Halorubrum orientale sp. nov., a halophilic archaeon isolated from Lake Ejnor, Inner Mongolia, China

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A motile, pleomorphic, red-pigmented archaeon, strain EJ-52^T, was isolated from water from Lake Ejnor, a saline lake in Inner Mongolia, China. Analysis of the almost-complete 16S rRNA gene sequence showed that the isolate was phylogenetically related to species of the genus *Halorubrum*, being most closely related to *Halorubrum saccharovorum* ATCC 29252^T (96.1 % sequence similarity), *Halorubrum lacusprofundi* JCM 8891^T (95.9 %), *Halorubrum tibetense* AS 1.3239^T (95.2 %), *Halorubrum alcaliphilum* AS 1.3528^T (95.2 %) and *Halorubrum vacuolatum* JCM 9060^T (95.1 %). The polar lipids of strain EJ-52^T were C₂₀C₂₀ derivatives of phosphatidylglycerol phosphate and phosphatidylglycerol phosphate methyl ester and a sulfated diglycosyl diether. Strain EJ-52^T requires at least 2.5 M NaCl for growth and grows optimally at 3.4 M NaCl. The strain grows at 25–50 °C, with optimal growth occurring at 35–45 °C. Mg²⁺ is not required. The DNA G + C content is 64.2 mol%. On the basis of the data obtained in this study, strain EJ52^T represents a novel species, for which the name *Halorubrum orientale* sp. nov. is proposed. The type strain is EJ-52^T (= CECT 7145^T = JCM 13889^T = CGMCC 1.6295^T).

Haloarchaea are aerobic members of the *Archaea* that are found in hypersaline habitats, mainly salt lakes and solar salterns (Rodríguez-Valera, 1988; Oren, 2000; Grant *et al.*, 2001). They are able to grow over a wide range of salt concentrations, i.e. between 8 and 30 % NaCl (Grant *et al.*, 2001). Currently, they are included in the order *Halobacteriales*, family *Halobacteriaceae*, with a large number of species being grouped in 20 genera (Grant *et al.*, 2001; Ventosa, 2006). The genus *Halorubrum* was formally proposed by McGinity & Grant (1995) to accommodate several species previously included in the genus *Halobacterium*: *Halorubrum saccharovorum* (Tomlinson &

Hochstein, 1976), *Halorubrum sodomense* (Oren, 1983), *Halorubrum trapanicum* (Petter, 1931) and *Halorubrum lacusprofundi* (Franzmann *et al.*, 1988). In addition, *Halorubrobacterium distributum* (Zvyagintseva & Tarasov, 1987) and *Halorubrobacterium coriense* (Kamekura & Dyall-Smith, 1995) were transferred to the genus *Halorubrum* by Oren & Ventosa (1996). 16S rRNA gene sequence studies showed that *Natronobacterium vacuolatum* (Mwatha & Grant, 1993) was phylogenetically related to the genus *Halorubrum*, and it was designated *Halorubrum vacuolatum* (Kamekura *et al.*, 1997). Lizama *et al.* (2002) described the species *Halorubrum tebenquichense*, isolated from Lake Tebenquiche, located in the Salar de Atacama, Chile. More recently, four novel species have been described: *Halorubrum terrestre* (Ventosa *et al.*, 2004), *Halorubrum tibetense* (Fan *et al.*, 2004), *Halorubrum xinjiangense* (Feng *et al.*, 2004) and *Halorubrum alkaliphilum* (Feng *et al.*, 2005).

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain EJ-52^T is AM235786.

A phase-contrast micrograph and TLC of polar lipids of strain EJ-52^T are available as supplementary figures in IJSEM Online.

Strain EJ-52^T was isolated from water from a saline lake in Inner Mongolia, China, and was subjected to 16S rRNA gene sequence analysis, polar lipid determination and phenotypic study. The results show that strain EJ-52^T is closely related to members of the genus *Halorubrum*.

Strain EJ-52^T was isolated from a sample of water from Lake Ejnor (45° 14' N 116° 31' E) by enrichment in liquid medium and subsequent plating on the same medium with added agar until clonal purity was obtained. The medium contained the following (l⁻¹): NaCl, 195 g; MgCl₂·6H₂O, 32.5 g; MgSO₄·7H₂O, 50.8 g; CaCl₂, 0.8 g; KCl, 5 g; NaHCO₃, 0.16 g; NaBr, 0.6 g; and yeast extract, 5 g. The pH was adjusted to 8 with 1 M NaOH. The water of the lake had a conductivity of 166 mS cm⁻¹, a pH of 8.5 and a temperature of 32 °C at the time of sampling in September 2003. Strain EJ-52^T grew at temperatures in the range 25–50 °C (optimum, 35–45 °C) and at pH values in the range 6.0–10.0 (optimum, pH 8.0). Routine cultivation was conducted at 37 °C and pH 8. The NaCl and MgCl₂ requirements for growth were determined in media containing 0.9–5.2 M NaCl or 0–0.5 M MgCl₂. Strain EJ-52^T was capable of growing in a wide range of NaCl concentrations, from 15 % (2.5 M) to 30 % (5 M); it grew optimally in the presence of 20 % (3.4 M) NaCl. The salt range and optimum for growth were found to be similar to those of most extremely halophilic archaea belonging to the *Halobacteriales* (Oren, 2000; Grant *et al.*, 2001). MgCl₂ was not required for growth.

Phenotypic tests were performed according to the proposed minimal standards for the description of novel taxa of the order *Halobacteriales* (Oren *et al.*, 1997). Cell motility and morphology of exponentially growing liquid cultures were examined using an Olympus BX41 microscope equipped with phase-contrast optics. The cells were found to be motile and pleomorphic (see Supplementary Fig. S1 available in IJSEM Online). Colony morphology was observed under optimal growth conditions on agar medium after incubation at 37 °C for 10 days. On agar plates, the colonies of strain EJ-52^T were circular, smooth, entire and red-pigmented. Anaerobic growth was tested in the presence of nitrate (5 g l⁻¹) or L-arginine (5 g l⁻¹) in filled stoppered tubes. The following characteristics were tested as described by Oren *et al.* (1997): hydrolysis of starch, gelatin, urea, DNA, aesculin, casein and Tween 80; nitrate reduction,

production of indole and H₂S, catalase and oxidase activities and utilization of sugars, alcohols, amino acids and organic acids as carbon and energy sources or carbon, nitrogen and energy sources. Strain EJ-52^T was found to be oxidase- and catalase-positive. Negative results were obtained for indole production from tryptophan and in methyl red, Voges–Proskauer and Simmons' citrate tests. Nitrate was reduced without the production of gas. Tween 80 and urea were hydrolysed, but starch, gelatin, casein, DNA and aesculin were not.

Susceptibility to antibiotics was determined on agar plates with antibiotic discs containing the following: ampicillin (10 µg), bacitracin (10 U), chloramphenicol (30 µg), erythromycin (15 µg), gentamicin (10 µg), nalidixic acid (30 µg), neomycin (10 µg), novobiocin (30 µg), penicillin G (10 U), rifampicin (30 µg), streptomycin (10 µg) and tetracycline (30 µg). The results for antibiotic susceptibility and for the utilization of various substrates are included in the species description.

Polar lipids were extracted with chloroform/methanol as described previously (Kamekura, 1993). TLC was performed using Merck HPTLC plates (silica gel 60; art. no. 5641) in the solvent system chloroform/methanol/acetic acid/water (85:22.5:10:4, by vol.). Glycolipids were detected as purple spots by spraying 0.5 % with α -naphthol in methanol/water (1:1) and then with sulfuric acid/ethanol (1:1), followed by heating at 160 °C. The polar lipids of strain EJ-52^T (see Supplementary Fig. S2 available in IJSEM Online) are C₂₀C₂₀ derivatives of phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester and the characteristic glycolipid of the neutrophilic species of *Halorubrum*, a sulfated mannosyl-glucosyl-glycerol diether (McGenity & Grant, 2001).

Chromosomal DNA of strain EJ-52^T was isolated and purified according to the method described by Marmur (1961). The G + C content of the genomic DNA was determined from the mid-point (*T*_m) of the thermal denaturation profile (Marmur & Doty, 1962) using the equation of Owen & Hill (1979), as described previously (Ventosa *et al.*, 2004), and was found to be 64.2 mol%. The 16S rRNA gene of strain EJ-52^T was amplified by means of a PCR with three universal primers, as described previously (López-García *et al.*, 2001; Arahal *et al.*, 1996), and an almost-complete

Fig. 1. Maximum-parsimony phylogenetic tree based on 16S rRNA gene sequences showing the position of strain EJ-52^T among the species of the genus *Halorubrum*. The sequence data used were obtained from the EMBL database (accession numbers are given in parentheses). Bar, 1 % sequence divergence.

Table 1. Some characteristics that distinguish strain EJ-52^T from *Halorubrum* species

Strains: 1, strain EJ-52^T; 2, *Hrr. saccharovororum* ATCC 29252^T; 3, *Hrr. alkaliphilum* AS 1.3528^T; 4, *Hrr. coriense* JCM 9275^T; 5, *Hrr. distributum* JCM 9100^T; 6, *Hrr. lacusprofundi* JCM 8891^T; 7, *Hrr. sodomense* ATCC 33755^T; 8, *Hrr. tebenquichense* DSM 14210^T; 9, *Hrr. terrestre* VKM B-1739^T; 10, *Hrr. tibetense* AS 1.3239^T; 11, *Hrr. trapanicum* JCM 10477^T; 12, *Hrr. vacuolatum* JCM 9060^T; 13, *Hrr. xinjiangense* AS 1.3527^T. Data were from Feng *et al.* (2004), Ventosa *et al.* (2004), Lizama *et al.* (2002), McGenity & Grant (2001), Feng *et al.* (2005) and this study. Symbols: +, positive; –, negative; +/–, doubtful; ND, not done/no data available.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13
Morphology	Pleomorphic rods	Rods	Short rods	Pleomorphic short rods and cup shapes	Pleomorphic rods	Pleomorphic rods	Rods	Irregular discs	Pleomorphic	Rods	Pleomorphic rods	Pleomorphic short rods	Short rods
Cell size (µm)	1.5–2 × 1–2	0.6–1.2 × 2.5	0.8–1.0 × 1.8–2.0	5–0.5	0.8–1 × 2.7–7	> 12	0.5 × 2.5	0.8–1.2 × 1.5–1.0	1.0–1.5 × 1.5–2.5	0.5–1 × 1.5–2.5	ND	0.7–10 × 1.5–3.0	0.8–1.2 × 1.8–2.6
Motility	+	+	+	+	+	+	+	ND	+	–	–	–	+
Colony colour	Red	Orange–red	ND	Orange–red	Orange–red	Red	Orange–red	Red–orange	Orange–red	Red	Pale orange	Bright pink	Red
NaCl range for growth (M)	2.5–5.0	1.5–5.2	1.8–5.2	2.0–5.2	1.7–5.2	1.5–5.2	0.5–4.3	2.5–5.2	2.5–5.2	1.7–5.2	0.7–1 × 1.5–3	2.5–5.2	2.0–5.2
pH optimum	8.0	7–7.5	9–10	7–7.5	7.5	7–7.5	7–7.5	ND	7.5	9.0–9.5	7–7.5	9.5	7.0–7.5
Temperature optimum (°C)	37	50	38	50	37–45	31–37	40	40	37–45	37–40	37	35–40	40
Nitrate reduction	+	–	+	ND	+	+/–	+/–	+	–	+	+	+	–
Utilization of:													
D-Fructose	+	+	+	ND	ND	–	+	+	–	ND	+	–	+
Lactose	–	+	–	+	ND	+	+	–	ND	–	–	ND	–
D-Galactose	–	+	–	+	–	+	–	+	ND	–	+	+	–
Maltose	+	+	+	ND	ND	–	+	–	ND	+	+	–	+
D-Glucose	+	+	+	+	–	+	+	+	–	+	+	+	+
Sucrose	ND	+	–	ND	–	ND	+	–	ND	–	+	+	+
H ₂ S production	–	+	+	ND	+	–	ND	+	–	–	ND	ND	+
DNA G+C content (mol%)	64.2	71.2	62.1	ND	63.6	65.3	67.4	63.2	64.4	63.3	64.3	62.7	68.0

nucleotide sequence (1409 bp) was determined. The ARB software package (Ludwig *et al.*, 2004) was used for analysis of the 16S rRNA gene sequence. Base-frequency filters were applied in the sequence comparison analysis and the effects on the results were evaluated. Alignment of the 16S rRNA gene sequence with all published sequences of haloarchaea clearly showed that strain EJ-52^T belonged to the genus *Halorubrum*, since it possessed 18 of the 19 signature bases of the genus *Halorubrum* (Kamekura *et al.*, 2004). The phylogenetic tree (Fig. 1) constructed by maximum parsimony (Saitou & Nei, 1987) indicated that strain EJ-52^T was related to *Hrr. saccharovororum* ATCC 29252^T (96.1%), *Hrr. lacusprofundi* JCM 8891^T (95.9%), *Hrr. tibetense* AS 1.3239^T (95.2%), *Hrr. alcaliphilum* AS 1.3528^T (95.2%) and *Hrr. vacuolatum* JCM 9060^T (95.1%). Similar topologies were obtained when other treeing methods (neighbour joining and maximum likelihood) were used. The low levels of similarity (<97% in all cases) between the 16S rRNA gene sequence of strain EJ-52^T and those of the species of *Halorubrum* suggest that the novel isolate could represent a novel species (Stackebrandt & Goebel, 1994; Vandamme *et al.*, 1996). Additionally, there are phenotypic differences between strain EJ-52^T and the species of *Halorubrum* (Table 1).

In conclusion, these data indicate that strain EJ-52^T represents a novel species of the genus *Halorubrum*, for which we propose the name *Halorubrum orientale* sp. nov.

Description of *Halorubrum orientale* sp. nov.

Halorubrum orientale (o.ri.en.ta'le. L. neut. adj. *orientale* of, or belonging to, the East, eastern, pertaining to the isolation of the type strain from China).

Cells are pleomorphic, 1.5–2 × 1–2 µm in size and motile. Colonies are small, entire, smooth and red-pigmented. Growth occurs in the presence of 2.5–5.0 M NaCl, with optimum growth occurring at 3.4 M NaCl. MgCl₂ is not required. Growth occurs between 25 and 50 °C (optimum, 35–45 °C) and at pH 6.0–10.0 (optimum, pH 8.0). Chemo-organotrophic. Oxidase- and catalase-positive. Nitrate is reduced to nitrite. Tween 80 and urea are hydrolysed, whereas starch, casein and gelatin are not. H₂S is not produced. Indole is not produced from tryptophan. The Voges–Proskauer test is negative. Arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase are not produced. Anaerobic growth with nitrate or arginine does not occur. Acid is not produced from lactose, glycerol, D-glucose, sucrose, D-fructose, D-arabinose, maltose, D-xylose, D-galactose, D-trehalose and D-mannose. Growth occurs on glycerol, D-fructose, maltose, D-glucose, starch, L-glutamate and fumarate as single carbon and energy sources. The following compounds are not used as sole carbon and energy sources: D-mannitol, D-sorbitol, lactose, D-arabinose, D-raffinose, D-ribose, D-xylose, succinate, propionate, malate and acetate. The following compounds are used as sole carbon, nitrogen or energy sources: L-asparagine, glycine, L-lysine, L-serine and L-threonine. Susceptible to novobiocin,

bacitracin, gentamicin, ampicillin, neomycin, erythromycin, chloramphenicol, nalidixic acid and streptomycin; resistant to penicillin G and rifampicin. The polar lipids are C₂₀C₂₀ derivatives of phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester and a sulfated mannosyl-glucosyl-glycerol diether. The DNA G+C content is 64.2 mol% (T_m method).

The type strain, EJ-52^T (=CECT 7145^T=JCM 13889^T=CGMCC 1.6295^T), was isolated from Lake Ejnor, a saline lake in Inner Mongolia, China.

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