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 AJ275T, AJ282T and ZG16T, 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 AJ275T, 56.7 mol% for AJ282T and 57.6 mol% for ZG16T. 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 AJ275T (=CGMCC 1.6493T =JCM 14606T =LMG 23976T), AJ282T (=CGMCC 1.6494T =JCM 14607T =LMG 23978T) and ZG16T (=CGMCC 1.6495T =JCM 14608T =LMG 23977T) 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 (Fränzmann et al., 1987; James et al., 1990; Mormile et al., 1999; Duckworth et al., 2000; Quillagumán et al., 2004), solar salterns (Bouchet et al., 2001; Lim et al., 2004; Martinez-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), seaweeds (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 AJ282T and AJ275T 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 ZG16T 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
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 et al., 2002).

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

| Strains: | 1, AJ282T; 2, ZG16T; 3, AJ275T; 4, *H. sulfidaeris* DSM 15722T; 5, *H. hydrothermalis* DSM 15725T; 6, *H. venusta* CGMCC 1.2315T; 7, *H. ventosae* DSM 15911T. +, 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.

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 et al., 2002).
et al., 2005) with primers 1 (5′-AGAGTTTGATCCTGCGT-
CAG-3′; positions 8–27 according to the Escherichia coli 16S
rRNA gene) and 2 (5′-GGTACCTTGTTACGACTT-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
neighbor-joining method with the MEGA3 program pack-
age (Kumar et al., 2004). The DNA G+C content was
determined by thermal denaturation (Tw) (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
AJ275T, AJ282T and ZG16T clustered within the genus
Halomonas (Fig. 1). Strain AJ275T exhibited the closest
phylogenetic affinity and highest sequence similarity to
Halomonas ventosae DSM 15911T (97.6 %). 16S rRNA gene
sequence similarity values between strain AJ275T and other
Halomonas species were below 96.5 %. The DNA G+C
content of strain AJ275T (65.9 mol%) was close to the upper
limit of typical values for Halomonas species (52–68 mol%;
Franzmann et al., 1998), but was notably lower than that of
H. ventosae DSM 15911T (73.4 mol%; Martinez-Cánovas
et al., 2004a). DNA–DNA hybridization was carried out at
80 °C. The DNA–DNA relatedness level between strain
AJ275T and H. ventosae DSM 15911T was 17 %. Phylogenetic
analysis based on 16S rRNA gene sequence comparison
showed that strains AJ282T and ZG16T 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 15722T and
H. hydrothermalis DSM 15725T. DNA–DNA relatedness
between the novel isolates and H. sulfidaeris DSM 15722T,
H. hydrothermalis DSM 15725T and Halomonas venusta
CGMCC 1.231T was less than 50 % (Table 2). In addition,
comparison of phenotypic properties (Table 1) also indi-
cated 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 propri-
ties, it is concluded that strains AJ275T, AJ282T and ZG16T
represent three novel species within the genus Halomonas,
Halomonas saccharovorans sp. nov., Halomonas arcis sp. nov.
and Halomonas subterranea sp. nov., respectively.

Description of Halomonas saccharovorans
sp. nov.

Halomonas saccharovorans (sac.char.o'viتناول.. L. n. saccharon
-t a kind of sugar; L. part. adj. evitans avoiding; N.L. part.
adj. saccharovorans sugar avoiding, because it uses very few
sugars).

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 iso-
lates and related taxa. Bootstrap values are based on 1000 replicates; only values
> 60 % are show. Bar, 0.01 substitutions per nucleotide position.](http://ijs.sgmjournals.org)
oxidation are positive. Negative for production of indole and urease. \( \text{H}_2\text{S} \) 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, melezoite, rhamnose, ribose, sorbose, sucrose, trehalose, xylose, adenitol, 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\( \text{o}_7 \text{c} \), 16:0, 18:0, and 18:1\( \text{o}_7 \text{c} \). The type strain is AJ275\( ^T \) (=CGMCC 1.6493\( ^T \)=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 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 \( \mu \text{m} \)). 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 \( ^{\circ} \text{C} \) (optimum growth at pH 7.0–8.0 and 30 \( ^{\circ} \text{C} \)). Catalase is produced, but not oxidase. Tween 20 and casein are hydrolysed. \( \text{H}_2\text{S} \) 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\( \text{o}_7 \text{c} \), 16:0, 18:0, 19:0 cyclo\( \text{o}_8 \text{c} \) and 16:1\( \text{o}_7 \text{c} \).

The type strain is AJ282\( ^T \) (=CGMCC 1.6494\( ^T \)=JCM 14607\( ^T \)=LMG 23978\( ^T \)), isolated 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. subterranean underground, subterranean, referring to the isolation of the organism from the subterranean brines).

Gram-negative and motile. Young cultures show rod-like cells (0.5–1.0×3.0–5.0 \( \mu \text{m} \)). 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 \( ^{\circ} \text{C} \) (optimum growth at pH 7.0–8.0 and 30 \( ^{\circ} \text{C} \)). Catalase is produced, but not oxidase. Tween 20, casein and urea are hydrolysed. \( \text{H}_2\text{S} \) 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 teomycin, but not to ampicillin, kanamycin, neomycin, neostyain, penicillin, rifampicin or streptomycin. Principal fatty acids (greater than 5%) are 18:1ω7c, 16:0, 18:0 and 19:0 cyclo ω8c.

The type strain is ZG16T (=CGMCC 1.6495T=JCM 14608T=LMG 23977T), isolated from hypersaline waters taken from a subterranean saline well on the Si-Chuan Basin, China. The DNA G+C content of strain ZG16T is 57.6 ± 1.1 mol% (Tm).

Acknowledgements

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References


