

Halomonas saccharevitans sp. nov., *Halomonas arcis* sp. nov. and *Halomonas subterranea* sp. nov., halophilic bacteria isolated from hypersaline environments of China

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Three strains of Gram-negative, aerobic, neutrophilic and halophilic bacteria were isolated from samples of a salt lake on the Qinghai–Tibet Plateau and a subterranean saline well in the Si-Chuan Basin of China. These isolates, designated AJ275^T, AJ282^T and ZG16^T, were investigated using a polyphasic approach. Based on 16S rRNA gene sequence analysis, the isolates could be affiliated to the genus *Halomonas*. Genomic DNA G+C contents were 65.9 mol% for AJ275^T, 56.7 mol% for AJ282^T and 57.6 mol% for ZG16^T. The results of DNA–DNA hybridizations, fatty acid analysis and physiological and biochemical tests allowed the isolates to be differentiated genotypically and phenotypically from closely related species. It is proposed that strains AJ275^T (=CGMCC 1.6493^T=JCM 14606^T=LMG 23976^T), AJ282^T (=CGMCC 1.6494^T=JCM 14607^T=LMG 23978^T) and ZG16^T (=CGMCC 1.6495^T=JCM 14608^T=LMG 23977^T) represent the type strains of three novel species in the genus *Halomonas*: *Halomonas saccharevitans* sp. nov., *Halomonas arcis* sp. nov. and *Halomonas subterranea* sp. nov., respectively.

The genus *Halomonas*, belonging to the family *Halomonadaceae* within the class *Gammaproteobacteria*, was originally proposed by Vreeland *et al.* (1980). During the past two decades, many *Halomonas* species have been isolated from different saline environments, such as saline or soda lakes (Franzmann *et al.*, 1987; James *et al.*, 1990; Mormile *et al.*, 1999; Duckworth *et al.*, 2000; Quillaguamán *et al.*, 2004), solar salterns (Bouchotroch *et al.*, 2001; Lim *et al.*, 2004; Martínez-Checa *et al.*, 2005; Lee *et al.*, 2005), saline sands or soils (Romano *et al.*, 1996; Martínez-Cánovas *et al.*, 2004a, b; García *et al.*, 2004), mineral pools (Romano *et al.*, 2005), marine environments (Yoon *et al.*, 2001; Kaye *et al.*, 2004), animals (Romanenko *et al.*, 2002), seafoods (Yoon *et al.*, 2002), artificial sewage treatments (Berendes *et al.*, 1996) and walls and mural paintings

(Heyrman *et al.*, 2002). Additionally, some bacteria that were assigned initially to other genera have been reclassified (Mellado *et al.*, 1995; Dobson & Franzmann, 1996). In total, 35 species of *Halomonas* have been described at the time of writing.

In this study, three novel halophilic bacteria are described using a polyphasic approach. Strains AJ282^T and AJ275^T were isolated from a water sample from Ayakekum salt lake (37° 33' N 89° 42' E; 3884 m altitude) located in Altun Mountain on the Qinghai–Tibet Plateau, China. Strain ZG16^T was isolated from subterranean hypersaline waters taken from a saline well located in Zigong (29° 3' N 105° 7' E) in the Si-Chuan Basin, China.

The medium (HM) used for isolation and maintenance of the strains was that described by Ventosa *et al.* (1982). The medium (pH 7.5) contained (% w/v): NaCl, 5.0; KCl, 0.2; MgSO₄·7H₂O, 0.1; CaCl₂·2H₂O, 0.036; NaBr, 0.023; NaHCO₃, 0.006; yeast extract (Difco), 1.0; peptone (Difco), 0.5; glucose, 0.1. Water samples were filtered through

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0.45 µm and 0.22 µm filters in sequence. The 0.22 µm membranes were added to HM medium and plated by using a tenfold dilution series method. Plates were incubated aerobically at 25 °C. After 3–7 days incubation, representative colonies were picked and maintained at 30 °C. Strains were purified by repeated restreaking; purity was confirmed by the uniformity of colony morphology. Cell morphology and motility were examined by optical microscopy (Olympus BX40). The optimal conditions for growth were determined in HM medium with 0–30% (w/v) NaCl. The pH range for growth was determined by adding the buffers 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 HM medium at a concentration of 50 mM. The temperature range for growth was determined by incubating the strains at 4–55 °C.

Phenotypic characteristics, including oxidase and catalase reactions, H₂S production, hydrolysis of aesculin, gelatin, casein, DNA, starch, Tween 20, Tween 80, tyrosine and urea, indole production, gluconate oxidation, phenylalanine deamination, substrate utilization and acid production from sugars, were tested in HM medium according to the methods of Mata *et al.* (2002). Antimicrobial susceptibility tests were performed in liquid HM medium containing 50 µg antimicrobial agent ml⁻¹. Detailed results are given in the species description.

Fatty acid methyl esters were obtained from cells grown in HM medium for 1 day at 30 °C and analysed by using GC/MS (Kuykendall *et al.*, 1988); data are given in Table 1. The 16S rRNA genes were amplified as described previously (Xu

Table 1. Differential phenotypic characteristics of the novel isolates and the type strains of related *Halomonas* species

Strains: 1, AJ282^T; 2, ZG16^T; 3, AJ275^T; 4, *H. sulfidaeris* DSM 15722^T; 5, *H. hydrothermalis* DSM 15725^T; 6, *H. venusta* CGMCC 1.2315^T; 7, *H. ventosae* DSM 15911^T. +, Positive; –, negative; ND, not determined; TR, trace. Data were from our comparative tests. All strains were positive for catalase and gluconate oxidation and negative for indole production and hydrolysis of gelatin and Tween 80.

Characteristic	1	2	3	4	5	6	7
Oxidase	–	–	+	+	+	+	+
H ₂ S formation	+	+	–	–	ND	–	–
Phenylalanine deamination	+	–	+	+	+	–	–
Hydrolysis of:							
Aesculin	–	–	–	–	–	–	+
Casein	+	+	–	+	–	–	–
Tween 20	+	+	+	+	–	–	+
Tyrosine	–	–	–	–	–	+	+
Urea	–	+	–	–	+	+	–
Utilization of:							
Citrate	+	+	–	+	+	+	+
Ethanol	+	–	–	+	–	+	–
Histidine	–	+	–	–	–	–	–
Isoleucine	–	–	+	+	+	+	ND
Malate	+	+	–	+	+	+	+
Acid production from:							
Galactose	+	+	–	+	ND	+	+
Glucose	+	+	–	+	+	+	+
Inositol	–	+	–	+	+	+	+
Maltose	+	+	–	+	ND	+	+
Sorbitol	+	+	–	–	–	+	+
Sucrose	+	+	–	–	ND	+	+
Susceptibility to:							
Nitrofurantoin	–	+	+	+	+	+	+
Penicillin	–	–	+	+	–	–	–
Major fatty acid methyl esters (%):							
16:1 ω 7c	8.94	3.31	10.37	5.75	3.84	9.84	11.77
16:0	17.89	21.30	20.60	18.12	17.79	9.38	21.62
18:1 ω 7c	30.03	29.46	30.65	42.89	36.46	42.36	27.11
18:0	11.41	12.42	14.78	TR	14.26	13.92	10.90
19:0 cyclo ω 8c	9.03	11.57	4.42	7.37	TR	TR	6.65

et al., 2005) with primers 1 (5'-AGAGTTTGATCCTGGCT-CAG-3'; positions 8–27 according to the *Escherichia coli* 16S rRNA gene) and 2 (5'-GGTTACCTTGTTACGACTT-3'; 1510–1492).

The sequence was compared with closely related sequences of reference organisms from the FASTA network service. Sequence data were aligned with CLUSTAL W 1.8 (Thompson *et al.*, 1994). Phylogenetic trees were constructed by the neighbour-joining method with the MEGA3 program package (Kumar *et al.*, 2004). The DNA G+C content was determined by thermal denaturation (T_m) (Marmur & Doty, 1962) using *E. coli* K12 DNA as the calibration standard. DNA–DNA hybridizations were performed by 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.

16S rRNA gene sequence analysis indicated that strains AJ275^T, AJ282^T and ZG16^T clustered within the genus *Halomonas* (Fig. 1). Strain AJ275^T exhibited the closest phylogenetic affinity and highest sequence similarity to *Halomonas ventosae* DSM 15911^T (97.6%). 16S rRNA gene sequence similarity values between strain AJ275^T and other *Halomonas* species were below 96.5%. The DNA G+C content of strain AJ275^T (65.9 mol%) was close to the upper limit of typical values for *Halomonas* species (52–68 mol%; Franzmann *et al.*, 1988), but was notably lower than that of *H. ventosae* DSM 15911^T (73.4 mol%; Martínez-Cánovas *et al.*, 2004a). DNA–DNA hybridization was carried out at 80 °C. The DNA–DNA relatedness level between strain AJ275^T and *H. ventosae* DSM 15911^T was 17%. Phylogenetic analysis based on 16S rRNA gene sequence comparison showed that strains AJ282^T and ZG16^T could be placed in a parallel branch with *Halomonas sulfidaeris* and *Halomonas hydrothermalis* with high bootstrap values (Fig. 1). The 16S rRNA gene sequence similarities of these two novel isolates

were around 97% to *H. sulfidaeris* DSM 15722^T and *H. hydrothermalis* DSM 15725^T. DNA–DNA relatedness between the novel isolates and *H. sulfidaeris* DSM 15722^T, *H. hydrothermalis* DSM 15725^T and *Halomonas venusta* CGMCC 1.2315^T was less than 50% (Table 2). In addition, comparison of phenotypic properties (Table 1) also indicated differences between the novel isolates and other *Halomonas* species, such as hydrolysis of substrates, acid production from sugars, sensitivity to antimicrobial agents and fatty acid composition.

Based on 16S rRNA gene sequence analysis, as well as DNA–DNA hybridization data and differential phenotypic properties, it is concluded that strains AJ275^T, AJ282^T and ZG16^T represent three novel species within the genus *Halomonas*, *Halomonas saccharevitans* sp. nov., *Halomonas arcis* sp. nov. and *Halomonas subterranea* sp. nov., respectively.

Description of *Halomonas saccharevitans* sp. nov.

Halomonas saccharevitans (sac.char.e'vi.tans. L. n. saccharon -i a kind of sugar; L. part. adj. evitans avoiding; N.L. part. adj. saccharevitans sugar avoiding, because it uses very few sugars).

Gram-negative. Aerobic. Oxidase- and catalase-positive. Motile cocci, 0.8–1.2 µm in diameter. Young cultures show ovoid-like cells (1–2 µm wide and 2–4 µm long). Colonies on complex agar medium are 1–2 mm in diameter, smooth, circular, elevated and light yellow after 2 days. Moderately halophilic. NaCl concentration for growth is between 0.5 and 15.0% (w/v), with optimum growth at 3.0–7.5%. Grows at pH 6.0–10.0 and 4–48 °C (optimum growth at pH 7.0–8.0 and 30 °C). Tween 20 is hydrolysed. Aesculin, casein, DNA, gelatin, starch, Tween 80 and tyrosine are not hydrolysed. Phenylalanine deamination and gluconate

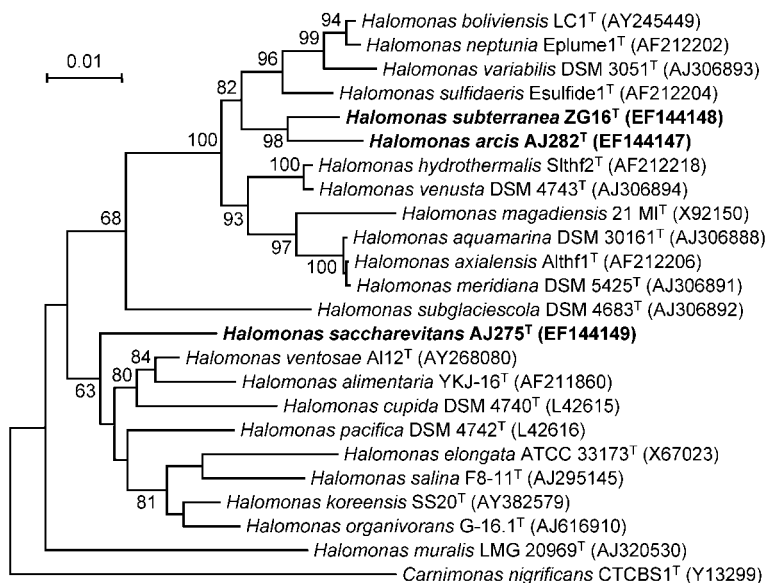


Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences, showing the phylogenetic relationships of the novel isolates and related taxa. Bootstrap values are based on 1000 replicates; only values >60% are shown. Bar, 0.01 substitutions per nucleotide position.

Table 2. DNA G+C content (T_m) and DNA–DNA hybridization between the novel strains and related species of the genus *Halomonas*

Strain	DNA G+C content (mol%)	DNA–DNA hybridization with: (%)	
		AJ282 ^T	ZG16 ^T
AJ282 ^T	56.7 ± 0.3	100	46
ZG16 ^T	57.6 ± 1.1	46	100
<i>H. sulfidaeris</i> DSM 15722 ^T	56.0*	48	49
<i>H. hydrothermalis</i> DSM 15725 ^T	56.3*	25	7
<i>H. venusta</i> CGMCC 1.2315 ^T	ND	25	ND

ND, Not determined.

*Data from Kaye *et al.* (2004).

oxidation are positive. Negative for production of indole and urease. H₂S is not produced from thiosulfate. Chemo-organotrophic. Casamino acids are required for growth. The following substrates are utilized for growth: glycerol, fumarate, alanine, aspartate, glutamate, isoleucine, serine and valine. No growth is observed on arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose, mannose, melezitose, rhamnose, ribose, sorbose, sucrose, trehalose, xylose, adonitol, ethanol, inositol, mannitol, sorbitol, salicin, acetate, citrate, formate, gluconate, malate, malonate, propionate, succinate, arginine, glycine, histidine, leucine, lysine, methionine or ornithine. Susceptible to ampicillin, carbenicillin, cefotaxime, chloramphenicol, erythromycin, nalidixic acid, nitrofurantoin, penicillin, polymyxin B and treptomycin, but not to kanamycin, neomycin, nystain, rifampicin or streptomycin. Principal fatty acids (greater than 5%) are 18:1 ω 7c, 16:0, 18:0 and 16:1 ω 7c.

The type strain is AJ275^T (=CGMCC 1.6493^T=JCM 14606^T=LMG 23976^T), isolated from a water sample taken from a salt lake on the Qinghai–Tibet Plateau, China. The DNA G+C content of strain AJ275^T is 65.9 ± 0.3 mol% (T_m).

Description of *Halomonas arcis* sp. nov.

Halomonas arcis (ar'cis. L. gen. n. *arcis* of a height, summit or peak, referring to the isolation of the organism from a salt lake on the Qinghai–Tibet Plateau).

Gram-negative and motile. Young cultures show rod-like cells (0.5–1.0 × 2.0–4.0 μm). Colonies on complex agar medium are smooth, circular, elevated and cream. Halotolerant. NaCl concentration for growth is between 0 and 20% (w/v), with optimum growth at 1–5% (w/v). Grows at pH 6.0–10.0 and 4–48 °C (optimum growth at pH 7.0–8.0 and 30 °C). Catalase is produced, but not oxidase. Tween 20 and casein are hydrolysed. H₂S is produced from thiosulfate. Aesculin, DNA, gelatin, starch, Tween 80 and tyrosine are not hydrolysed. Phenylalanine deamination and gluconate oxidation are positive. Indole and urease production are negative. Chemo-organotrophic. Casamino acids are

required for growth. Acid is produced from galactose and glucose and, to a lesser extent, from arabinose, fructose, maltose, mannitol, melezitose, sorbitol, sucrose and trehalose. No growth is observed on cellobiose, lactose, mannose, rhamnose, ribose or xylose. The following substrates are utilized for growth: xylose, ethanol, glycerol, acetate, citrate, fumarate, gluconate, malate, malonate, propionate, succinate, alanine, arginine, aspartate, glutamate, lysine, ornithine and valine. Susceptible to chloramphenicol, erythromycin, nalidixic acid, polymyxin B and treptomycin, but not to ampicillin, kanamycin, neomycin, nitrofurantoin, nystain, penicillin, rifampicin or streptomycin. Principal fatty acids (greater than 5%) are 18:1 ω 7c, 16:0, 18:0, 19:0 cyclo ω 8c and 16:1 ω 7c.

The type strain is AJ282^T (=CGMCC 1.6494^T=JCM 14607^T=LMG 23978^T), isolated from a water sample taken from a salt lake located in Altun Mountain on the Qinghai–Tibet Plateau, China. The DNA G+C content of strain AJ282^T is 56.7 ± 0.3 mol% (T_m).

Description of *Halomonas subterranea* sp. nov.

Halomonas subterranea (sub.ter.ra'ne.a. L. fem. adj. *subterranea* underground, subterranean, referring to the isolation of the organism from the subterranean brines).

Gram-negative and motile. Young cultures show rod-like cells (0.5–1.0 × 3.0–5 μm). Colonies on complex agar medium are smooth, circular, elevated and cream. Halotolerant. NaCl concentration for growth is between 0 and 15% (w/v), with optimum growth at 1–5% (w/v). Grows at pH 6.0–10.0 and 4–48 °C (optimum growth at pH 7.0–8.0 and 30 °C). Catalase is produced, but not oxidase. Tween 20, casein and urea are hydrolysed. H₂S is produced from thiosulfate. Aesculin, DNA, gelatin, starch, Tween 80 and tyrosine are not hydrolysed. Gluconate oxidation is positive. Indole production and phenylalanine deamination are negative. Chemo-organotrophic. Casamino acids are required for growth. Acid is produced from arabinose, galactose and glucose and, to a lesser extent, from fructose, inositol, maltose, mannitol, melezitose, sorbitol, sucrose and trehalose. No growth is observed on cellobiose, lactose,

mannose, rhamnose, ribose, sorbose or xylose. The following substrates are utilized for growth: xylose, glycerol, acetate, citrate, fumarate, gluconate, malate, succinate, alanine, arginine, aspartate, glutamate, histidine and lysine. Susceptible to chloramphenicol, erythromycin, nalidixic acid, nitrofurantoin, polymyxin B and treptomycin, but not to ampicillin, kanamycin, neomycin, nystain, penicillin, rifampicin or streptomycin. Principal fatty acids (greater than 5%) are 18:1 ω 7c, 16:0, 18:0 and 19:0 cyclo ω 8c.

The type strain is ZG16^T (=CGMCC 1.6495^T=JCM 14608^T=LMG 23977^T), isolated from hypersaline waters taken from a subterranean saline well on the Si-Chuan Basin, China. The DNA G+C content of strain ZG16^T is 57.6 \pm 1.1 mol% (T_m).

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