Halorubrum ezzemoulense sp. nov., a halophilic archaeon isolated from Ezzemoul sabkha, Algeria

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A novel extremely halophilic archaeon was isolated from Ezzemoul sabkha, Algeria. The strain, designated 5.1^T, was neutrophilic, motile and Gram-negative. At least 15% (w/v) NaCl was required for growth. The isolate grew at pH 6.5–9.0, with optimum growth at pH 7.0–7.5. Mg²⁺ was required for growth. Polar lipids were C₂₀C₂₀ derivatives of phosphatidylglycerol and phosphatidylglycerol phosphate methyl ester, and phosphatidylglycerol sulfate and sulfated diglycosyl diether. The genomic DNA G + C content of strain 5.1^T was 61.9 mol% (T_m). Phylogenetic analysis based on comparison of 16S rRNA gene sequences revealed that strain 5.1^T clustered with *Halorubrum* species. The results of DNA–DNA hybridization and biochemical tests allowed genotypic and phenotypic differentiation of strain 5.1^T from other *Halorubrum* species. The name *Halorubrum ezzemoulense* sp. nov. (type strain 5.1^T = CECT 7099^T = DSM 17463^T) is proposed.

The extremely halophilic, aerobic archaea are classified within the family *Halobacteriaceae*. They are found in hypersaline environments such as the Dead Sea, salt lakes, salterns and sabkhas, but have also been isolated from lower-salinity environments where the NaCl concentration is sufficient to prevent their lysis (Elshahed *et al.*, 2004). The current divisions are based on 16S rRNA gene sequences and chemotaxonomic criteria, particularly polar lipid composition. To date, 20 genera of haloarchaea are recognized.

Members of the genus *Halorubrum* (McGenity & Grant, 1995, 2001) are widely distributed in hypersaline environments and play important roles in the carbon and nitrogen cycles in this ecosystem. Currently, the genus *Halorubrum* comprises 12 species with validly published names: *Halorubrum sodomense* (Oren, 1983), *Halorubrum lacusprofundi* (Franzmann *et al.*, 1988), *Halorubrum saccharovororum* (Tomlinson & Hochstein, 1976), *Halorubrum trapanicum* (Petter, 1931; McGenity & Grant, 1995), *Halorubrum tebenquichense* (Lizama *et al.*, 2002), *Halorubrum tibetense* (Fan *et al.*, 2004), *Halorubrum terrestre* (Ventosa *et al.*, 2004), *Halorubrum xinjiangense* (Feng *et al.*, 2004) and *Halorubrum alkaliphilum* (Feng *et al.*, 2005). Oren & Ventosa (1996) reclassified *Halorubrobacterium coriense* (Kamekura & Dyll-Smith, 1995) and *Halorubrobacterium*

distributum (Zvyagintseva & Tarasov, 1987; Kamekura & Dyll-Smith, 1995) as *Halorubrum coriense* and *Halorubrum distributum*, respectively. *Natronobacterium vacuolatum* (Mwatha & Grant, 1993) was also reclassified as *Halorubrum vacuolatum* by Kamekura *et al.* (1997).

In this paper, a novel halophilic archaeon designated strain 5.1^T belonging to the genus *Halorubrum* is described. Strain 5.1^T was isolated from water samples collected from Ezzemoul sabkha, in north-east Algeria. Halophilic medium modified from the formulation of Oren (1983) was used, comprising (per litre distilled water): 125 g NaCl, 100 g MgCl₂·6H₂O, 5 g K₂SO₄, 0.1 g CaCl₂·2H₂O, 1 g yeast extract, 1 g Casamino acids and 2 g soluble starch. The pH was adjusted to 7.0 and incubation was at 37 °C. The following reference strains were used for comparative purposes: *Hrr. sodomense* DSM 3755^T, *Hrr. saccharovororum* DSM 1137^T, *Hrr. lacusprofundi* DSM 5036^T, *Hrr. coriense* DSM 10284^T, *Hrr. tebenquichense* CECT 5317^T, *Hrr. trapanicum* NRC 34021^T, *Hrr. distributum* JCM 9100^T, *Hrr. xinjiangense* JCM 12388^T and *Hrr. terrestre* JCM 10247^T. The reference strains were cultured in medium HE, at a final concentration of approximately 25% (w/v) salts (Torreblanca *et al.*, 1986) and supplemented with 0.1% (w/v) glucose.

Phenotypic analysis of strain 5.1^T was performed according to the proposed minimal standard for the description of new taxa in the order *Halobacteriales* (Oren *et al.*, 1997). Cell

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 5.1^T is DQ118426.

morphology was examined using transmission electron microscopy. Gram staining was performed by using acetic-acid-fixed samples, as described by Dussault (1955). Growth rates in various salt concentrations were determined in medium supplemented with 0, 7.5, 10, 15, 20, 25 or 30% (w/v) NaCl and the requirement for Mg^{2+} was tested using agar plates containing final concentrations of 0, 0.005, 0.01, 0.02, 0.1, 0.5, 0.7 or 0.8 M $MgCl_2$. The pH range for growth was determined using solid medium at pH 5.0, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0. Temperatures tested included 4, 22, 30, 37, 40, 50, 55 and 60 °C. Anaerobic growth in the presence of L-arginine and nitrate was tested as described by Hartmann *et al.* (1980). Controls without arginine and nitrate were included and incubation was performed in the dark. Reduction of nitrate was detected by using the sulfanilic acid and α -naphthylamine reagent (Smibert & Krieg, 1981). Tests for formation of indole and hydrolysis of starch and aesculin were performed as described by González *et al.* (1978). Hydrolysis of gelatin and Tween 80 was tested as outlined by Gutiérrez & González (1972). Catalase and oxidase activities were tested according to Gerhardt *et al.* (1994). Utilization of carbohydrates, sugar alcohols, amino acids and organic acids were carried out as described by Oren *et al.* (1997). Acid production from carbohydrates and sugar alcohols was detected using a phenol red solution. Antibiotic susceptibility was determined by spreading bacterial suspensions on culture plates and applying disks impregnated with the antibiotics tested (Bonelo *et al.*, 1984).

Lipids were extracted from the strains using a modified Bligh and Dyer extraction procedure (Kates, 1972). The lipids were separated by thin-layer chromatography with single development on silica gel in a chloroform/methanol/acetic acid/water (85:22.5:10:4, by vol.) solvent system. In addition, two-dimensional chromatography was performed using chloroform/methanol/water (65:25:4, by vol.) in the first dimension and chloroform/methanol/acetic acid/water (80:12:15:4, by vol.) in the second dimension. Glycolipid spots were detected by spraying the plates with 0.5% α -naphthol in 50% methanol, followed by 5% sulfuric acid in ethanol and heating at 150 °C. Phospholipids were visualized with an ammonium molybdate/sulfuric acid spray.

Genomic DNA was extracted and purified by using the method of Lind & Ursing (1986). Purity was assessed from A_{260}/A_{280} and A_{230}/A_{260} ratios. The G+C content was determined from the mid-point value (T_m) of the thermal denaturation profile (Marmur & Doty, 1962), with a Perkin Elmer UV-Vis Lambda 3B spectrophotometer at 260 nm, programmed for temperature increases of 1.0 °C min⁻¹. The T_m was determined by using the graphic method described by Ferragut & Leclerc (1976) and the G+C content was calculated from this temperature by using the equation of Owen & Hill (1979). DNA–DNA hybridization studies were performed as described by Ziemke *et al.* (1998). DNA was double-labelled using DIG-11-dUTP and biotin-16-dUTP (Boehringer Mannheim). The labelling reaction

was carried out using a Boehringer Mannheim nick-translation kit.

16S rRNA genes were amplified by PCR with a primer set designed to complement the highly conserved regions of the *Halobacterium salinarum* and *Halorubrum* species 16S rRNA genes: forward primer F8 (5'-TTGATCCTGCCGG-AGGCCATTG-3') and reverse primer R1462 (5'-ATCCA-GCGCAGATTCCCCTAC-3'), corresponding to positions 8–30 and 1462–1441, respectively. PCR and sequencing were performed as described previously (Lizama *et al.*, 2002). Multiple sequence alignments were performed using CLUSTAL W 1.8 (Thompson *et al.*, 1994). A phylogenetic tree was constructed by using the neighbour-joining method with the MEGA 3 program package (Kumar *et al.*, 2004).

Fig. 1. Electron micrograph showing negatively stained cells (a) and transmission electron micrograph (b) of strain 5.1^T. Bars, 1 µm.

On the basis of pigmentation, NaCl-dependent growth, antibiotic susceptibility and 16S rRNA gene sequences, strain 5.1^T was identified as representing a member of the family *Halobacteriaceae*. Colonies on standard growth agar medium were circular, convex and with entire margins. Light-red-coloured colonies, about 0.5–1.0 mm in diameter, were formed after 7 days of incubation at 37 °C. Cells of strain 5.1^T were motile, single, small rods. Pleomorphic forms (irregular rods and coccoid and triangular forms) were also observed in liquid culture. Mean cell dimensions of the rod forms were 0.6 × 1.5–3.0 µm (Fig. 1). Cells stained Gram-negative and lysed in distilled water. Strain 5.1^T grew in media containing 15–25 % (w/v) NaCl, with optimum growth at 20 % (w/v) NaCl. The strain grew at pH 6.5–9.0, with optimum growth at pH 7.0–7.5. Strain 5.1^T grew at temperatures between 22 and 50 °C. Mg²⁺ was required for growth and optimum growth occurred at 37–40 °C.

A number of sugars and other organic compounds stimulated growth of strain 5.1^T. Growth was observed with glucose, sucrose, arabinose, maltose, mannitol, glycerol, acetate, oxalate, citrate and malate. Lactose, xylose, rhamnose, galactose, raffinose, cellobiose, mannose, fructose, starch, inositol, sorbitol, dulcitol, adonitol, salicin, cellulose, malonate, fumarate, formate and amino acids were not utilized. Acid was produced in the presence of glucose, lactose, xylose, galactose and rhamnose. The isolate did not grow anaerobically with L-arginine or nitrate, but reduced nitrate to nitrite. Strain 5.1^T was catalase- and oxidase-positive, but indole was not produced. The strain was resistant to penicillin, chloramphenicol, ampicillin, rifampicin, streptomycin and neomycin, but was sensitive to novobiocin and bacitracin.

Strain 5.1^T contained C₂₀C₂₀ derivatives of phosphatidylglycerol and phosphatidylglycerol phosphate methyl ester, and phosphatidylglycerol sulfate and a single glycolipid, chromatographically identical to S-DGD-3 of *Hrr. coriense* DSM 10284^T.

The 16S rRNA gene sequence of strain 5.1^T was determined and comparison with the 16S rRNA gene sequences of

members of the family *Halobacteriaceae* placed strain 5.1^T in the genus *Halorubrum* (Fig. 2). Strain 5.1^T was related to *Hrr. coriense* (97 % 16S rRNA gene sequence similarity), *Hrr. sodomense* (97 % similarity), *Hrr. trapanicum* (96 %), *Hrr. distributum* (96 %), *Hrr. xinjiangense* (96 %), *Hrr. terrestre* (95 %), *Hrr. tebenquichense* (94 %), *Hrr. saccharovororum* (93 %) and *Hrr. lacusprofundi* (93 %).

The G + C content of the DNA of strain 5.1^T was 61.9 mol% (*T_m*) (mean of three independent determinations). DNA–DNA reassociation values were determined between strain 5.1^T and the type strains of closely related *Halorubrum* species. Strain 5.1^T showed relatively low DNA–DNA reassociation values with *Hrr. sodomense* DSM 3755^T (32.4 %), *Hrr. distributum* JCM 9100^T (21.3 %), *Hrr. coriense* DSM 10284^T (18.2 %), *Hrr. trapanicum* NRC 34021^T (17.5 %) and *Hrr. xinjiangense* JCM 12388^T (16.9 %), and less than 15 % with *Hrr. saccharovororum* DSM 1137^T, *Hrr. lacusprofundi* DSM 5036^T, *Hrr. tebenquichense* CECT 5317^T and *Hrr. terrestre* JCM 10247^T. Characteristics that differentiated strain 5.1^T from other *Halorubrum* species are listed in Table 1.

On the basis of low levels of DNA–DNA hybridization, polar lipid composition and other phenotypic differences among the novel and previously described taxa, we conclude that strain 5.1^T should be considered as representing a novel species of the genus *Halorubrum*.

Description of *Halorubrum ezzemoulense* sp. nov.

Halorubrum ezzemoulense (ez.ze.mou.len'se. N.L. neut. adj. *ezzemoulense* pertaining to Ezzemoul sabkha, where the type strain was isolated).

Cells stain Gram-negative, are motile, lyse in distilled water and are pleomorphic. Cells occur mostly as single rods and exhibit a range of morphological types, including irregular rod-shaped, cocci and triangular forms. Rods are 0.6 × 1.5–3.0 µm. Colonies on standard agar growth medium are 0.5–1.0 mm in diameter, circular, convex, entire and red. Chemo-organotrophic, aerobic growth occurs at 15–25 %

Fig. 2. Phylogenetic tree showing the position of strain 5.1^T among species of the genus *Halorubrum*. The 16S rRNA gene sequence of *Methanospirillum hungatei* DSM 864^T was used as an outgroup. Numbers at branch points indicate the level of bootstrap support, based on 100 resamplings. Only values greater than 50 are shown. Bar, 5 substitutions per 100 nucleotides.

Table 1. Differential characteristics of *Halorubrum ezzemoulense* sp. nov. strain 5.1^T and other *Halorubrum* species

Strains: 1, strain 5.1^T; 2, *Hrr. coriense* DSM 10284^T; 3, *Hrr. sodomense* DSM 3755^T; 4, *Hrr. trapanicum* NRC 34021^T; 5, *Hrr. saccharovororum* ATCC 29252^T; 6, *Hrr. lacusprofundi* JCM 8891^T; 7, *Hrr. tebenquichense* CECT 5317^T; 8, *Hrr. vacuolatum* JCM 9060^T; 9, *Hrr. tibetense* AS 1.3239^T; 10, *Hrr. terrestre* JCM 10247^T; 11, *Hrr. alkaliphilum* JCM 12358^T; 12, *Hrr. distributum* JCM 9100^T; 13, *Hrr. xinjiangense* JCM 12388^T. Data were taken from Grant *et al.* (2001), Lizama *et al.* (2002), Ventosa *et al.* (2004), Fan *et al.* (2004), Feng *et al.* (2004, 2005) and this study. +, Positive; −, negative; ND, not determined.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13
Motility	+	+	+	−	+	+	ND	−	−	+	+	+	+
Gas vacuoles	−	−	−	−	−	−	ND	+	−	−	−	−	−
NaCl range for growth (M)	2.5–4.3	2.0–5.2	0.5–4.3	2.5–5.2	1.5–5.2	1.5–5.2	2.5–5.2	2.5–5.2	1.7–5.2	2.5–5.2	1.8–5.2	1.7–5.2	2.0–5.2
Mg ²⁺ requirement	+	+	+	ND	+	+	−	−	−	ND	−	ND	−
Growth at pH 10.5	−	−	−	−	−	−	−	+	+	−	+	−	−
Temperature range for growth (°C)	22–50	30–56	20–50	ND	30–56	4–40	35–50	20–50	22–45	28–50	20–44	26–50	10–54
Hydrolysis of starch	−	ND	+	−	−	−	−	−	−	−	−	−	−
Utilization of:													
Glucose	+	+	+	+	+	+	+	+	+	−	+	−	+
Sucrose	+	+	+	+	+	ND	ND	+	+	−	−	−	+
Lactose	−	+	−	−	+	+	ND	ND	−	ND	−	ND	−
Galactose	−	ND	−	+	+	+	+	+	−	ND	−	−	−
Acetate	+	ND	ND	ND	ND	+	+	+	+	ND	−	ND	−
Succinate	−	ND	ND	ND	ND	ND	ND	+	+	ND	−	ND	−
Glycerol	+	+	+	+	+	−	+	ND	ND	−	+	+	−
Acid from glucose	+	ND	+	+	+	−	−	ND	−	−	+	+	+
C ₂₀ C ₂₀ and C ₂₀ C ₂₅ core lipids	−	−	−	−	−	−	ND	+	+	−	+	−	−
Presence of PGS*	+	+	+	+	+	+	−	−	−	+	−	+	+
Presence of S-DGD*	+	+	+	+	+	+	+	−	−	+	−	+	+
G + C content (mol%)†	61.9 ^a	ND	67.4 ^b	64.3 ^b	71.2 ^b	65.8 ^b	63.2 ^a	62.7 ^a	63.3 ^a	64.4 ^a	62.1 ^a	63.6	68.0 ^b

*PGS, Phosphatidylglycerol sulfate; S-DGD, sulfated diglycosyl diether.

†G + C content was measured by: *a*, *T_m*; or *b*, buoyant density.

(w/v) NaCl, pH 6.5–9.0 and 22–50 °C. Optimum NaCl concentration, pH and temperature for growth are 20 % (w/v), pH 7.0–7.5 and 37–40 °C, respectively. Mg^{2+} is required for growth. Catalase- and oxidase-positive. Anaerobic growth with nitrate or L-arginine does not occur. Nitrate is reduced to nitrite. Indole is not produced. Gelatin is not liquefied. Starch, aesculin and Tween 80 are not hydrolysed. Lactose, xylose, rhamnose, galactose, raffinose, cellobiose, mannose, fructose, starch, inositol, sorbitol, dulcitol, adonitol, salicin, cellulose, malonate, fumarate, formate and amino acids are not utilized. Glucose, sucrose, maltose, arabinose, mannitol, glycerol, acetate, oxalate, citrate and malate are used as carbon sources. Acids are produced from glucose, lactose, xylose, galactose and rhamnose. Sensitive to novobiocin and bacitracin, but resistant to penicillin, chloramphenicol, ampicillin, rifampicin, streptomycin and neomycin. Polar lipids are $C_{20}C_{20}$ derivatives of phosphatidylglycerol and phosphatidylglycerol phosphate methyl ester. Also contains phosphatidylglycerol sulfate and a sulfated diglycosyl diether. The G + C content of genomic DNA of the type strain is 61.9 mol% (T_m).

The type strain, 5.1^T (=CECT 7099^T = DSM 17463^T), was isolated from Ezzemoul sabkha in Algeria.

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