Halomonas sabkhae sp. nov., a moderately halophilic bacterium isolated from an Algerian sabkha

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A novel moderately halophilic bacterium belonging to the genus *Halomonas* was isolated from brine samples collected from Ezzemoul sabkha in north-eastern Algeria. The cells of strain 5-3^T were Gram-negative, rod-shaped and non-motile. The strain was catalase- and oxidase-positive and produced an exopolysaccharide. Growth occurred at NaCl concentrations of 5–25 % (optimum at 7.5 %), at 30–50 °C (optimum at 37–40 °C) and at pH 6.0–9.0 (optimum at pH 7.5). The major fatty acids were $C_{12:0}$ 3-OH, $C_{16:1}\omega7c$/iso-$C_{15:0}$ 2-OH, $C_{16:0}$, $C_{18:1}\omega7c$ and $C_{19:0}\omega8c$ cyclo. The G+C content of the genomic DNA was 57.0 mol% (T_m). The affiliation of strain 5-3^T with the genus *Halomonas* was confirmed by 16S rRNA gene sequence comparisons. The most closely related species was *Halomonas halmophila*, which showed a 16S rRNA gene sequence similarity of 99.7 %. However, the level of DNA–DNA relatedness between the novel isolate and the related *Halomonas* species was less than 31.4 %. On the basis of the data from this polyphasic study, strain 5-3^T represents a novel species of the genus *Halomonas*, for which the name *Halomonas sabkhae* sp. nov. is proposed. The type strain is 5-3^T (=CECT 7246^T=DSM 19122^T=LMG 24084^T).

On the basis of phylogenetic analyses, the family *Halomonadaceae* (Dobson & Franzmann, 1996; Franzmann *et al.*, 1988) contains the genera *Halomonas* (the type genus) (Vreeland *et al.*, 1980), *Carnimonas* (Garriga *et al.*, 1998), *Chromohalobacter* (Ventosa *et al.*, 1989), *Cobetia* (Arahal *et al.*, 2002a) and *Zymobacter* (Okamoto *et al.*, 1993). Species of the genus *Halomonas* have been isolated from a wide variety of hypersaline environments, including salt lakes (Great Salt Lake, Antarctic lakes), soda lakes, saline soils, salterns, deep-sea environments and hydrothermal vent habitats (Dobson & Franzmann, 1996; Fendrich, 1988; Franzmann *et al.*, 1987; Kaye *et al.*, 2004; Martinez-Cánovas *et al.*, 2004; Poli *et al.*, 2007; Quillaguamán *et al.*, 2004; Reddy *et al.*, 2003; Vreeland *et al.*, 1980). In this study, a novel exopolysaccharide-producing strain was isolated and characterized, and can be classified as a novel species of the genus *Halomonas*.

Strain 5-3^T was isolated from brine samples collected from Ezzemoul sabkha in north-eastern Algeria. The novel strain was isolated using a halophilic medium (Oren *et al.*, 1995) with the following composition (l^{-1} distilled water): 125 g NaCl, 160 g $MgCl_2 . 6H_2O$, 5 g K_2SO_4, 0.1 g $CaCl_2 . 2H_2O$, 1 g yeast extract, 1 g Casamino acids and 2 g soluble starch. The pH of the medium was adjusted to 7.0 with NaOH and the incubation temperature was 37 °C.

The medium used for the growth and characterization of strain 5-3^T had the following composition (l^{-1} distilled water): 5 g yeast extract, 5 g proteose peptone no. 3 (Difco) and 1 g glucose, supplemented with a 7.5 % (w/v) sea-salt solution (Rodriguez-Valera *et al.*, 1981; Subov, 1931). Cellular morphology was determined as described previously (Kharroub *et al.*, 2006). Gram staining was performed by using samples fixed with acetic acid, as described by Dussault (1955). NaCl tolerance was determined in growth medium prepared with 0, 3, 5, 7.5, 10, 15, 20, 25 or 30 % (w/v) NaCl. The pH range for growth was

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 5-3^T is EF442769.

A maximum-parsimony phylogenetic tree, based on 16S rRNA gene sequences, for strain 5-3^T, species of the genus *Halomonas* and other taxa comprising Gram-negative, halophilic bacteria is available with the online version of this paper.

tested from pH 5.0 to pH 10.0. The growth temperatures tested included 4, 22, 30, 37, 40, 50, 55 and 60 °C. Nitrate respiration was tested as outlined by Mata *et al.* (2002). Nitrate reduction was tested using the sulfanilic acid and α-naphthylamine method (Smibert & Krieg, 1981). Tests for the formation of indole and the hydrolysis of aesculin, gelatin, starch, Tween 20 and Tween 80 were performed as described by Kharroub *et al.* (2006). Growth on MacConkey agar supplemented with a 7.5 % (w/v) sea-salt solution was assessed. Tyrosine hydrolysis was determined by checking for clear zones on a solid medium (Mata *et al.*, 2002) supplemented with 5 g tyrosine l^{-1}; pigment production was also tested using this medium. The Voges–Proskauer, ONPG and methyl red tests and tests for urea hydrolysis, lysine decarboxylase and ornithine decarboxylase were performed according to the procedures of Larpent & Larpent-Gourgaud (1985), using supplementation with a 7.5 % (w/v) sea-salt solution. Catalase production was tested using 10 % (v/v) H_2O_2. The oxidase reaction was tested on filter paper moistened with a 1 % (w/v) aqueous solution of *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine. The utilization of organic compounds by strain 5-3^T was tested in basal medium in which the yeast extract and proteose peptone concentrations were reduced to 0.01 %, glucose was omitted and the medium further amended by the addition of 0.5 g NH_4Cl l^{-1}, 0.05 g KH_2PO_4 l^{-1}, 1 % of the respective sugar or alcohol, or 0.1 % of the respective amino acid, with buffering at pH 7.0. The production of acids from sugars and alcohols was tested in the basal medium supplemented with 5 g test substrate l^{-1} without buffer; the pH of the cultures was measured with a pH electrode. Sensitivity to various antibiotics was determined using the standard disc diffusion assay.

Analysis of the fatty acid methyl esters was carried out by the Analytical Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) by means of high-resolution GLC. The fatty acid methyl esters were obtained from fresh wet biomass; the identification and quantification of the fatty acid methyl esters were performed by using Microbial Identification System (MIS) software (MIDI).

Genomic DNA was extracted as described by Marmur (1961). The 16S rRNA genes were amplified with PCRs using primers 16F27 (5′-AGAGTTTGATCMTGGCTCAG-3′) and 16R1525 (5′-AAGGAGGTGWTCCARCC-3′). PCRs were carried out under the conditions described by Saiki *et al.* (1988). PCR products were purified with a Microcon-100 concentrator (Amicon). Sequences were determined using an ABI PRISM *Taq* DyeDeoxy DNA sequencer (Applied Biosystems). The primers described by Lane (1991) were used as sequencing primers. Phylogenetic analysis was carried out using MEGA, version 3.1 (Kumar *et al.*, 2004), after multiple alignment of the data by CLUSTAL W, version 1.8 (Thompson *et al.*, 1994). Distances and clustering were determined using the neighbour-joining and maximum-parsimony methods with bootstrap analyses based on 1000 replications.

The genomic DNA G+C content was determined from the mid-point value (T_m) of the thermal denaturation profile (Marmur & Doty, 1962). The T_m was determined by using the graphic method described by Ferragut & Leclerc (1976) and the DNA G+C content was calculated from this temperature by using the equation of Owen & Hill (1979).

DNA–DNA hybridization was performed by using the method of Lind & Ursing (1986) with the modifications of Ziemke *et al.* (1998).

The cells of strain 5-3^T were non-motile, Gram-negative rods approximately 0.6–0.8 × 0.9–1.5 μm in size. The colonies that formed on standard agar medium were cream, smooth, circular, convex and approximately 3 mm in diameter. Strain 5-3^T grew in media containing 5–25 % (w/v) NaCl, with an optimum at 7.5 % (w/v) NaCl. Growth occurred at temperatures in the range 30–50 °C, with an optimum at 37–40 °C. The optimal pH for growth was 7.5 and growth was observed at pH values of 6.0 to 9.0.

The phenotypic characteristics of strain 5-3^T are summarized in the species description and are compared with those of the type strains of related *Halomonas* species in Table 1.

Strain 5-3^T synthesized straight-chain saturated and unsaturated fatty acids in combination with hydroxyl fatty acids. The predominant fatty acids were as follows (%): $C_{18:1}\omega7c$ (50.17), $C_{16:0}$ (27.04), $C_{12:0}$ 3-OH (5.42), $C_{19:0}\omega8c$ cyclo (5.39), $C_{16:1}\omega7c$/iso-$C_{15:0}$ 2-OH (5.08), $C_{10:0}$ (1.64), $C_{12:0}$ (1.15) and $C_{18:0}$ (1.15).

The 16S rRNA gene sequence obtained for strain 5-3^T consisted of 1540 bp. The fragment analysed contained the 15 signature nucleotides defined for the family *Halomonadaceae*, as described by Dobson & Franzmann (1996). It clustered with those for group 1 species of the genus *Halomonas*, as described by Arahal *et al.* (2002b), and displayed the highest levels of 16S rRNA gene sequence similarity with respect to *Halomonas halmophila* ATCC 19717^T (99.7 %), *Halomonas almeriensis* CECT 7050^T (98 %), *Halomonas eurihalina* DSM 5720^T (97.3 %) and *Halomonas elongata* DSM 2581^T (96.6 %). The phylogenetic tree constructed using the neighbour-joining algorithm is shown in Fig. 1. The phylogenetic tree constructed using the maximum-parsimony algorithm is available as Supplementary Fig. S1 in the online version of this paper. The G+C content of the genomic DNA of strain 5-3^T was 57.0 mol% (T_m), which is somewhat lower than the value reported for the most closely related *Halomonas* species (Table 1).

DNA–DNA hybridization experiments between strain 5-3^T and *H. halmophila* ATCC 19717^T, *H. eurihalina* DSM 5720^T, *H. almeriensis* CECT 7050^T and *H. elongata* DSM 2581^T revealed levels of relatedness of 50.6, 38.1, 43.5 and 31.4 %, respectively. These levels of DNA–DNA hybridization are low enough to justify the classification of strain 5-3^T as a novel species within the genus *Halomonas*, for which we propose the name of *Halomonas sabkhae* sp. nov.

Table 1. Differential characteristics of strain 5-3^T and closely related *Halomonas* type strains

Taxa: 1, strain 5-3^T; 2, *H. halmophila* ATCC 19717^T; 3, *H. almeriensis* M8^T; 4, *H. eurihalina* F9-6^T; 5, *H. elongata* 1H9^T. Data are from this study and from Dobson *et al.* (1990), Mata *et al.* (2002), Martinez-Checa *et al.* (2005) and Vreeland *et al.* (1980). +, Positive; −, negative; NR, not reported.

Characteristic	1	2	3	4	5
Cell size (μm)	0.6–0.8 × 0.9–1.5	0.3–0.6 × 0.9–1.3	2–2.5 × 0.75	0.8–1.0 × 2.0–2.5	NR
Motility	−	+	−	−	+
Exopolysaccharide	+	−	+	+	−
Oxidase	+	+	−	−	−
Temperature range (°C)	30–50	15–45	15–37	4–45	4–45
pH range	6–9	5–10	6–10	5–10	5–9
Hydrolysis of:					
Aesculin	−	−	−	+	−
Gelatin	−	−	−	+	−
Starch	−	−	−	−	−
Tween 20	−	+	−	+	−
Tween 80	−	−	−	+	−
Tyrosine	+	−	−	+	−
ONPG	−	−	−	+	+
Urea	−	−	−	+	+
Facultatively anaerobic	+	−	−	−	+
Reduction of nitrate	+	−	−	+	+
Growth on:					
L-Arabinose	−	−	−	+	+
D-Fructose	+	+	−	+	+
D-Galactose	+	+	−	+	+
D-Glucose	+	+	−	+	+
Lactose	+	+	−	+	+
Maltose	+	+	−	+	+
D-Salicin	−	−	−	+	−
D-Xylose	−	+	−	+	+
Fumarate	−	−	−	+	+
Propionate	−	−	−	+	+
Succinate	−	−	+	−	+
Adonitol	−	−	+	−	+
D-Mannitol	−	−	+	−	+
L-Alanine	−	+	+	+	+
L-Histidine	−	+	+	−	+
L-Lysine	−	−	+	−	+
Acid from D-glucose	+	+	−	−	+
DNA G+C content (mol%)	57.0	63.0	59.1–65.7	63.5	60.5

Description of *Halomonas sabkhae* sp. nov.

Halomonas sabkhae (sab′khae. N.L. gen. n. *sabkhae* of sabkha, the Arabic name for a salt flat of the type usually found near sand dunes).

Cells are Gram-negative rods (0.6–0.8 × 0.9–1.5 μm) and are non-motile. Colonies are circular, smooth, convex and have cream pigmentation. Produces an exopolysaccharide. Catalase- and oxidase-positive. Moderately halophilic, being capable of growth in 5–25 % (w/v) NaCl (optimum, 7.5 %, w/v). Grows at pH 6.0–9.0, with an optimum at pH 7.5. The temperature range for growth is 30–50 °C (optimum, 37–40 °C). Utilizes D-glucose, D-galactose, D-fructose, lactose, maltose and sucrose as carbon sources. L-Arabinose, D-xylose, D-mannose, L-rhamnose, D-salicin, adonitol, dulcitol, glycerol, inositol, D-mannitol, D-sorbitol, acetate, benzoate, citrate, formate, fumarate, lactate, malonate, oxalate, propionate, succinate, L-alanine, L-arginine, L-asparagine, L-cysteine, glycine, L-histidine, L-leucine, L-lysine, L-methionine, L-tryptophan and L-tyrosine are not utilized. Acid is produced from D-glucose and D-mannitol, but not from D-galactose, D-fructose, lactose, maltose, sucrose, L-arabinose or L-rhamnose. Grows anaerobically in the presence of nitrate. Does not hydrolyse aesculin, gelatin, starch, Tween 20 or Tween 80. Negative for lysine decarboxylase, ornithine decarboxylase, urease and β-galactosidase. Nitrate is reduced to nitrite. Indole, methyl red and Voges–Proskauer tests are negative. Grows on MacConkey agar and hydrolyses tyrosine, but does not produce pigment from tyrosine. Cells are

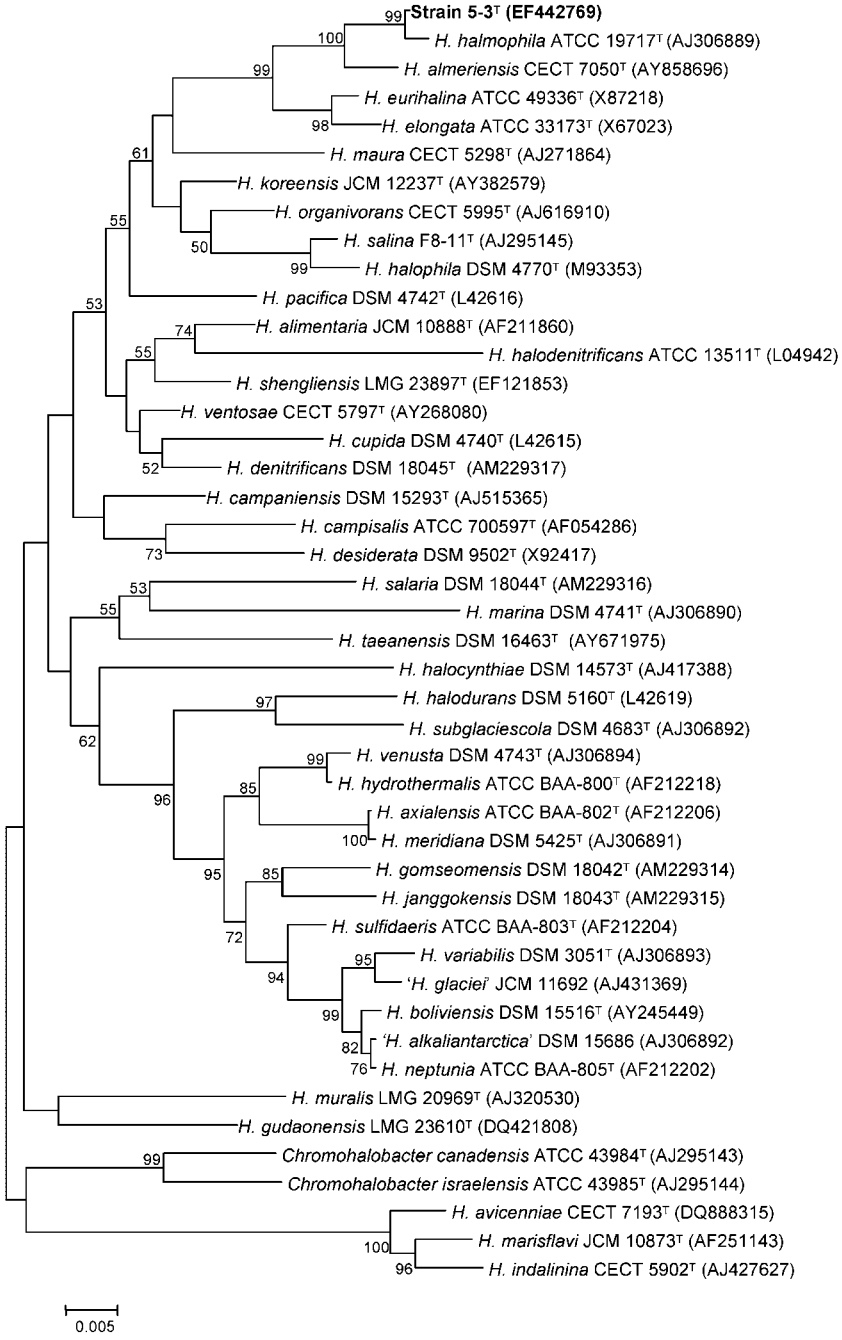

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships of strain 5-3^T and species of the genus *Halomonas* plus other taxa comprising Gram-negative, halophilic bacteria. Bootstrap percentages (based on 1000 replications) are shown if greater than 50 %. Bar, 0.005 substitutions per site.

resistant to novobiocin (30 μg), bacitracin (10 IU), but sensitive to ampicillin (10 μg), kanamycin (30 μg), chloramphenicol (30 μg), penicillin G (10 IU), carbenicillin (100 μg) and rifampicin (30 μg). The major fatty acids are $C_{12:0}$ 3-OH, $C_{16:1}\omega7c$/iso-$C_{15:0}$ 2-OH, $C_{16:0}$, $C_{18:1}\omega7c$ and $C_{19:0}\omega8c$ cyclo. The DNA G+C content is 57.0 mol% (T_m).

The type strain, 5-3^T (=CECT 7246^T=DSM 19122^T=LMG 24084^T), was isolated from brines of Ezzemoul sabkha (Algeria).

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