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Halomonas caseinilytica sp. nov., a halophilic bacterium isolated from a saline lake on the Qinghai–Tibet Plateau, China

Yue-Hong Wu,[1] Xue-Wei Xu,[1,2,3] Ying-Yi Huo,[1] Peng Zhou,[1] Xu-Fen Zhu,[1] Hui-Bin Zhang[4] and Min Wu[1]

Correspondence
Min Wu
wumin@zju.edu.cn

[1]College of Life Sciences, Zhejiang University, Hangzhou 310058, PR China

[2]Laboratory of Marine Ecosystem and Biogeochemistry, State Oceanic Administration, Hangzhou 310012, PR China

[3]Second Institute of Oceanography, State Oceanic Administration, Hangzhou 310012, PR China

[4]Altun Mountain National Nature Reserve Administration, Kuerle 841000, PR China

A halophilic, Gram-negative bacterial strain, designated AJ261^T, which was isolated from a soil sample from a salt lake on the Qinghai–Tibet Plateau, was subjected to a polyphasic taxonomic study. The isolate grew optimally in the presence of 3–5 % NaCl and used various carbohydrates as sole carbon and energy sources. The genomic DNA G+C content was 63.0 mol%. The predominant fatty acids were $C_{18:1}\omega7c$, $C_{16:0}$ and $C_{12:0}$. A phylogenetic analysis based on 16S rRNA gene sequences indicated that the isolate had the highest sequence similarity with respect to type strains of *Halomonas elongata* (98.2 %), *Halomonas eurihalina* (98.1 %) and *Halomonas halmophila* (97.2 %). The DNA–DNA relatedness of strain AJ261^T with respect to *H. elongata* NBRC 15536^T, *H. eurihalina* CGMCC 1.2318^T and *H. halmophila* DSM 5349^T was 42, 25 and 26 %, respectively. Overall, the phenotypic, genotypic and phylogenetic results demonstrate that strain AJ261^T represents a novel species within the genus *Halomonas*, for which the name *Halomonas caseinilytica* is proposed. The type strain is AJ261^T (=CGMCC 1.6773^T =JCM 14802^T).

The family *Halomonadaceae* of the *Gammaproteobacteria* includes four genera of halophilic bacteria, *Halomonas*, *Chromohalobacter*, *Cobetia* and *Modicisalibacter*, and three genera of non-halophilic bacteria, *Carnimonas*, *Halotalea* and *Zymobacter* (Franzmann *et al.*, 1989; Ventosa *et al.*, 1989; Okamoto *et al.*, 1993; Dobson & Franzmann, 1996; Garriga *et al.*, 1998; Arahal *et al.*, 2002, 2007; Ntougias *et al.*, 2007; Ben Ali Gam *et al.*, 2007). The genus *Halomonas* was proposed by Vreeland *et al.* (1980), with *Halomonas elongata* as the type species. As of November 2007, the genus *Halomonas* contained 48 species (Euzéby, 1997) and is the largest genus in the family *Halomonadaceae*.

This study describes a novel halophilic bacterium, designated AJ261^T, which belongs to the genus *Halomonas*. Strain AJ261^T was isolated from a soil sample from the Ayakekum salt lake (37° 33′ N 89° 42′ E; 3884 m altitude) on the Qinghai–Tibet Plateau, China. The lake environment has been described previously (Xu *et al.*, 2005). The samples were incubated with halophilic medium (HM) (Ventosa *et al.*, 1982) for approximately 3 days and the liquid was plated by using a 10-fold dilution series.

HM contained the following (l^{-1} distilled water): 50.0 g NaCl, 2.0 g KCl, 1.0 g $MgSO_4$, 0.36 g $CaCl_2 . 2H_2O$, 0.23 g NaBr, 0.06 g $NaHCO_3$, trace $FeCl_3$, 10.0 g yeast extract (Difco), 5.0 g peptone (Difco) and 1.0 g glucose (pH 7.5). After 1 week incubation at 30 °C, a representative colony was picked and maintained on HM. The strain was purified by repeated restreaking; purity was confirmed by the uniformity of cell morphology. Cell morphology and motility were examined by using optical (BX40; Olympus), transmission electron (H-600; Hitachi) and scanning electron microscopy (Tecnai 10; Philips) (see Supplementary Fig. S1, available in IJSEM Online).

The optimal conditions for growth were determined in HM with different salt concentrations (0, 0.5, 1, 3, 5, 7.5, 10, 15, 20, 25 and 30 %, w/v). The pH range for growth was determined by adding MES (pH 5.0–6.0), PIPES (pH 6.5–7.0), Tricine (pH 7.5–8.5) and CHES (pH 9.0–10.0) to the HM at a concentration of 25 mM. The temperature range

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain AJ261^T is EF527874.

Micrographs of strain AJ261^T and detailed fatty acid compositions of strain AJ261^T and related type strains are available as supplementary material with the online version of this paper.

for growth was determined by using incubation temperatures ranging from 4 to 48 °C. Biochemical and nutritional tests were tested in HM according to the methods of Mata *et al.* (2002). Antimicrobial-susceptibility tests were performed in liquid HM containing the antimicrobial agent at 50 μg ml^{-1}. Detailed results are given in the species description.

The 16S rRNA gene was amplified and analysed as described previously (Xu *et al.*, 2007). The almost-complete 16S rRNA gene sequence (1446 bp) of strain AJ261^T was determined. The sequence was compared with closely related sequences of reference organisms from the FASTA network service. Strain AJ261^T showed the highest levels of sequence similarity with respect to type strains of *H. elongata* (98.2 %), *Halomonas eurihalina* (98.1 %), *Halomonas koreensis* (97.3 %) and *Halomonas halmophila* (97.2 %) and showed less than 97.0 % sequence similarity with respect to other *Halomonas* species. Sequence data were aligned with CLUSTAL W 1.8 (Thompson *et al.*, 1994). Phylogenetic trees were constructed using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony methods (Fitch, 1971) with the MEGA3 program package (Kumar *et al.*, 2004) and using the maximum-likelihood method (Felsenstein, 1981) with the PHYLIP 3.6 program. Evolutionary distances were calculated according to the algorithm of the Kimura two-parameter model (Kimura, 1980) for the neighbour-joining method. Strain AJ261^T always showed the closest phylogenetic affinity to *H. elongata*, *H. eurihalina*, *H. halmophila* and *H. almeriensis*, with high levels of bootstrap support (Fig. 1).

Fatty acid methyl esters were prepared from lipids extracted from cells grown in HM for 24 h at 30 °C and were analysed by using GC/MS (Kuykendall *et al.*, 1988); data are given in Supplementary Table S1 (available with IJSEM Online). The unsaturated fatty acid content of strain AJ261^T was higher than those of *H. elongata* NBRC 15536^T and *H. eurihalina* CGMCC 1.2318^T. The G+C content of genomic DNA was determined by thermal denaturation (T_m) (Marmur & Doty, 1962) using *Escherichia coli* K-12 DNA as the calibration standard. DNA–DNA hybridizations were performed using the thermal denaturation and renaturation method of De Ley *et al.* (1970), as modified by Huß *et al.* (1983), using a Beckman DU 800 spectrophotometer. The hybridization temperature used was 79 °C and the experiments were carried out in triplicate. The DNA–DNA relatedness values between strain AJ261^T and *H. elongata* NBRC 15536^T, *H. eurihalina* CGMCC 1.2318^T and *H. halmophila* DSM 5349^T were 42, 25 and 26 %, respectively. In addition, comparisons of phenotypic properties (Table 1) also indicated differences between strain AJ261^T and recognized *Halomonas* species, such as cell morphology, colony pigmentation, motility, salt or temperature range for growth, methyl red test results, hydrolysis of substrates and utilization of carbohydrates.

On the basis of phenotypic, genotypic and phylogenetic data, therefore, strain AJ261^T represents a novel species of the genus *Halomonas*, for which the name *Halomonas caseinilytica* sp. nov. is proposed.

Description of *Halomonas caseinilytica* sp. nov.

Halomonas caseinilytica (ca.se.i.ni.ly′ti.ca. N.L. n. *caseinum* casein; Gr. adj. *lutikos* able to loosen, able to dissolve; N.L. fem. adj. *lytica* dissolving; N.L. fem. adj. *caseinilytica* casein-dissolving).

Cells are Gram-negative, motile, short rods or oval in shape, 0.8–2.0 × 0.4–0.6 μm, and occur singly or in pairs. Colonies on complex agar medium are 1–2 mm in diameter, smooth, circular, elevated and light yellow after 2 days. Moderately halophilic. No growth occurs in the absence of salt. The total salts concentration for growth is 0.5–15 % (w/v), with an optimum at 3–5 %. Grows at

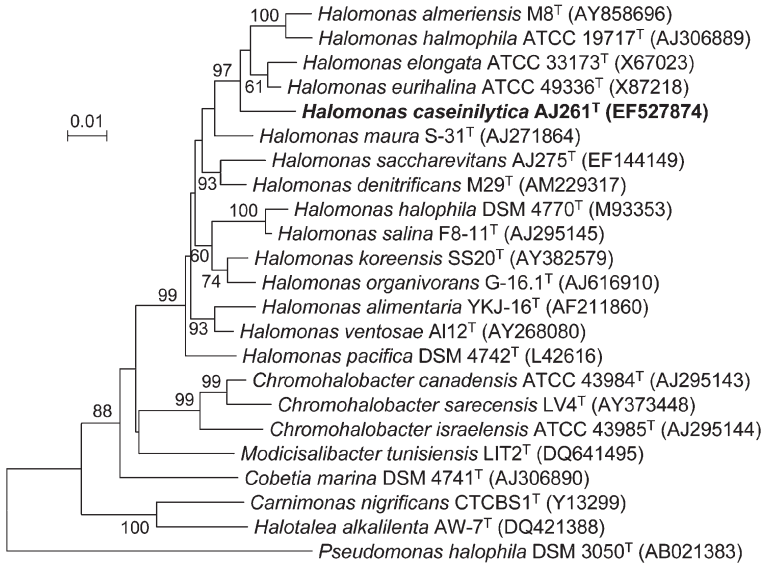

Fig. 1. Neighbour-joining tree, based on 16S rRNA gene sequences, showing the phylogenetic relationships of strain AJ261^T and related taxa. Bootstrap percentages (based on 1000 replicates) >60 % are shown at branch points. Filled circles indicate branches of the tree that were also formed using the maximum-parsimony and maximum-likelihood methods. Bar, 0.01 substitutions per nucleotide position.

Table 1. Phenotypic characteristics that serve to differentiate strain AJ261^T from related *Halomonas* species

Taxa: 1, AJ261^T; 2, *H. elongata* NBRC 15536^T; 3, *H. eurihalina* CGMCC 1.2318^T (data in columns 1–3 from this study unless indicated); 4, *H. almeriensis* (Martínez-Checa *et al.*, 2005); 5, *H. halmophila* (Dobson *et al.*, 1990; Mata *et al.*, 2002). +, Positive; −, negative; ND, no data available.

Characteristic	1	2	3	4	5
Morphology	Short rod	Rod[a]*	Rod[a]	Short rod	Short rod
Colony pigmentation	Light yellow	White	Cream	Cream–white	Cream
Exopolysaccharide	+	−[a]	+[a]	+	−
Motility	+	+	−	−	+
Oxidase	−	−	−	−	+
Salt range (%, w/v)	0.5–15	0–20[a]	0.5–25[a]	5–25	3–25
Temperature range (°C)	4–48	4–45[a]	4–45[a]	15–37	15–45
Methyl red	+	−	−	−	ND
ONPG	+	−	+	−	+
Urease	+	+	+	−	−
H_2S formation	+	+	+	−	+
Growth on MacConkey agar	+	−	+	+	ND
Gluconate oxidation	−	+	−	+	−
Hydrolysis of:					
Casein	+	−	−	−	−
Gelatin	+	+	+	−	−
Tween 20	+	ND	+	−	+
Utilization of:					
Adonitol	−	+	+	−	−
Arabinose	+	+	+	−	−
Cellobiose	+	+	+	−	+
Ethanol	−	+	−	−	−
Galactose	+	+	+	−	+
Histidine	+	+	+	−	+
Lactose	+	+	+	−	+
Mannose	+	+	+	−	+
Raffinose	+	+	−	ND	−
Ribose	−	+	+	ND	ND
Xylose	+	+	+	−	+
DNA G+C content (mol%)	63.0	60.5[b]	59.1–65.7[c]	63.5	63

*Data from: *a*, Mata *et al.* (2002); *b*, Vreeland *et al.* (1980); *c*, Mellado *et al.* (1995).

pH 5.0–9.0 and 4–48 °C (optimum growth at pH 7.0–8.0 and 30 °C). Does not grow anaerobically in the presence of nitrate, nitrite or fumarate. Oxidase-negative and catalase-positive. Grows on MacConkey agar, but not on cetrimide agar. Produces exopolysaccharide and poly-β-hydroxyalkanoate. Aesculin, casein, gelatin, Tween 20 and tyrosine are hydrolysed. DNA, starch and Tween 80 are not hydrolysed. Negative for gluconate oxidation, indole production, phenylalanine deamination, lecithinase, ornithine decarboxylase and urease. Positive for lysine decarboxylase, ONPG, in the methyl red test and for fermentation of D-glucose. Reduces selenite and nitrate. H_2S is formed from thiosulfate or L-cysteine. Chemo-organotrophic. The following substrates are utilized for growth: L-arabinose, cellobiose, D-fructose, D-galactose, glucose, glycerol, inositol, lactose, maltose, mannitol, D-mannose, raffinose, rhamnose, L-sorbitol, sucrose, trehalose, D-xylose, acetate, citrate, fumarate, gluconate, lactate, malate, propionate, pyruvate, succinate, L-alanine, L-arginine, L-aspartate, L-glutamate, glycine, L-isoleucine, L-histidine, L-ornithine, L-serine and L-valine. The following compounds are not utilized as sole carbon and energy sources: adonitol, L-cysteine, ethanol, formate, malonate, L-methionine, ribose, sorbose and starch. Acid is produced from L-arabinose, D-fructose, D-galactose, glucose, inositol, lactose, maltose, mannitol, D-mannose, rhamnose, L-sorbitol, sucrose and trehalose. Susceptible to chloramphenicol, kanamycin, nalidixic acid, neomycin, nitrofurantoin, polymyxin B, rifampicin and tetracycline, but not to ampicillin, bacitracin, carbenicillin, cefotaxime, erythromycin, penicillin, novobiocin, nystatin or streptomycin. The major fatty acids are $C_{18:1}\omega7c$, $C_{16:0}$ and $C_{12:0}$/$C_{12:0}$ 3-OH. The DNA G+C content of the type strain is 63.0±0.1 mol% (T_m).

The type strain, AJ261^T (=CGMCC 1.6773^T =JCM 14802^T), was isolated from a soil sample collected from a salt lake on the Qinghai–Tibet Plateau, China.

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