Natronoarchaeum mannanilyticum gen. nov., sp. nov., an aerobic, extremely halophilic archaeon isolated from commercial salt

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Strain YSM-123^T was isolated from commercial salt made from Japanese seawater in Niigata prefecture. Optimal NaCl and Mg²⁺ concentrations for growth were 4.0–4.5 M and 5 mM, respectively. The isolate was a mesophilic and slightly alkaliphilic haloarchaeon, whose optimal growth temperature and pH were 37 °C and pH 8.0–9.0. Phylogenetic analysis based on 16S rRNA gene sequence analysis suggested that strain YSM-123^T is a member of the phylogenetic group defined by the family *Halobacteriaceae*, but there were low similarities to type strains of other genera of this family (≤90 %); for example, *Halococcus* (similarity <89 %), *Halostagnicola* (<89 %), *Natronolimnobius* (<89 %), *Halobiforma* (<90 %), *Haloterrigena* (<90 %), *Halovivax* (<90 %), *Natrialba* (<90 %), *Natronobacterium* (<90 %) and *Natronococcus* (<90 %). The G+C content of the DNA was 63 mol%. Polar lipid analysis revealed the presence of phosphatidylglycerol, phosphatidylglycerophosphate methyl ester, disulfated diglycosyl diether and an unknown glycolipid. On the basis of the data presented, we propose that strain YSM-123^T should be placed in a new genus and species, *Natronoarchaeum mannanilyticum* gen. nov., sp. nov. The type strain of *Natronoarchaeum mannanilyticum* is strain YSM-123^T (=JCM 16328^T =CECT 7565^T).

The extremely halophilic, aerobic archaea that require least 1.5 M NaCl for growth are placed in the family *Halobacteriaceae*, order *Halobacteriales*, class ‘*Haloarchaea*’ (Grant *et al.*, 2001). The current classification of this family is based mainly on three taxonomic characters: 16S rRNA gene sequence, polar lipid composition and DNA–DNA hybridization (Oren *et al.*, 1997; Grant *et al.*, 2001). At the time of writing, the aerobic, extremely halophilic archaea are classified within 27 different genera. The type species of these genera are *Halobacterium salinarum* (Elazari-Volcani, 1957), *Halococcus morrhuae* (Kocur & Hodgkiss, 1973), *Natronobacterium*

gregoryi (Tindall *et al.*, 1984), *Haloarcula vallismortis* (Torreblanca *et al.*, 1986), *Haloferax volcanii* (Torreblanca *et al.*, 1986), *Natronococcus occultus* (Tindall *et al.*, 1984), *Halobaculum gomorrense* (Oren *et al.*, 1995), *Halorubrum saccharovororum* (McGenity & Grant, 1995), *Natrialba asiatica* (Kamekura & Dyll-Smith, 1995), *Natronomonas pharaonis* (Kamekura *et al.*, 1997), *Halogeometricum borinquense* (Montalvo-Rodríguez *et al.*, 1998), *Natrinema pellirubrum* (McGenity *et al.*, 1998), *Haloterrigena turkmenica* (Ventosa *et al.*, 1999), *Natronorubrum bangense* (Xu *et al.*, 1999), *Halorhabdus utahensis* (Wainø *et al.*, 2000), *Halobiforma haloterrestris* (Hezayen *et al.*, 2002), *Halomicrobium mukohataei* (Oren *et al.*, 2002), *Halosimplex carlsbadense* (Vreeland *et al.*, 2002), *Halalkalicoccus tibetensis* (Xue *et al.*, 2005), *Natronolimnobius baerhuensis* (Itoh *et al.*, 2005), *Halovivax asiaticus* (Castillo *et al.*, 2006a), *Halostagnicola larsenii* (Castillo *et al.*, 2006b), *Haladaptatus paucihalophilus* (Savage *et al.*, 2007), *Halopiger xanaduensis* (Gutiérrez *et al.*, 2007),

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strain YSM-123^T are AB501361 (*rnaA*) and AB501360 (*rnaB*).

Results of TLC of polar lipids of strain YSM-123^T and related haloarchaea are available as supplementary material with the online version of this paper.

Haloplanus natans (Bardavid *et al.*, 2007), *Haloquadratum walsbyi* (Burns *et al.*, 2007) and *Halosarcina pallida* (Savage *et al.*, 2008).

Most salt that is sold in Japan is made from seawater concentrated in salterns and then evaporated by heating. In this paper, we describe a halophilic archaeal strain, YSM-123^T, which was isolated from commercial salt made by the heating process in Niigata, Japan. Strain YSM-123^T was isolated from a salt sample that was produced and sold in Japan using medium 168M [containing (l⁻¹): 200 g NaCl, 2.0 g KCl, 20 g MgSO₄·7H₂O, 0.5 g Casamino acids, 0.5 g yeast extract, 2.0 g locust-bean gum, 0.3 g sodium citrate, 0.1 g sodium glutamate, 36 mg FeCl₂·4H₂O, 0.36 mg MnCl₂·4H₂O; adjusted to pH 7.2 with 1 M KOH]. Solid media contained 20 g Bacto agar. The medium was based on JCM-168 medium (Japan Collection of Micro-organisms, RIKEN BioResource Center). Carbon sources were reduced and locust-bean gum was then added. Salt samples dissolved in sterile water were spread on 168M medium agar plates and then incubated in plastic bags at 37 °C. After 2–8 weeks of incubation, clear red-coloured colonies developed, and these were transferred to a new plate. The strain was isolated and purified by repeated streaking.

Cells of strain YSM-123^T grown under optimal conditions were Gram-negative, non-motile and extremely pleomorphic (Fig. 1). The cells were typically 1–5 µm in diameter in liquid media. On agar medium, the strain formed clear, red microcolonies.

Phenotypic tests were carried out in accordance with the proposed minimum standards for the description of new taxa in the order *Halobacteriales* (Oren *et al.*, 1997) and included anaerobic growth in the presence of nitrate or

Fig. 1. Phase-contrast micrograph of cells of strain YSM-123^T. Bar, 10 µm.

arginine, catalase and oxidase activities and the utilization of sugars, alcohols and organic acids.

Medium A [containing (l⁻¹): 220 g NaCl, 2.0 g KCl, 1.0 MgCl₂·6H₂O, 1.0 g K₂HPO₄, 0.5 g Casamino acids, 0.5 g yeast extract, 0.3 g sodium citrate, 0.1 g sodium glutamate, 3.0 g glucose, 18 mg FeCl₂·4H₂O, 0.18 mg MnCl₂·4H₂O] was used for culture maintenance. The pH was adjusted to 8.8 with 1 M KOH. Medium B [containing (l⁻¹): 220 g NaCl, 2.0 g KCl, 1.0 g MgCl₂·6H₂O, 1.0 g K₂HPO₄, 0.05 g yeast extract, 0.5 g NH₄Cl, 18 mg FeCl₂·4H₂O, 0.18 mg MnCl₂·4H₂O, pH 8.8; 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 spreading bacterial suspensions on agar of medium A. Discs impregnated with the antibiotics used were applied to the plates.

Detailed results of the physiological and biochemical tests as well as antibiotic susceptibility tests are given in the species description.

Total lipids were extracted by a modified method of Kamekura (1993). Polar lipids were extracted with methanol/chloroform (2 : 1, v/v). Extracts were then 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). Lipid spots were detected by spraying with the following reagents: 0.5 % α -naphthol in 50 % methanol, followed by 5 % H₂SO₄ in ethanol and heating of the plates at 150 °C. Polar lipid analysis showed that the strain contained diether derivatives of phosphatidylglycerol and phosphatidylglycerophosphate methyl ester. Disulfated diglycosyl diether and an unknown glycolipid were also produced (Supplementary Fig. S1, available in IJSEM Online).

The 16S rRNA gene of strain YSM-123^T was amplified by PCR with the forward primer 5'-ATTCCGGTTGATCC-TGCCGG-3' and the reverse primer 5'-AGGAGGTGA-TCCAGCCGCAG-3' using LA-Taq (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 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 by the dye-termination method (BigDye cycle sequencing kit version 1.1) using an ABI 377 sequencer. Similarity values were calculated by NCBI BLAST. Phylogenetic analysis and tree construction was performed using the programs CLUSTAL_X (Larkin *et al.*, 2007), RAXML (Stamatakis *et al.*, 2005) and TreeView (Page, 1996).

Phylogenetic analysis based on the 16S rRNA gene sequence suggested that strain YSM-123^T has two 16S rRNA genes, *rrnA* and *rrnB*, which share 99 % sequence similarity. The NCBI BLAST program indicated that YSM-123^T was a member of the phylogenetic group defined by

Fig. 2. Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences showing the positions of strain YSM-123^T and related haloarchaeal strains. GenBank accession numbers are shown in parentheses. Bar, 0.03 changes per nucleotide position. Bootstrap values are percentages of 1000 replicates.

the family *Halobacteriaceae*, but there were low similarities to type strains of other genera of this family ($\leq 90\%$); for example, *Halococcus* (similarity $< 89\%$), *Halostagnicola* ($< 89\%$), *Natronolimnobius* ($< 89\%$), *Halobiforma* ($< 90\%$), *Haloterrigena* ($< 90\%$), *Halovivax* ($< 90\%$), *Natrialba* ($< 90\%$), *Natronobacterium* ($< 90\%$) and *Natronococcus* ($< 90\%$). Tree reconstruction using the maximum-likelihood (Fig. 2) and neighbour-joining (not shown) methods showed similar phylogenetic placement for both genes of YSM-123^T.

The G + C content of the DNA was determined by HPLC on a Cosmosil 5C18 (150 $\times$ 4 mm) column, according to Tamaoka & Komagata (1984). The elution buffer was 0.02 M ammonium dihydrogen phosphate/acetonitrile (40 : 1, v/v). The DNA G + C content was calculated to be 63 mol%.

The phenotypic and chemotaxonomic data and phylogenetic data based on 16S rRNA gene sequence comparison support the placement of strain YSM-123^T in a novel genus

Table 1. Characteristics that distinguish *Natronoarchaeum* gen. nov. from related haloarchaeal genera

Genera: 1, *Natronoarchaeum* gen. nov. (strain YSM-123^T); 2, *Halobiforma*; 3, *Haloterrigena*; 4, *Halovivax*; 5, *Natrialba*; 6, *Natronobacterium*; 7, *Natronococcus*. Data were derived from Castillo *et al.* (2006c, 2007), Cui *et al.* (2006), Gutiérrez *et al.* (2008), Hezayen *et al.* (2001), Kanal *et al.* (1995), Montalvo-Rodríguez *et al.* (2000), Oren *et al.* (2009), Roh *et al.* (2007), Romano *et al.* (2007), Tapingkae *et al.* (2008), Xin *et al.* (2000), Xu *et al.* (2001, 2005a, b, c) and this study. Members of all genera contain phosphatidylglycerol and phosphatidylglycerophosphate methyl ester. None of the genera contain diglycosyl diether or sulfated tetraglycosyl diether.

Characteristic	1	2	3	4	5	6	7
Morphology*	C/P	C/P	C/R	R/PR	R	R	C
Pigmentation†	CR	RD	RD/LP	PP	WH/RD	RD	RD
Cell size (μm)	1–5	0.4–2.6 $\times$ 2–8	0.4–1.5 $\times$ 2–13	0.4–0.8 $\times$ 3.5–5	0.5–1 $\times$ 1–5	0.5–0.7 $\times$ 10–15	1–2
NaCl optimum (M)	4.0–4.5	3.4	3.0–3.5	3.4	3.5–4.0	3.0	2.5–3.8
pH optimum	8.0–9.0	7.5	7.0–7.5	7.0–7.5	6.6–9.5	9.5	9.0–9.5
Temperature optimum ($^{\circ}\text{C}$)	37	36–45	40–50	37	35–40	37	35–45
DNA G + C content (mol%)	63	64.9–66.9	59.2–66.6	60.3–65	60.3–64.3	65	63.5–64
Polar lipids‡							
PGS	–	–	–	–	–	–	+
S-DGD	–	–	–/+	–	–	–	–
S ₂ -DGD	+	–	–/+	–	–/+	–	–
TGD	–	+	–	–	–	–	–
S-TGD	–	+	–	–	–	–	–
Glycolipids	+	+	+	+	–/+	–	–

*C, Cocci; P, pleomorphic; R, rods.

†CR, Clear red; LP, light pink; PP, pale pink; RD, red; WH, white.

‡PGS, Phosphatidylglycerosulfate; S-DGD, sulfated diglycosyl diether; S₂-DGD, disulfated diglycosyl diether; S-TGD, sulfated triglycosyl diether; TGD, triglycosyl diether.

and species within the haloarchaea. On the basis of these results, particularly the lack of significant 16S rRNA gene similarity with representatives of other genera, we propose that strain YSM-123^T should be placed in the new genus and species *Natronoarchaeum mannanilyticum* gen. nov., sp. nov.

Description of *Natronoarchaeum* gen. nov.

Natronoarchaeum [Na.tro'no.ar.chae'um. N.L. n. *natron* (arbitrarily derived from Arabic n. *natrun* or *natron*) soda, sodium carbonate; N.L. pref. *natrono-* pertaining to soda; N.L. neut. n. *archaeum* archaeon from Gr. adj. *archaios* -ê-on ancient; N.L. neut. n. *Natronoarchaeum* the soda archaeon].

Cells are Gram-negative, non-motile and extremely pleomorphic. Colonies on agar are small and are pigmented clear red. Chemo-organotrophic, aerobic, halophilic and slightly alkaliphilic, requiring at least 2 M NaCl for growth. The major polar lipids are phosphatidylglycerol, phosphatidylglycerophosphate methyl ester and disulfated diglycosyl diether (S₂-DGD). Phylogenetically, a member of the *Halobacteriales*. The type species is *Natronoarchaeum mannanilyticum*. Recommended three-letter abbreviation: *Nac*.

Description of *Natronoarchaeum mannanilyticum* sp. nov.

Natronoarchaeum mannanilyticum (man.na.ni.ly'ti.cum. N.L. neut. n. *mannanum* mannan; N.L. neut. adj. *lyticum* from Gr. adj. *lytikos* able to loosen, able to dissolve; N.L. neut. adj. *mannanilyticum* mannan-dissolving).

The description is identical to that given for the genus, with the following additions. Cells are typically 1–5 µm in diameter in liquid medium. Cells lyse in distilled water. On agar medium, under optimal conditions, forms clear red microcolonies (0.5–1.0 mm in diameter after 10 days of growth at 37 °C). Grows in 2.0–5.3 (saturated) M NaCl (optimum 4.0–4.5 M), at pH 5.8–9.5 (optimum pH 8.0–9.0) and at 20–55 °C (optimum 37 °C). Mg²⁺ is required for growth, with maximum growth at 5 mM. Phenotypic characteristics are summarized in Table 1 in comparison with other haloarchaea. Catalase- and oxidase-negative. Does not reduce nitrate to nitrite. Anaerobic growth does not occur with nitrate, arginine or DMSO. Indole production from tryptophan is positive. Susceptible to bacitracin, novobiocin and rifampicin and resistant to ampicillin, chloramphenicol, erythromycin, gentamicin, kanamycin, neomycin, penicillin, streptomycin, tetracycline and vancomycin. Utilizes cellobiose, glucose, lactose, mannose, raffinose, locust-bean gum, yeast extract, Casamino acids, peptone, sodium glutamate and pyruvic acid as single or complex carbon sources for growth. Hydrolyses starch. Does not hydrolyse gelatin, Tween 80 or casein. Contains an unknown glycolipid in addition to the polar lipids described for the genus. The DNA G+C content of the type strain is 63 mol% as determined by

HPLC. The type strain has two 16S rRNA genes that show low sequence similarity (≤90%) to those of other members of the family *Halobacteriaceae*.

The type strain, YSM-123^T (=JCM 16328^T =CECT 7565^T), was isolated from a commercial salt sample made in Niigata prefecture, Japan.

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