Halorubrum californiense sp. nov., an extreme archaeal halophile isolated from a crystallizer pond at a solar salt plant in California, USA

Peter T. Pesenti,¹ Masoumeh Sikaroodi,¹ Patrick M. Gillevet,¹
Cristina Sánchez-Porro,² Antonio Ventosa² and Carol D. Litchfield¹

Correspondence
Antonio Ventosa
ventosa@us.es

¹Department of Environmental Science and Policy, George Mason University, Manassas, VA 20110, USA

²Department of Microbiology and Parasitology, Faculty of Pharmacy, University of Seville, 41012 Seville, Spain

A motile, rod-shaped, pink-pigmented, extremely halophilic archaeon, strain SF3-213^T, was isolated from a crystallizer pond at the Cargill Solar Salt Plant, Newark, California (USA). Analysis of the almost-complete 16S rRNA gene sequence showed that the isolate was phylogenetically related to species of the genus *Halorubrum*, with a close relationship to *Halorubrum trapanicum* NRC 34021^T (98.6 % similarity), *Halorubrum sodomense* ATCC 33755^T (98.3 %) and *Halorubrum xinjiangense* AS 1.3527^T (98.2 %). The polar lipids of strain SF3-213^T were C₂₀C₂₀ derivatives of phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, phosphatidylglycerol sulfate and a sulfated diglycosyl-diether. Strain SF3-213^T grew in 2.5–5.0 M NaCl. The temperature and pH ranges for growth were 25–42 °C and 6.8–8.5, respectively. Optimal growth occurred at 3.5–4.5 M NaCl, 37 °C and pH 7.3. Mg²⁺ was required for growth. The DNA G + C content was 69.4 mol%. DNA–DNA hybridization values lower than 70 % were obtained between strain SF3-213^T and the closely related species of the genus *Halorubrum*. Based on the data presented in this study, strain SF3-213^T represents a novel species for which the name *Halorubrum californiense* sp. nov. is proposed; the type strain is SF3-213^T (=CECT 7256^T=DSM 19288^T=JCM 14715^T).

Solar salterns harbour a diverse community of halophilic micro-organisms that represent the three domains of life: *Archaea*, *Bacteria* and *Eukarya* (Oren, 2002a). The archaeal members of the order *Halobacteriales*, family *Halobacteriaceae*, are significant components of higher salinity pans (Benlloch *et al.*, 2002). The genus *Halorubrum* (McGenity & Grant, 2001) is a member of the family and species of this genus are widely distributed in hypersaline environments, notably solar salterns (Kamekura 1998; Ochsenreiter *et al.*, 2002; Oren, 2002a, b). The generic name *Halorubrum* was first proposed by Grant & Ross (1986) for a distinct grouping of species within the genus *Halobacterium*. However, the genus *Halorubrum* was not officially recognized until the subsequent work of McGenity and Grant, which proposed the transfer of four species of *Halobacterium* to the new genus *Halorubrum* (McGenity & Grant, 1995). The transfer was based on

distinct phylogenetic clustering of the 16S rRNA sequences of all four species, with *Halorubrum saccharovororum* as the type species (McGenity & Grant, 1995). At the time of submission, 19 species were recognized within the genus *Halorubrum*: *Hrr. saccharovororum* (Tomlinson & Hochstein, 1976), *Hrr. lacusprofundi* (Franzmann *et al.*, 1988), *Hrr. coriense* (Kamekura & Dyall-Smith, 1995; McGenity & Grant, 1995), *Hrr. distributum* (Oren & Ventosa, 1996), *Hrr. sodomense* (Oren, 1983; Oren & Ventosa, 1996), *Hrr. tebenquichense* (Lizama *et al.*, 2002), *Hrr. terrestre* (Ventosa *et al.*, 2004), *Hrr. trapanicum* (McGenity & Grant, 1995), *Hrr. xinjiangense* (Feng *et al.*, 2004), *Hrr. alkaliphilum* (Feng *et al.*, 2005), *Hrr. tibetense* (Fan *et al.*, 2004), *Hrr. vacuolatum* (Kamekura *et al.*, 1997; Mwatha & Grant, 1993), *Hrr. lipolyticum* (Cui *et al.*, 2006), *Hrr. aidingense* (Cui *et al.*, 2006), *Hrr. ezzemoulense* (Kharroub *et al.*, 2006), *Hrr. orientale* (Castillo *et al.*, 2006), *Hrr. arcis* (Xu *et al.*, 2007), *Hrr. litoreum* (Cui *et al.*, 2007) and *Hrr. ejinorensis* (Castillo *et al.*, 2007). In this study, a novel strain that belongs to the genus *Halorubrum* is described and, based on phylogenetic, phenotypic and genotypic features and polar lipid composition, it is shown to represent a novel species.

Abbreviations: PG, phosphatidylglycerol; PGP-Me, phosphatidylglycerol phosphate methyl ester; PGS, phosphatidylglycerol sulfate; S-DGD, sulfated diglycosyl-diether.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain SF3-213^T is EF139654.

Strain SF3-213^T was isolated from water samples taken from a crystallizer pond at the Cargill Solar Salt Plant, Newark, California, USA (37° 29' N 122° 1' W) in spring 1997. Samples were isolated on agar plates under aerobic conditions at 37 °C using a modified Casamino acid medium (MCAT) (Litchfield *et al.*, 2005) containing the following ingredients (l⁻¹): Casamino acids, 7.3 g; yeast extract, 0.51 g; sodium citrate, 3 g; solar salt, 250 g; MgSO₄·7H₂O, 20.0 g; and FeCl₃, 0.02 g. The pH was adjusted to 7.3 with 1 M NaOH. The isolation methods have been described previously (Litchfield *et al.*, 2005). Salt and temperature ranges were determined using a microtitre plate format with three replicates of each treatment read using a Packard SpectraCount microplate reader at 590 nm. Salinity tolerance was tested at 0.5, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 and 5.0 M NaCl. Separate plates with the above salt concentrations were incubated at 10, 25, 37 and 42 °C and also read on the SpectraCount. The pH range was tested in buffered media at pH 5.0, 6.0, 6.5, 6.8, 7.0, 7.3, 7.8, 8.0, 8.5, 9.0 and 9.5. Strain SF3-213^T grew over the temperature range 25–42 °C (optimum 37 °C), with no growth at 20 °C or below, and at pH 6.8–8.5 (optimum pH 7.3) when tested in buffered media. Routine cultivation was accomplished at 37 °C and pH 7.3. Strain SF3-213^T grew over a wide range of NaCl concentrations (2.5–5.0 M) with optimal growth at 3.5–4.5 M NaCl. Mg²⁺ was required for growth (optimum at 0.2 M) and growth was observed over the range 0.17–1.7 M.

A polyphasic taxonomic approach was followed for the phenotypic, chemotaxonomic and genotypic characterization of strain SF3-213^T. All tests were performed in accordance with the proposed minimal standards for the description of new taxa in the order *Halobacteriales* (Oren *et al.*, 1997). The type species *Hrr. saccharovororum* NCIMB 2081^T was used as a reference; positive and negative controls were also used. Cell motility, morphology and Gram staining were examined using a Leica DM LB2 phase-contrast-equipped light microscope with an integrated Qimage camera (Retiga 1300) running BioQuant Nova Prime image analysis software v.6.90. Cells were taken from liquid media during early exponential growth. They were motile and rod-shaped by the hanging drop method and stained Gram-negative. Colony morphology was observed after growth at 37 °C for 7 days on MCAT agar with 4.5 M solar salt. Colonies of strain SF3-213^T were circular, 1.0–1.5 mm in diameter and slightly raised with light pink pigmentation. Anaerobic growth was tested with L-arginine in screw-topped sealed vials. Phenotypic characteristics were tested as described by Oren *et al.* (1997) and included the following tests: hydrolysis of starch, gelatin, casein, chitin, cellulose and Tween 80; nitrate reduction; production of H₂S, indole, catalase and Kovac's oxidase; and the utilization of sugars and amino acids as carbon and energy sources. In parallel, API 20 NE test strips (bioMérieux) were used for confirmation with a slight modification to the inoculum concentration to reduce high salt interference.

Strain SF3-213^T was positive for catalase and Kovac's oxidase. Indole was produced from tryptophan. Nitrate was reduced to nitrite. H₂S was not produced. Casein was hydrolysed, but starch, gelatin, chitin, cellulose and Tween 80 were not. In addition, the API 20 strips gave positive tests for aesculin and 4-nitrophenyl-β-D-galactopyranoside hydrolysis. The assimilation of sugars as single carbon sources was tested with modified Bushnell–Haas medium (Brown & Braddock, 1990) supplemented with 0.05 % (w/v) yeast extract, 2 % (w/v) MgCl₂, 0.01 % (w/v) sugar (D-glucose, D-fructose, lactose, D-galactose or mannose) and 25 % NaCl. Strain SF3-213^T was positive for the assimilation of D-glucose, lactose and D-galactose and negative for D-fructose and mannose. No acid was detected via pH meter readings for any of the sugars. Methyl red and Voges–Proskauer tests were negative. Utilization of various substrates and tests are summarized in the species description and Table 1.

Antibiotic susceptibilities were determined by the Kirby–Bauer (Bauer *et al.*, 1966) method on large MCAT agar plates (3.5 M NaCl) with antibiotic discs containing the following concentrations: ampicillin (10 µg), bacitracin (10 U), chloramphenicol (5 µg), erythromycin (15 µg), kanamycin (30 µg), nalidixic acid (30 µg), rifampicin (5 µg) and streptomycin (10 µg). The results are included in the species description.

Polar lipids were extracted using a chloroform/methanol system as described previously (Litchfield & Oren, 2001). One-dimensional TLC was performed on 19-channel silica gel-G plates (Whatman) in a solvent system of chloroform/methanol/acetic acid/water (85:22.5:10:4, by vol.) in lined tanks. For two-dimensional TLC, single sheet silica gel-G plates were employed with chloroform/methanol/acetic acid/water (80:12:15:4, by vol.) as the solvent system in the first dimension and chloroform/methanol/water (65:25:4, by vol.) in the second dimension (Litchfield & Oren, 2001). Polar lipid fractions were detected with spray reagents: orcinol for glycolipids followed by heating to 110 °C; ammonium molybdate for phospholipids (Tindall, 1990); and cresyl violet for sulfolipids (Soto *et al.*, 2000). The polar lipids of strain SF3-213^T are C₂₀C₂₀ derivatives of phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (PGP-Me) and phosphatidylglycerol sulfate (PGS). In addition, a single sulfated diglycosyl-diether (S-DGD) was also detected. This polar lipid profile is typical of neutrophilic species of the genus *Halorubrum* (McGenity & Grant, 2001).

Methods employed for genomic DNA extraction/preparation, PCR amplification of the 16S rRNA gene sequence and gene sequencing have been described previously (Litchfield *et al.*, 2005). The haloarchaeal primers used for the PCR were HK 1F (ATTCCGGTTGATCCTGCCGG) and H1492R (GTTACCTTGCCCGTTCCCTTC) (Litchfield *et al.*, 2005). The almost complete 16S rRNA gene sequence (1436 bp) of strain SF3-213^T was determined and com-

Table 1. Distinguishing characteristics of strain SF3-213^T and other species of the genus *Halorubrum*

Strains: 1, SF3-213^T; 2, *Hrr. saccharovororum* NCIMB 2081^T (tested in this study); 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; 14, *Hrr. ezzemoulense* CECT 7099^T; 15, *Hrr. lipolyticum* JCM 13559^T; 16, *Hrr. aidingense* JCM 13560^T; 17, *Hrr. orientale* CECT 7145^T; 18, *Hrr. arcis* AJ201^T; 19, *Hrr. litoreum* CGMCC 1.5336^T; 20, *Hrr. ejinorensense* CECT 7194^T. Data for strains 3–20 are from Feng *et al.* (2004, 2005), Ventosa *et al.* (2004), Lizama *et al.* (2002), McGenity & Grant (1995), Cui *et al.* (2006, 2007), Castillo *et al.* (2006, 2007) and Xu *et al.* (2007). +, Positive; –, negative; +/–, doubtful; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Morphology	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	Pleomorphic	Rods	Rods	Pleomorphic rods	Rods	Rods	Rods
Cell size (µm)	0.5 × 1.0–1.5	0.6–1.2 × 2.5	0.8–1.0 × 1.8–2.0	5.0–0.5	0.8–1.0 × 2.7–7	>12	0.5 × 2.5–5.0	0.8–1.2 × 1.5–1.0	1.0–1.5 × 1.5–2.5	0.5–1.0 × 1.5–2.5	0.7–1.0 × 1.5–3.0	0.7–10.0 × 1.5–3.0	0.8–1.2 × 1.8–2.6	0.6 × 1.5–3.0	0.9–1.2 × 1.8–5.5	0.9–1.0 × 3.6–6.4	1.5–2.0 × 1.0–2.0	0.5–1.0 × 2.0–4.0	0.3–0.5 × 2.0–5.0	1.0–1.5 × 5.0–8.0
Motility	+	+	+	+	+	+	+	ND	+	–	–	–	+	+	+	+	+	+	+	–
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	2.5–5.2	2.5–5.2	2.0–5.2	2.5–4.3	1.7–4.8	1.7–4.3	2.5–5.0	2.2–5.2	2.0–5.1	2.5–5.0
pH optimum	7.3	7.0–7.5	9–10	7.0–7.5	7.5	7.0–7.5	7.0–7.5	ND	7.5	9.0–9.5	7.0–7.5	9.5	7.0–7.5	7.0–7.5	7.5	7.5	8.0	7.54	7.0–7.5	7.5
Nitrate reduction	+	–	+	ND	+	+/–	+/–	+	–	+	+	+	–	+	+	+	+	+	+	+
Indole production	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	ND	ND	–
Casein hydrolysis	+	–	–	ND	–	–	ND	ND	–	ND	ND	ND	ND	ND	–	–	–	–	–	–
Mg ²⁺ requirement	+	+	–	+	ND	+	+	–	ND	–	ND	–	–	+	–	+	–	–	+	ND
Utilization of:																				
D-Glucose	+	+	+	+	–	+	+	+	–	+	+	+	+	+	+	+	+	+	+	–
D-Galactose	+	+	–	+	–	+	–	+	ND	–	+	+	–	–	+	+	–	–	+	–
Lactose	+	+	–	+	ND	+	+	–	ND	–	–	ND	–	ND	ND	ND	–	–	+	–
D-Fructose	–	+	+	ND	ND	–	+	+	–	ND	+	–	+	–	+	+	–	–	–	–
Mannose	–	+	+	+	+	+	ND	+	–	+	+	ND	–	–	+	+	ND	–	–	ND
PGS present	+	+	–	+	+	+	+	–	+	–	+	–	+	+	+	+	–	+	+	–
DNA G + C content (mol%)	69.4	71.2	62.1	ND	63.6	65.3	67.4	63.2	64.4	63.3	64.3	62.7	68.7	61.9	65.9	64.2	64.2	65.7	64.9	64

pared via BLAST to those of other members of the family *Halobacteriaceae*. The ARB software package (Ludwig *et al.*, 2004) was used for 16S rRNA gene sequence analysis. Several treeing methods (maximum-likelihood, distance matrix and maximum-parsimony) were performed on the basis of the recommendations of Ludwig *et al.* (1998). Strain SF3-213^T showed the highest level of similarity with species of the genus *Halorubrum*, with the closest relationships to *Hrr. trapanicum* NRC 34021^T (98.6 % similarity), *Hrr. sodomense* ATCC 33755^T (98.3 %) and *Hrr. xinjiangense* AS 1.3527^T (98.2 %). The phylogenetic tree obtained by using the maximum-parsimony method shows strain SF3-213^T within the species of the genus *Halorubrum* (Fig. 1). These results were consistent with those obtained using other algorithms to construct phylogenetic trees. In addition, the 16S rRNA gene sequence of strain SF3-213^T matched the signature sequences for the genus *Halorubrum* at positions 199–229, 656–672 and 1242–1259 (positions based on the sequence of *Escherichia coli*) (Grant *et al.*, 2001).

Chromosomal DNA of strain SF3-213^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 value (T_m) of the thermal denaturation profile (Marmur & Doty, 1962) by using the equation of Owen & Hill (1979) as described previously in detail by Ventosa *et al.* (1999). The DNA G+C content of strain SF3-213^T was 69.4 mol%, a relatively high value when compared with the close relatives mentioned above

(Table 1). DNA–DNA hybridization studies were performed according to the competition procedure of the membrane method (Johnson, 1994) as described in detail by Mormile *et al.* (1999). Strain SF3-213^T showed low DNA–DNA hybridization values to the closely related type strains of *Hrr. distributum* JCM 10118^T (44 %), *Hrr. trapanicum* JCM 10477^T (37 %), *Hrr. terrestre* VKM B-1739^T (33 %), *Hrr. tebenquichense* JCM 12290^T (24 %), *Hrr. saccharovororum* ATCC 29252^T (21 %), *Hrr. sodomense* JCM 8880^T (18 %) and *Hrr. xinjiangense* JCM 12388^T (4 %). These data show that strain SF3-213^T represents a different species, since the threshold value to separate two species is 70 % (Stackebrandt & Goebel, 1994).

The above phenotypic, genotypic, chemotaxonomic and phylogenetic data indicate that strain SF3-213^T represents a novel species within the genus *Halorubrum* for which the name *Halorubrum californiense* sp. nov. is proposed. Differential characteristics of strain SF3-213^T and other species of the genus *Halorubrum* are shown in Table 1.

Description of *Halorubrum californiense* sp. nov.

Halorubrum californiense (ca.li.for.ni.en'se. N.L. neut. adj. *californiense* pertaining to California, USA, where the strain was isolated from a solar saltern).

Cells are Gram-negative and short-rod-shaped (0.5 × 1.0–1.5 µm). Motile. Colonies are small, round, 1.0–1.5 mm in diameter, pink, smooth and slightly raised. Extreme halophile. Growth occurs optimally at 3.5–4.5 M NaCl

Fig. 1. Maximum-parsimony phylogenetic tree based on 16S rRNA gene sequences showing the position of strain SF3-213^T among related species. Bootstrap values (%) at the branches are based on 1000 replicates. The sequence data used were obtained from GenBank (accession numbers are given in parentheses). Bar, 1 % sequence divergence.

and over the range 2.5–5.0 M NaCl. The pH range for growth is 6.8–8.5, with optimum growth at pH 7.3. The temperature range is 25–42 °C; optimal temperature for growth is 37 °C. Magnesium is required for growth, with an optimum of 0.2 M and growth over the range 0.17–1.7 M. Chemo-organoheterotrophic and strictly aerobic. Positive for catalase and Kovac's oxidase. Casein and aesculin are hydrolysed, but gelatin, starch, cellulose, chitin and Tween 80 are not. H₂S is not produced. Nitrate is reduced to nitrite. Indole is produced. Methyl red and Voges–Proskauer tests are negative. D-Glucose, lactose and D-galactose are used as single carbon sources. Mannose, D-fructose and acetate are not utilized. No acid production from sugars. Sensitive to bacitracin, kanamycin and nalidixic acid. Resistant to ampicillin, chloramphenicol, erythromycin, rifampicin and streptomycin. The polar lipids are C₂₀C₂₀ diether derivatives of PG, PGP-Me and PGS and a single glycolipid, S-DGD.

The type strain, SF3-213^T (=CECT 7256^T=DSM 19288^T=JCM 14715^T), was isolated from the crystallizer pond at the Cargill Solar Salt Plant in Newark, California, USA. The DNA G+C content of the type strain is 69.4 mol% (T_m).

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