The Journal of Microbiology (2011) Vol. 49, No. 1, pp. 24-28
DOI 10.1007/s12275-011-0197-z

Halomonas alkalitolerans sp. nov., a Novel Moderately Halophilic Bacterium Isolated from Soda Meadow Saline Soil in Daqing, China[§]

Shuang Wang[1], Qian Yang[1]*, Zhi-Hua Liu[1], Lei Sun[2], Dan Wei[2], Jun-Zheng Zhang[1], Jin-Zhu Song[1], Yun Wang[1], Jia Song[1], Jin-Xia Fan[1], Xian-Xin Meng[2], and Wei Zhang[2]

[1]*Department of Life Science and Engineering, Harbin Institute of Technology, Harbin 150001, P. R. China*
[2]*Soil Fertilizer and Environment Energy Institute of Heilongjiang Academy of Agricultural Sciences, Harbin 150086, P. R. China*

(Received May 26, 2010 / Accepted September 28, 2010)

A moderately halophilic bacterial strain 15-13^T, which was isolated from soda meadow saline soil in Daqing City, Heilongjiang Province, China, was subjected to a polyphasic taxonomic study. The cells of strain 15-13^T were found to be Gram-negative, rod-shaped, and motile. The required growth conditions for strain 15-13^T were: 1-23% NaCl (optimum, 7%), 10-50°C (optimum, 35°C), and pH 7.0-11.0 (optimum, pH 9.5). The predominant cellular fatty acids were $C_{18:1}$ *ω7c* (60.48%) and $C_{16:0}$ (13.96%). The DNA G+C content was 67.6 mol%. Phylogenetic analysis based on 16S rRNA gene sequence comparisons indicated that strain 15-13^T clustered within a branch comprising species of the genus *Halomonas*. The closest phylogenetic neighbor of strain 15-13^T was *Halomonas pantelleriensis* DSM 9661^T (98.9% 16S rRNA gene sequence similarity). The level of DNA-DNA relatedness between the novel isolated strain and *H. pantelleriensis* DSM 9661^T was 33.8%. On the basis of the phenotypic and phylogenetic data, strain 15-13^T represents a novel species of the genus *Halomonas*, for which the name *Halomonas alkalitolerans* sp. nov. is proposed. The type strain for this novel species is 15-13^T (=CGMCC 1.9129^T =NBRC 106539^T).

***Keywords*:** *Halomonas alkalitolerans* sp. nov., 16S rRNA gene sequence, fatty acid composition, DNA-DNA hybridization

The genus *Halomonas* is the largest genus in the family *Halomonadaceae* and was first proposed by Vreeland *et al*. (1980). Taxonomically, the genus *Halomonas* is very heterogeneous and, at the time of writing, contained 62 species (http://www.bacterio.cict.fr/). The type species in this genus is *Halomonas elongata*. Members of the genus *Halomonas* are ubiquitous and moderately halophilic/halotolerant bacteria. Most species of *Halomonas* have been isolated from saline environments and are able to grow in a wide range of pH and are therefore considered alkalitolerant (Kaye *et al*., 2004; Quillaguamán *et al*., 2004; and references therein). Furthermore, there are examples of species that grow optimally at alkaline pH (Berendes *et al*., 1996; Romano *et al*., 1996, 2005, 2006; Mormile *et al*., 1999; Duckworth *et al*., 2000; Heyrman *et al*., 2002; Boltyanskaya *et al.*, 2007; Wang *et al.*, 2007; Wu *et al.*, 2008). Here, we describe the features of a novel bacterial strain isolated from soda meadow saline soil and show that it belongs to the genus *Halomonas*.

Materials and Methods

Isolation of strains and culture conditions

Strain 15-13^T was isolated from soda meadow saline soil in Daqing City, China (46°34'N 125°07'E). The isolation procedure was the same as that described previously (Wang *et al*., 2010), using modified S-G agar medium (Sehgal and Gibbons, 1960) containing 10% NaCl (w/v). Unless otherwise indicated, morphological and physiological characterization of strains was performed on complete medium (DSM medium 752), as described by the Deutsche Sammlung von Microorganismen und Zellkulturen (DSMZ, http://www.dsmz.de), containing (per L DW): yeast extract (Difco), 1.0 g; Na_3-citrate, 3.0 g; KCl, 2.0 g; $MgSO_4{\cdot}7H_2O$, 1.0 g; NaCl, 100.0 g; Na_2CO_3, 3.0 g; $MnCl_2{\cdot}4H_2O$, 0.36 mg and $FeSO_4{\cdot}7H_2O$, 50 mg. The isolated strain was grown aerobically on DSM medium 752 containing 10% NaCl (w/v) at 35°C and maintained as a glycerol suspension (20%, v/v) at -70°C. This organism was then submitted to the China General Microbiological Culture Collection (=CGMCC 1.9129^T) and the National Institute of Technology and Evaluation Biological Resource Center, Japan (=NBRC 106539^T).

Phenotypic and biochemical characteristics

Cell morphology was examined using an optical microscope (Olympus BX5-1) and a transmission electron microscope (Hitachi H-7650), with cells from exponentially growing cultures. Gram staining was performed as described by Dussault (1955) and motility was examined using semi-solid agar. The optimal conditions for growth were determined in liquid DSM medium 752 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 in medium 752 by adding MES [2-(N-morpholine) ethane sulfonic acid] (pH 5.0-6.0, 25 mM), PIPES [N,N'-piperazine diethane sulfonic acid] (pH 6.5-7.0, 25 mM), tricine (pH 7.5-9.0, 25 mM) and Na_2CO_3/$NaHCO_3$ (pH 9.5-10.0). The temperature range for growth was determined by incubation of cells at 10, 15, 20, 25, 30, 35, 37, 40, 45, and 50°C. Nutritional assays were performed using modified Koser medium (Ventosa *et al*., 1982) containing (per L DW): NaCl, 75.0 g; KCl, 2.0 g; $MgSO_4{\cdot}7H_2O$, 0.2 g;

* For correspondence. E-mail: yangq@hit.edu.cn; Tel/Fax: +86-451-8641-2952
§ Supplemental material for this article may be found at http://www.springer.com/content/120956

KNO_3, 1.0 g; $(NH_4)_2HPO_4$, 1.0 g, and KH_2PO_4, 0.5 g, according to the proposed minimal standards for describing new taxa in the family *Halomonadaceae* (Arahal *et al*., 2007). Other biochemical tests (anaerobic growth, oxidase, and catalase reactions; productions of H_2S, indole, and exopolysaccharide; methyl red and Voges-Proskauer tests; hydrolysis of aesculin, gelatin, starch, casein, DNA, tyrosine, Tween 20, 40, 60, and 80; o-nitrophenol-β-D-galactoside (ONPG), hemolysis, and lecithovitellin tests; growth on MacConkey or cetrimide agar; phenylalanine deaminase, lysine and ornithine decarboxylases; and urease activity and sensitivity to different antimicrobials) were performed in DSM medium 752 containing 10% NaCl (w/v) according to the methods of Mata *et al*. (2002).

Chemotaxonomic characterization

The whole cell fatty acid profiles of strain 15-13^T, and the type strains, *Halomonas pantelleriensis* DSM 9661^T and *H. elongata* ATCC 33173^T, were analyzed according to the instructions of the Microbial Identification System (MIDI; Microbial ID Inc.) after cultivation on DSM medium 276 agar (pH 7.5) from the DSMZ for two days at 30°C.

Molecular characterization

To determine the DNA G+C content of strain 15-13^T, the genomic DNA of the strain was prepared according to the method of Marmur (1961) and the purity was checked spectrophotometrically. The DNA G+C content was determined by thermal denaturation (*Tm*) (Marmur and Doty, 1962) using the genomic DNA of *Escherichia coli* strain K-12 as the standard for calibration. The phylogenetic position of strain 15-13^T was determined by 16S rRNA gene sequence analysis. The 16S rRNA gene was amplified using 27F and 1492R (Reysenbach *et al*., 2000) as the forward and reverse primers, respectively. Sequence similarity analysis was performed by comparing the 16S rRNA gene sequence of strain 15-13^T with sequences from the GenBank database using the BLAST program (http:/www.ncbi.nlm.nih.gov/blast/). Sequence data were aligned using the CLUSTAL W2 software (http://www.ebi. ac.uk/Tools/clustalw2/). Phylogenetic trees were constructed using the neighbor-joining, maximum parsimony and minimum evolution methods with the MEGA3 program (Kumar *et al*., 2004). To evaluate the stability of the phylogenetic tree, a bootstrap analysis (1,000 replicates) was performed with the SEQBOOT, DNADIST, NEIGHBOR, and CONSENSE programs in the PHYLIP software package (Felsenstein, 2004). DNA-DNA hybridization was performed using the thermal denaturation and renaturation method of De Ley *et al*. (1970), modified by Huß *et al*. (1983). The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 15-13^T is FJ950737.

Results and Discussion

Morphological analysis of the cells of strain 15-13^T revealed that they were Gram-negative rods and motility assays confirmed that these cells were motile. On DSM medium 752, colonies appeared creamy, circular, and opaque. Strain 15-13^T was strictly aerobic and grew at 1-23% (w/v) NaCl, with optimum growth at 3% (w/v) NaCl. The temperature and pH ranges for growth of this strain were 10-50°C and 7.0-11.0, respectively. Optimum growth occurred at pH 9.5 and 35°C

Table 1. Differential characteristics of strain 15-13^T and the closely related *Halomonas* type strain

Characteristic	1	2	3
Cell size (μm)	0.5-0.7×1.2-1.8	0.5-0.9×0.8-2.0	0.6-0.8×2.8-5.2
EPS	+	-	-
NaCl range (%, w/v)	1-23	3-25	0-20
Optimum NaCl (%, w/v)	7	5	3-8
Optimum pH	9.5	9.0	8.0
Growth on[a]			
D-Galactose	-	+	+
D-Mannose	-	+	+
L-Rhamnose	-	+	+
Hydrolysis of			
Tween 40	+	-	-
Tween 60	+	-	-
Growth on[b]			
L-Lysine	+	-	+
L-Serine	+	-	+
L-Histidine	+	-	+
L-Valine	-	+	+
Susceptible to			
Carbenicillin	+	-	-
Novobiocin	-	+	-
Lincomycin	+	-	+
Rifampicin	-	+	+
DNA G+C content (mol%)	67.6	65.0	60.5

Strains/species: 1, 15-13^T; 2, *Halomonas pantelleriensis* DSM 9661^T; 3, *Halomonas elongata* ATCC 33173^T. Data for strains 15-13^T and *H. pantelleriensis* DSM 9661^T were obtained in our laboratory. Data for the utilization of substrates and the DNA G+C content of strain *H. elongata* were taken from Mata *et al*. (2002) and Vreeland *et al*. (1980). The other data for strain *H. elongata* were obtained in our laboratory. The three strains are Gram-negative rods, produce H_2S, catalase positive, oxidase negative, and negative for the hydrolysis of gelatin, starch, Tween 20 and 80. +, Positive; -, negative; NR, not reported. EPS, exopolysaccharide.

[a] When supplied as the sole carbon and energy source.

[b] When supplied as the sole carbon, nitrogen and energy source.

Table 2. Cellular fatty acid content (%) of strain 15-13^T and related type strains

Fatty acid	1	2	3
$C_{10:0}$	3.77±0.10	3.81±0.11	4.80±0.11
$C_{10:0}$ 3-OH	-	-	0.92±0.01
$C_{12:0}$	5.15±0.08	4.83±0.08	6.05±0.06
$C_{12:0}$ 3-OH	6.99±0.12	7.04±0.98	9.79±0.17
Summed feature 3	7.04±0.01	5.27±0.04	7.71±0.05
$C_{16:0}$	13.96±0.16	9.62±0.13	20.04±0.21
$C_{15:0}$ iso 3-OH	-	1.29±0.12	-
$C_{17:0}$ iso 3-OH	-	25.17±0.52	-
$C_{18:1}$ *ω7c*	60.48±1.01	40.82±0.64	46.78±0.57
$C_{19:0}$ cyclo *ω8c*	1.43±0.04	0.57±0.01	1.80±0.02

Taxa: 1, strain 15-13^T; 2, *Halomonas pantelleriensis* DSM 9661^T; 3, *Halomonas elongata* ATCC 33173^T. Values<0.5% are not shown; -, not detected. Summed features represent groups of two or three fatty acids that could not be separated by GLC (gas-liquid chromatography) with the MIDI system; summed feature 3 consists of $C_{16:1}$ *ω7c* / $C_{16:1}$ *ω6c*.

in DSM medium 752 containing 7% (w/v) NaCl. In addition, strain 15-13^T displayed some phenotypic properties that were different from those of related *Halomonas* species (Table 1).

The predominant cellular fatty acids of strain 15-13^T were $C_{18:1}$ ω7*c* (60.48%) and $C_{16:0}$ (13.96%). The contents of $C_{10:0}$, $C_{12:0}$, $C_{12:0}$ 3-OH, summed feature 3 ($C_{16:1}$ *ω7c* / $C_{16:1}$ *ω6c*), and $C_{19:0}$ cyclo *ω8c* were less than 10%. The fatty acid content of strain 15-13^T clearly discriminated it from closely related *Halomonas* type strains (Table 2).

The DNA G+C content of strain 15-13^T was 67.6 mol% (determined using the *Tm* method), which lay within the range specified for the genus *Halomonas* (52-68 mol%; Vreeland, 2005), but was distinct from that of the most closely related species *H. pantelleriensis* (65.0 mol%; Romano *et al*., 1996). The 16S rRNA gene from strain 15-13^T was 1,533-bp in length. The sequence shared 98.9% similarity with the corresponding gene from *H. pantelleriensis* DSM 9661^T and less than 97.0% similarity with those of related species of the genus *Halomonas* (96.7% with *Halomonas campaniensis* 5AG^T, 96.7% with *Halomonas lutea* KCTC 12847^T, 96.5% with *Halomonas muralis* LMG 20969^T, 96.2% with *Halomonas ventosae* DSM 15911^T). Whole-genome DNA-DNA hybridization was carried out between strain 15-13^T and its most closely related species *H. pantelleriensis* DSM 9661^T. The DNA-DNA relatedness between these two strains was 33.8%, which is well below the threshold value of approximately 70% recommended by Wayne *et al*. (1987) for assigning strains to the same species. The phylogenetic tree constructed using the neighbor-joining algorithm is shown in Fig. 1. The phylogenetic trees constructed with the minimum-evolution and maximum-parsimony algorithms are available as Supplementary data Fig. 1 at JM Online.

Based on the phylogenetic data, the DNA-DNA hybridization data and the phenotypic differences between the novel and closely related species of *Halomonas*, we suggest that strain 15-13^T should be classified as the type strain of a novel species within the genus *Halomonas*, and assigned the name, *Halomonas alkalitolerans* sp. nov.

Description of *Halomonas alkalitolerans* sp. nov.

Halomonas alkalitolerans (al.ka.li.to'le.rans. Arabic article *al* the; Arabic n. *qaliy* ashes of saltwort; N.L. n. *alkali* alkali; L. part. adj. *tolerans* tolerating; N.L. part. adj. *alkalitolerans* alkali-tolerating).

Cells are Gram-negative, motile, aerobic, short rods (0.5-0.7 ×1.2-1.8 μm), that produce exopolysaccharide. Growth occurs at 10-50°C (optimum growth at 35°C), pH 7.0-11.0 (optimum, pH 9.5) and NaCl concentrations of 1-23% (w/v) (optimum, 7%). No growth occurs in the absence of salt. Cells are chemo-organotrophic and undergo respiratory metabolism, with oxygen as the terminal electron acceptor. The cells do not grow anaerobically in the presence of nitrate, nitrite or fumarate. Cells are oxidase-negative and catalase-positive, and negative for indole, methyl red, and Voges-Proskauer. Tween 40, 60 and aesculin are hydrolyzed, but these cells do not hydrolyze starch, gelatin, casein, Tween 20, 80, DNA, lecithin or blood. The cells hydrolyze tyrosine, producing a pigment, produce H_2S from L-cysteine, and grow on MacConkey and cetrimide agar supplemented with 10% NaCl. Tests for the activity of urease, lysine, ornithine decarboxylases, ONPG, and phenylalanine deaminase are negative. Glucose, D-xylose, maltose, sucrose, D-fructose, D-trehalose, D-ribose, aesculin, sorbitol, D-mannitol, glycerol, citrate, fumarate, acetate, malate, pyruvate, succinate, lactate, propionate, glutamate, and potassium gluconate can be used as sole carbon and energy sources, but not lactose, L-sorbinose, D-galactose, D-cellobiose, D-mannose, D-melezitose, D-raffinose, D-arabinose, L-arabinose, L-rhamnose, *N*-acetylglucosamine, starch, dextrin, malonate, adonitol, xylitol, ethanol, formate, D-salicin, and *myo*-inositol. L-lysine, L-proline, L-alanine, L-serine, L-aspartic acid, L-glutamic acid, L-arginine, and L-histidine can be used as sole carbon, nitrogen, and energy sources, but not L-threonine, DL-isoleucine, glycine, L-leucine, L-methionine, and L-valine. Cells are susceptible to (μg per disc, unless otherwise indicated): carbenicillin (100), cefoxitin (30), cefotaxime (30), chloramphenicol (30), erythromycin (15), streptomycin (10), lincomycin (2), nalidixic acid (30), norfloxacin (10), nitrofurantoin (300), ciprofloxacin (5), trimetoprim-sulfametoxazol (1.25-23.75), and polymyxin B (300 UI per disc). Cells are resistant to the following antibiotics: amoxicillin (25), ampicillin (10), neomycin (30), novobiocin (30), tetracycline (30), vancomycin (30), kanamycin (30), gentamycin (10), tobramycin (10), rifampicin (5), penicillin G (10 IU per disc), and bacitracin (0.04 IU per disc). The predominant fatty acids (greater

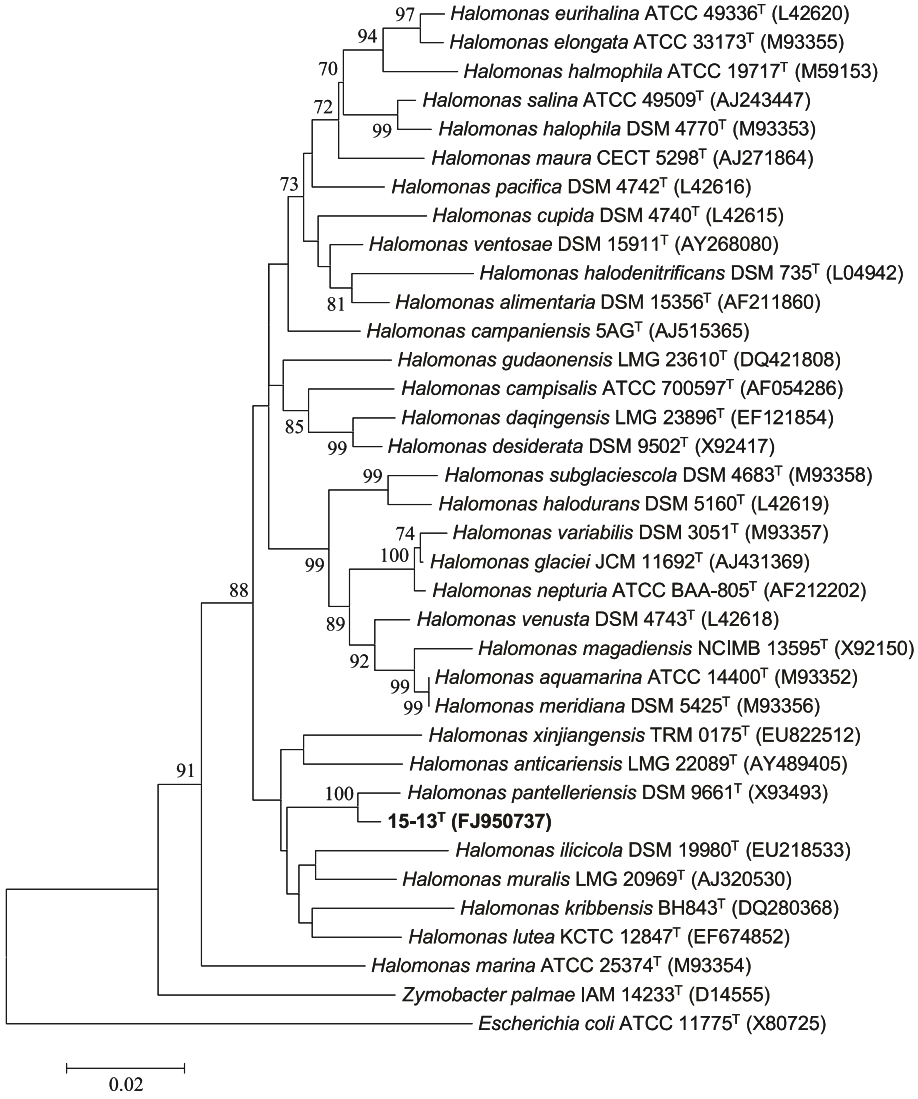

Fig. 1. Neighbor-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationship between strain 15-13^T and related taxa. The tree was constructed using MEGA3 software (Kumar *et al*., 2004). Bootstrap values (%) are based on 1,000 replicates and are shown for branches with more than 70% bootstrap support. Bar, 0.02 expected changes per site.

than 10%) are $C_{18:1}$ $\omega 7c$ (60.48%) and $C_{16:0}$ (13.96%), and the DNA G+C content of the type strain is 67.6 mol% (*Tm* method).

In summary, the findings presented here reveal the features of a novel bacterial strain within the genus *Halomonas*, designated *H. alkalitolerans* sp. nov., isolated from soda meadow saline soil in Daqing City, Heilongjiang Province of China. We describe the characteristic features of the type strain, 15-13^T (=CGMCC 1.9129^T =NBRC 106539^T).

Acknowledgements

This work was supported by grants from the 863 High-tech Project of China (2006AA10Z424) and the Key Project of Science and Technology Development of Harbin, China (2007AA6CN094). We thank the Institute of Microbiology at the Chinese Academy of Sciences for technical assistance. We thank Mr. Yuguang Zhou (CGMCC) for providing the type strain of the *Halomonas* species. We are also grateful to Prof. Susheng Yang (China Agricultural University) for his valuable help.

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