Halomicrobium katesii sp. nov., an extremely halophilic archaeon

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Two extremely halophilic archaea, strains Al-5^T and K-1, were isolated from Lake Tebenquiche (Atacama Saltern, Chile) and Ezzemoul sabkha (Algeria), respectively. Cells of the two strains were short-rod-shaped and Gram-negative; colonies were orange-pigmented. They grew optimally at 37–40 °C and pH 7.0–7.5 in the presence of 25 % (w/v) NaCl. Magnesium was not required. Polar lipid analysis revealed the presence of phosphatidylglycerol and phosphatidylglycerophosphate methyl ester, the absence of phosphatidylglycerosulfate, and the presence of sulfated diglycosyl diether and diether diglycosyl as the sole glycolipids. DNA G + C contents of strains Al-5^T and K-1 were 52.4 and 52.9 mol% (*T_m* method), respectively. 16S rRNA gene sequence comparison with database sequences showed that strains Al-5^T and K-1 were most closely related to *Halomicrobium mukohataei* DSM 12286^T (similarities of 97.5 and 96.9 %, respectively). DNA–DNA hybridization indicated that strains Al-5^T and K-1 were members of a single species. However, DNA–DNA relatedness to *Halomicrobium mukohataei* was 55.7 ± 2.5 %. A comparative analysis of phenotypic characteristics and DNA–DNA hybridization between the isolates and *Halomicrobium mukohataei* DSM 12286^T supported the conclusion that Al-5^T and K-1 represent a novel species within the genus *Halomicrobium*, for which the name *Halomicrobium katesii* sp. nov. is proposed. The type strain is Al-5^T (=CECT 7257^T=DSM 19301^T).

The extremely halophilic archaea are found in hypersaline environments such as the Dead Sea, the Great Salt Lake, sabkhas and natural and artificial salterns. They are classified within the family *Halobacteriaceae*. Halobacteria are considered to be the most extremely halophilic micro-organisms and require at least 1.5 M NaCl for their growth (Grant *et al.*, 2001). Currently, members of the aerobic, extremely halophilic archaea are classified in 26 genera:

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of isolates Al-5^T and K-1 are EF533994 and EU409597, respectively.

A phylogenetic tree, based on 16S rRNA gene sequences and constructed using the maximum-parsimony algorithm, of isolates Al-5^T and K-1 and related species is available with the online version of this paper.

Halobacterium (Grant, 2001a), *Haloarcula* and *Haloferax* (Torreblanca *et al.*, 1986), *Halobaculum* (Oren *et al.*, 1995), *Halococcus* (Grant, 2001b), *Halogeometricum* (Montalvo-Rodriguez *et al.*, 1998), *Halorubrum* (McGenity & Grant, 1995), *Haloterrigena* (Ventosa *et al.*, 1999), *Natrialba* (Kamekura & Dyll-Smith, 1995), *Natrinema* (McGenity *et al.*, 1998), *Natronobacterium* and *Natronococcus* (Tindall *et al.*, 1984), *Natronomonas* (Kamekura *et al.*, 1997), *Natronorubrum* (Xu *et al.*, 1999), *Halomicrobium* (Oren *et al.*, 2002), *Halorhabdus* (Wainø *et al.*, 2000), *Halosimplex* (Vreeland *et al.*, 2002), *Halobiforma* (Hezayen *et al.*, 2002), *Halalkalicoccus* (Xue *et al.*, 2005), *Natronolimnobi* (Itoh *et al.*, 2005), *Halo*vivax and *Halostagnicola* (Castillo *et al.*, 2006a, b), *Haladaptatus* (Savage *et al.*, 2007), *Haloquadratum* (Burns *et al.*, 2007), *Halopiger* (Gutiérrez *et al.*, 2007) and *Haloplanus* (Bardavid *et al.*, 2007).

The genus *Halomicrobium* was proposed by Oren *et al.* (2002) and currently contains only one species, *Halomicrobium mukohataei*, which was first isolated from the soils of the Argentine salt flats (Ihara *et al.*, 1997). In this study, the characterization of two halophilic strains that represent a novel species of the genus *Halomicrobium* is described.

Strain Al-5^T was isolated from Lake Tebenquiche of the Atacama Saltern, Chile, and strain K-1 was isolated from water samples collected from Ezzemoul sabkha located in the north-east of Algeria. They were isolated independently from each other on hypersaline agar medium containing (per litre): 5 g proteose-peptone no. 5 (Difco), 5 g yeast extract (Difco), 1 g glucose with 25 % (w/v) total salts. The stock of total salts at 30 % (w/v) was prepared as described by Subov (1931): 234 g NaCl, 42 g MgCl₂·6H₂O, 60 g MgSO₄·7H₂O, 1 g CaCl₂·2H₂O, 6 g KCl, 0.2 g NaHCO₃, 0.7 g NaBr, 0.005 g FeCl₃ and 1000 ml distilled water. The medium was brought to pH 7.0 and incubation was at 40 °C. Phenotypic tests of strains Al-5^T and K-1 were performed according to the proposed minimal standards for the description of new taxa in the order *Halobacteriales* (Oren *et al.*, 1997). Cell morphology was examined by transmission electron microscopy of exponentially growing cells in broth containing 25 % (w/v) NaCl and Gram staining was performed by using acetic-acid-fixed samples as described by Dussault (1955). Colony morphology was observed on solid medium after incubation at 40 °C for 5 days. The requirement for NaCl was determined in media containing 0–30 % (w/v) NaCl. Tolerance to pH was tested in solid medium at pH 5.0, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0 and 10.0. The requirement for magnesium was tested in media containing final concentrations of 0.0, 0.005, 0.1, 0.2, 0.4, 0.8 and 3.2 % (w/v) MgCl₂ at the optimum NaCl concentration for growth. The temperature range for growth was tested by incubating cultures on agar plates at 30–55 °C. Reduction of nitrate was tested by using the sulfanilic acid and α -naphthylamine reagent (Smibert & Krieg, 1981). Anaerobic growth in the presence of L-arginine and nitrate was tested as described by Oren *et al.* (1997). Controls without arginine and nitrate were included and incubation was performed in the dark. Catalase and oxidase activities were tested according to Gerhardt *et al.* (1994). Tests for formation of indole and hydrolysis of starch, gelatin and aesculin were as described previously (González *et al.*, 1978). Hydrolysis of Tweens 20, 40, 60 and 80 was tested as outlined by Gutiérrez & González (1972). Utilization of carbohydrates and other compounds as carbon sources and acid production from these compounds were determined as described by Oren *et al.* (1997). The determination of antibiotic susceptibility was performed by spreading bacterial suspensions on plates containing medium with 25 % (w/v) NaCl and applying antibiotic discs [penicillin G, 10 U; kanamycin, 30 mg; tetracycline, 30 mg; erythromycin, 15 mg; streptomycin, 10 mg; bacitracin, 10 U; novobiocin, 30 mg; polymyxin B, 300 U; ampicillin, 10 mg; neomycin, 30 mg; chlor-

amphenicol, 30 mg; gentamicin, 10 mg; and carbenicillin, 50 mg (Lizama *et al.*, 2001)].

Cells of strain Al-5^T were non-motile, short-rod-shaped, 1.80–2.25 × 2.25–2.80 µm in size and both novel strains stained Gram-negative. Cell lysis occurred in distilled water. Colonies on standard growth agar medium were small and circular with entire margins and smooth surfaces. The two novel strains formed orange-coloured colonies about 2.0 mm in diameter after incubation for 5 days on the medium given above at 40 °C.

Strains Al-5^T and K-1 grew in the presence of NaCl concentrations ranging from 20 % (3.4 M) to 30 % (5.2 M). They grew optimally in the presence of 25 % (4.3 M) NaCl. Magnesium was not required for growth. The growth pH range was 6.5–10.0 (optimum growth at pH 7.0–7.5) and the isolates grew at 35–50 °C (optimum growth at 37–40 °C). They reduced nitrate to nitrite and anaerobic growth with arginine or nitrate did not occur. They were positive for catalase and oxidase, but did not form indole. Both strains hydrolysed aesculin, starch, and Tweens 20, 40, 60 and 80. Strain Al-5^T hydrolysed gelatin, but strain K-1 did not. Acid production from D-fructose and D-arabinose was observed. The results of the antibiotic susceptibility and substrate utilization tests are included in the species description.

Polar lipids were extracted from the strains using a modified Bligh and Dyer extraction procedure (Kates, 1972). They were separated by TLC using a single development on silica gel in a chloroform/methanol/acetic acid/water (85:22.5:10:4, by vol.) solvent system. In addition, two dimensional chromatography was performed by using chloroform/methanol/water (65:25:4, by vol.) in the first dimension and chloroform/methanol/acetic acid/water (80:12:15:4, by vol.) in the second dimension. Glycolipid spots were detected by spraying the plates with 0.5 % (w/v) α -naphthol in 50 % methanol and then with 5 % H₂SO₄ in ethanol and heating them at 150 °C. The polar lipid profile of strains Al-5^T and K-1 was similar to that of *Halomicrobium mukohataei* DSM 12286^T, having phosphatidylglycerol, phosphatidylglycerophosphate methyl ester, sulfated diglycosyl diether and diglycosyl diether, but the two novel strains did not contain phosphatidylglycerosulfate.

DNA was isolated and purified by the method described by Lind & Ursing (1986). The 16S rRNA genes of strains Al-5^T and K-1 were amplified by PCR using universal primers as described previously (Arahal *et al.*, 1996). Multiple sequence alignments were performed using CLUSTAL W version 1.8 (Thompson *et al.*, 1994). Phylogenetic trees were constructed on the basis of the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods using the MEGA3 program package (Kumar *et al.*, 2004). DNA G + C content was determined from the mid-point value (T_m) of the thermal denaturation profile (Marmur & Doty, 1962) with a Perkin-Elmer Lambda 3B spectrophotometer at 260 nm. DNA–DNA hybridization studies were performed by the non-radioactive method

described by Ziemke *et al.* (1998). DNA was double-labelled using DIG-11-dUTP and biotin-16-dUTP (Boehringer Mannheim). The labelling reaction was carried out using the Boehringer Mannheim nick-translation kit.

The 16S rRNA gene sequences of strains Al-5^T (1419 bp) and K-1 (1308 bp) were determined. The two sequences shared a high degree of similarity (99.5 %). Phylogenetic analysis based on 16S rRNA gene similarities showed that strains Al-5^T and K-1 were closely related to *Halomicrobium mukohataei* DSM 12286^T (similarities of 97.5 and 96.9 %, respectively). Phylogenetic analysis using the neighbour-joining algorithm revealed that strains Al-5^T and K-1 were related to *Halomicrobium mukohataei* DSM 12286^T, forming a branch in the cluster with a bootstrap value of 100 % (Fig. 1). The phylogenetic position of both strains was also confirmed in a tree generated using the maximum-parsimony algorithm (see Supplementary Fig. S1 available in IJSEM Online).

The DNA G+C contents of strains Al-5^T and K-1 were 52.4 and 52.9 mol%, respectively, as determined by the thermal denaturation method. These are lower than the values reported for members of the family *Halobacteriaceae* (Grant *et al.*, 2001), but close to the value found for *Halobacterium noricense* DSM 15987^T (54.3 mol%) (Gruber *et al.*, 2004). DNA–DNA hybridization between the two isolates showed that isolate Al-5^T had a relatedness value of 94.3 ± 0.3 % to isolate K-1. This high relatedness value indicates that the two strains are representatives of the same species. DNA–DNA reassociation between strain Al-5^T and the related strain *Halomicrobium mukohataei* DSM 12286^T was 55.7 ± 2.5 %.

On the basis of their phenotypic and chemotaxonomic characteristics, including low levels of DNA–DNA related-

ness to *Halomicrobium mukohataei* DSM 12286^T, the new strains described herein are considered to represent a novel species of the genus *Halomicrobium*, for which the name *Halomicrobium katesii* sp. nov. is proposed. Table 1 shows the features that distinguish this novel species from *Halomicrobium mukohataei* DSM 12286^T.

Description of *Halomicrobium katesii* sp. nov.

Halomicrobium katesii (ka.tes.i.i. N.L. gen. masc. n. *katesii* of Kates, named in honour of Professor Morris Kates, for his contributions on halophilic micro-organisms).

Gram-negative, non-motile and short-rod-shaped ($1.80\text{--}2.25 \times 2.25\text{--}2.80$ µm). Colonies are orange, circular with an entire margin and the surface is smooth with a diameter of 2.0 mm on medium containing 25 % (w/v) NaCl after incubation for 5 days at 40 °C. Growth occurs at 20–30 % (3.4–5.2 M) NaCl, 35–50 °C and pH 6.5–10.0; optimal conditions are 37–40 °C, pH 7.0–7.5 and 25 % (4.3 M) NaCl. Magnesium is not required. Reduces nitrate to nitrite and indole is not produced. Anaerobic growth with nitrate or arginine does not occur. Catalase- and oxidase-positive. Aesculin, starch and Tweens 20, 40, 60 and 80 are hydrolysed. Gelatin is hydrolysed by strain Al-5^T, but not by strain K-1. The following substrates are utilized for growth: D-glucose, D-fructose, D-galactose, maltose, cellobiose, starch, trehalose, adonitol, glycerol, D-mannitol, D-sorbitol, acetate and pyruvate. No growth occurs on lactose, D-xylose, D-mannose, L-arabinose, raffinose, D-salicin, L-sorbose, L-rhamnose, sucrose, dulcitol, inositol, succinate, malate, gluconate, citrate, formate, oxalate, propionate, benzoate, L-alanine, L-arginine, L-aspartate, L-asparagine, L-cysteine, glycine, L-glutamate, L-histidine, L-leucine, L-lysine, L-methionine, L-ornithine, L-tryptophan, L-tyrosine or L-valine. Acid is produced from D-fructose

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strains Al-5^T and K-1 with respect to other species of the family *Halobacteriaceae*. Numbers at nodes indicate bootstrap values (based on 1000 replications). Bar, 0.02 substitutions per nucleotide position.

Table 1. Characteristics that distinguish *Halomicrobium katesii* sp. nov. from *Halomicrobium mukohataei* DSM 12286^T

Both species utilize D-glucose, D-galactose and maltose and hydrolyse starch. +, Positive; –, negative; NR, not reported.

Characteristic	<i>Halomicrobium katesii</i> Al-5 ^T	<i>Halomicrobium mukohataei</i> DSM 12286 ^T
Pigmentation	Orange	Red–orange*
Cell shape	Short rods	Short or longer rods†
Size (µm)	1.80–2.25 × 2.25–2.80	1–3 or 3.0–8.0 × 0.5†
Mg ²⁺ required for growth (mM)	0	3*
NaCl range (M)	3.4–5.2	2.5–4.5*
pH range	6.5–10.0	6.2–8.0†
Temperature range (°C)	35–50	NR
Hydrolysis of:		
Aesculin	+	NR
Tween 80	+	–†
Gelatin liquefaction	+	–†
Utilization of:		
Sucrose	–	+†
Glycerol	–	+†
Presence of PGS‡	–	+*
DNA G+C content (mol%)§	52.4–52.9	65*

*Data from Ihara *et al.* (1997).

†Data from Oren *et al.* (2002).

‡PGS, Phosphatidylglycerosulfate.

§Determined by the *T_m* method.

and D-arabinose. Cells are resistant to penicillin G (10 U), chloramphenicol (30 mg), kanamycin (30 mg), ampicillin (10 mg), tetracycline (30 mg), erythromycin (15 mg), streptomycin (10 mg), polymyxin B (300 U), neomycin (30 mg), gentamicin (10 mg) and carbenicillin (50 mg), but susceptible to bacitracin (10 U) and novobiocin (30 mg). The polar lipids present are phosphatidylglycerol, phosphatidylglycerophosphate methyl ester, sulfated diglycosyl diether and diglycosyl diether. Phosphatidylglycerosulfate is absent. DNA G+C content is 52.4–52.9 mol% (*T_m*).

The type strain, Al-5^T (=CECT 7257^T=DSM 19301^T), was isolated from Lake Tebenquiche in the Atacama Saltern, Chile. Strain K-1, isolated from water samples collected from Ezzemoul sabkha, Algeria, is a reference strain.

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