$, 10 g K_2SO_4, 0.1 g K_2HPO_4, 0.1 g yeast extract (as a vitamin source), 1.0 g NH_4Cl, 18 mg $FeCl_2.4H_2O$, 0.18 mg $MnCl_2.4H_2O$, pH 5.5 and supplemented with 0.5 % (w/v) carbon source, was used to perform phenotypic tests including utilization of single or complex carbon sources. Antibiotic-sensitivity tests were performed by using BD Sensi-Discs with solid medium A. 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 or nitrite was detected by using the sulfanilic acid and α-naphthylamine reagent (Smibert & Krieg, 1994). H_2S formation was determined by black sulfide precipitate in medium containing 0.5 % (w/v) sodium subsulfite or cysteine. Anaerobic growth tests were carried out in a screw-cap vial using medium A supplemented with 2.5 g DMSO, arginine or nitrate l^{-1}. The air in the vial was substituted by nitrogen gas. Other enzymic activities were tested by the API ZYM system (bioMérieux) at 17 % (w/v) NaCl, 37 °C and for 24 h. All chemicals were obtained from Wako Pure Chemical if not otherwise specified.

Detailed results of the physiological and biochemical tests, as well as the antibiotic-susceptibility tests, are given in the species description below. Phenotypic characteristics in comparison with those of other haloarchaea are summarized in Table 1.

Total lipids were extracted by the modified method of Kamekura (1993). Polar lipids were extracted with methanol/chloroform (2 : 1, v/v). Extracts were dissolved in a small volume of chloroform, applied to silica gel TLC plates and separated by single development with chloroform/methanol/acetic acid/water (85 : 22.5 : 10 : 4, v/v). Lipid spots were detected by spraying with 0.5 % α-naphthol in 50 % methanol, followed by 5 % H_2SO_4 in ethanol, and heating of the plates at 150 °C. Polar lipid analysis showed that strain YSM-79^T contained the diether derivatives of phosphatidylglycerol (PG) and phosphatidylglycerophosphate methyl ester (PGP-Me), sulfated diglycosyl diether-1 (S-DGD-1) and five unidentified glycolipids (see Supplementary Fig. S2, available in IJSEM Online).

The G + C content of the DNA was determined by HPLC on a Cosmosil 5C18 (150 × 4 mm; Nacalai Tesque) column, according to Tamaoka & Komagata (1984). The composition of the elution buffer was 0.02 M ammonium dihydrogen phosphate/acetonitrile (40 : 1, v/v). The DNA G + C content of strain YSM-79^T was 64 mol%.

The 16S rRNA gene of strain YSM-79^T was amplified by PCR with the forward primer 5′-ATTCCGGTTGATCCTGCCGG-3′ and the reverse primer 5′-AGGAGGTGATCCAGCCGCAG-3′ using LA *Taq* polymerase (TaKaRa). PCR was performed for 28 cycles with denaturation for 30 s at 94 °C, annealing for 45 s at 55 °C and polymerization for 2 min at 72 °C. The amplified gene was cloned into the pTA2 plasmid vector (TArget clone; TOYOBO) and recombinant plasmids were reproduced in *Escherichia coli* HB101 (TaKaRa). The sequence of the 16S rRNA gene was determined from 40 clones by the dye-termination method (BigDye Terminator cycle sequencing kit ver. 3.1; Applied Biosystems) using an ABI 3130XL sequencer (Applied Biosystems).

Phylogenetic analysis and tree construction were performed by using the following programs. Similarity values were calculated by the NCBI BLAST program; the sequences of related strains retrieved from GenBank were aligned by using CLUSTAL_X 2.0.11 (Larkin *et al.*, 2007). Then, the gaps in the alignment were removed manually. Maximum-likelihood analyses were performed with RAxML 7.0.3 (Stamatakis *et al.*, 2005) with the general time-reversible plus gamma (GTR + Γ) model. The phylogenetic tree was drawn by using TreeView (Page, 1996). The BLAST analysis indicated that strain YSM-79^T (1420 bp; GenBank accession no. AB454051) is a member of the phylogenetic group defined as the family *Halobacteriaceae*, with 16S rRNA gene sequence similarities of <94 % to *Halobacterium noricense* A1^T (<94 %), *Halobacterium salinarum* DSM 3754^T (<94 %) and the type species of the genera *Halomicrobium* (*Halomicrobium mukohataei* JCM 9738^T; <92 %), *Halarchaeum*

Table 1. Differential characteristics between strain YSM-79^T and representatives of closely related genera within the family *Halobacteriaceae*

Data for reference genera are from Gruber *et al.* (2004), Inoue *et al.* (2011), Kharroub *et al.* (2008), Minegishi *et al.* (2010), Oren *et al.* (2002, 2009), Yachai *et al.* (2008) and Yang *et al.* (2006). +, Positive; −, negative; ND, not determined. Fractions in columns indicate (no. of positive species)/(no. of species within the genus). All genera were positive for cell lysis in distilled water and the presence of PG and PGP-Me.

Characteristic	YSM-79^T	*Halobacterium*	*Halarchaeum*	*Halomicrobium*
Cell shape	Short rod	Rod	Pleomorphic	Rod
Motility	+	+	−	+/−
Pigmentation	Clear red	Red or pink	Non-pigmented	Red–orange
Cell size (μm)	0.5–1.0 × 0.5–2.0	0.5–1.2 × 1–6	2.0	0.5–2.8 × 1–8
Growth optima:				
NaCl (M)	2.5–3.0	2.2–4.5	3.6–4.1	3.0–4.3
Mg^{2+} (M)	0.2–0.3	0.05–0.6	0.05	0–0.3
pH	5.2–6.3	7.0–7.5	4.4–4.5	7.0–7.5
Temperature (°C)	40	37–50	37	37–45
Utilization of any sugar for growth	−	+	+	+
Hydrolysis of:				
Starch	−	−	−	+
Gelatin	−	3/4	−	1/2
Tween 80	+	2/4	−	1/2
Casein	−	+	−	ND
Oxidase activity	+	3/4	−	+
Anaerobic growth with DMSO	−	3/4	−	ND
Nitrate reduction	−	2/4	−	+
Indole production	−	+	−	−
Polar lipids*:				
PGS	−	+	−	1/2
S-DGD-1	+	−	−	+
TGD-1	−	+	−	−
S-TGD-1	−	+	−	−
S-TeGD	−	+	−	−
No. of unknown glycolipids	5	−	4	−
DNA G+C content (mol%)	64	54.5–71.2	61.4	52.4–65

*PGS, Phosphatidylglycerosulfate; TGD-1, triglycosyl diether-1; S-TGD-1, sulfated triglycosyl diether; S-TeDG, sulfated tetraglycosyl diether-1.

(*Halarchaeum acidiphilum* MH1-52-1^T; <91 %), *Halosimplex* (*Halosimplex carlsbadense* 2-9-1^T; <91 %) and *Haladaptatus* (*Haladaptatus paucihalophilus* DX253^T; <91 %). A maximum-likelihood tree with examples of other related genera is shown in Fig. 1.

Determination of the *rpoB′* gene sequence and its analysis were done according to Minegishi *et al.* (2010). The *rpoB′* gene, from the 3′ terminal of *rpoB″* to the 5′ terminal of *rpoA*, was amplified by PCR with the forward primer (HrpoB2 1420F) 5′-TGTGGGCTNGTGAAGAACTT-3′ and the reverse primer (HrpoA 153R) 5′-GGGTCCATC-AGCCCCATGTC-3′ using LA *Taq* polymerase. The conditions for gene amplification were as follows: initial denaturation step (7 min, 96 °C), 35 cycles of denaturation (1 min, 96 °C), annealing (1 min, 48 °C) and extension step (2.5 min, 72 °C), followed by final terminal extension step (7 min, 72 °C). PCR products were purified by agarose-gel electrophoresis and then sequenced with the following primer set: forward primers HrpoB-458F (5′-TTACSATGGGNKCRGGGATG-3′), HrpoB-721F (5′-TT-CTTCCGNCANTACGAGGG-3′) and HrpoB-1148F (5′-AGGAGGACATGCCNTTYACC-3′), and reverse primers HrpoB-671R (5′-GCGTCCTCGATGTTGAANCCC-3′), HrpoB-1166R (5′-GTRAASGGCATGTCCTCCTG-3′) and HrpoB-1457R (5′-ACCATGTGRTASAGYTTSTG-3′). All other comparative sequences were obtained from GenBank. Any gaps in an alignment equivalent to the N-terminal 15 bp of strain YSM-79^T were deleted and the resulting 1812 bp *rpoB′* sequences were used for the following analysis. Sequence similarity values between strain YSM-79^T and related taxa were calculated by GENETYX ver. 10 (Genetyx Corporation); phylogenetic analysis and tree construction were performed with CLUSTAL_X 2.0.11 for sequence alignment, RAxML 7.0.3 with the GTR + Γ model and TreeView.

The *rpoB′* gene sequence of strain YSM-79^T (1827 bp; GenBank accession no. AB550269) formed a branch with

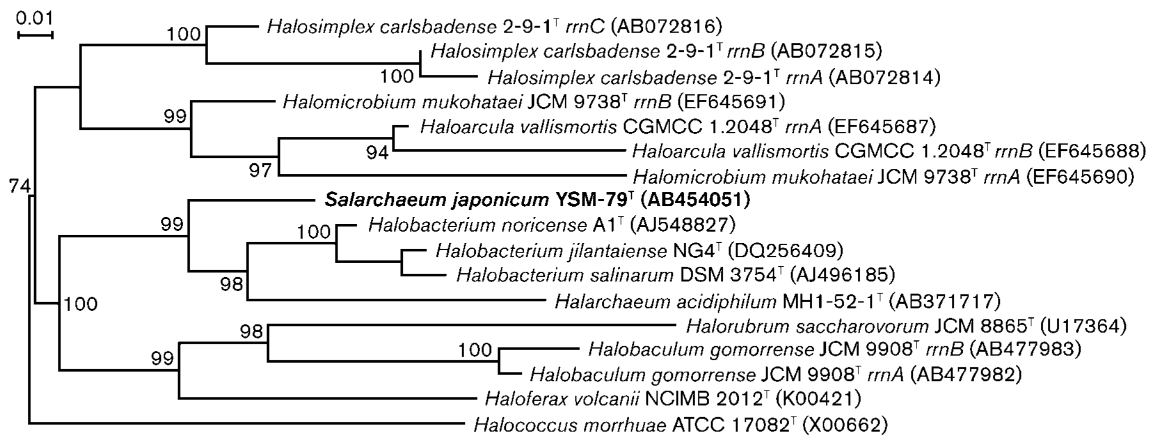

Fig. 1. Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences, showing the positions of strain YSM-79^{T} and some other related haloarchaeal strains. GenBank accession numbers are shown in parentheses. Bar, 0.01 changes per nucleotide position. Bootstrap values are percentages of 1000 replications.

Halarchaeum and *Halobacterium* spp. on the maximum-likelihood phylogenetic tree (see Supplementary Fig. S3, available in IJSEM Online), similar to the 16S rRNA gene sequence analysis, with an *rpoB′* gene sequence similarity value of <88 % to members of the genera *Halobacterium* and *Halarchaeum*. The result supported the view that strain YSM-79^{T} was clearly different from representatives of other genera in the family *Halobacteriaceae*.

The phenotypic, chemotaxonomic and phylogenetic data, based on both 16S rRNA gene and *rpoB′* gene sequence comparison, support the placement of strain YSM-79^{T} in a novel genus and species within the haloarchaea, for which we propose the name *Salarchaeum japonicum* gen. nov., sp. nov. The type strain is YSM-79^{T} (=JCM 16327^{T} =CECT 7563^{T}).

Description of *Salarchaeum* gen. nov.

Salarchaeum [Sal.ar.chae′um. L. n. *sal*, *salis* salt; N.L. neut. n. *archaeum* (from Gr. adj. *archaios* ancient) ancient one, archaeon; N.L. neut. n. *Salarchaeum* salt-requiring archaeon].

Cells are Gram-negative, motile, short rods. Colonies on agar are very small and clear red. Chemo-organotrophic, does not utilize sugar as a single carbon source. Aerobic, halophilic and slightly acidophilic, requiring at least 1.5 M NaCl for growth. The major polar lipids are PG, PGP-Me, S-DGD-1 and five unidentified glycolipids. Phylogenetically a member of the order *Halobacteriales*. The type species is *Salarchaeum japonicum*. Recommended three-letter abbreviation: *Sar*.

Description of *Salarchaeum japonicum* sp. nov.

Salarchaeum japonicum (ja.po′ni.cum. N.L. neut. adj. *japonicum* Japanese, pertaining to Japan, referring to the place of isolation of the type strain).

The description of *Salarchaeum japonicum* is as that given above for the genus, with the following additions.

Cells are Gram-negative, motile, short rods, 0.5–1.0 × 0.5–2.0 μm in liquid media. On agar plates, under optimal conditions clear red microcolonies are formed (0.1 mm in diameter after 1 week growth at 37 °C). Growth occurs at NaCl concentrations of 1.5–5.3 M (saturated), optimum 2.5–3.0 M; at pH 5.0–8.8, optimum pH 5.2–6.3; and at 20–55 °C, optimum 40 °C. Mg^{2+} is required for growth, with maximum growth occurring at 200–300 mM. Cells lyse in distilled water. Catalase- and oxidase-positive. Does not reduce nitrate or nitrite under aerobic conditions. Anaerobic growth with nitrate, arginine and DMSO does not occur. Indole production from tryptophan is negative. H_2S production from thiosulfate or cysteine is negative. Susceptible to novobiocin; resistant to ampicillin, anisomycin, bacitracin, chloramphenicol, erythromycin, gentamicin, kanamycin, neomycin, penicillin, rifampicin, streptomycin, tetracycline and vancomycin. Utilizes sodium fumarate, sodium malate, sodium succinate, sodium glutamate, yeast extract, Casamino acids, peptone (Oxoid) and neopeptone (BD Difco) as single or complex carbon sources for growth. Does not utilize arabinose, cellobiose, fructose, galactose, glucose, glycerol, inositol, lactose, maltose, mannitol, mannose, raffinose, ribitol, ribose, sodium acetate, sodium citrate, sodium pyruvate, sodium lactate, sorbitol, sucrose, trehalose, xylose, glycine, L-alanine, L-arginine chloride, L-lysine chloride, sodium L-aspartate or Bacto peptone. Hydrolyses Tween 80. Does not hydrolyse starch, gelatin or casein. The following enzymic activities are positive: esterase (C4), esterase lipase (C8), lipase (C14, weak), alkaline phosphatase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, trypsin and α-chymotrypsin. Ornithine decarboxylase, lysine decarboxylase, valine arylamidase, leucine arylamidase, cystine arylamidase, α-galactosidase, *β*-galactosidase, *β*-glucuronidase, α-glucosidase, *β*-glucosidase, *N*-acetyl-*β*-glucosaminidase, α-mannosidase and α-fucosidase activities are negative.

The type strain is YSM-79^{T} (=JCM 16327^{T} =CECT 7563^{T}), isolated from commercial salt made from seawater

in Yonaguni island, Okinawa, Japan. Its DNA G+C content is 64 mol% as determined by HPLC.

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