Published in final edited form as:
Int J Syst Evol Microbiol. 2009 August ; 59(Pt 8): 1908–1913. doi:10.1099/ijs.0.000463-0.

Halorubrum chaoviator sp. nov., a haloarchaeon isolated from sea salt in Baja California, Mexico, Western Australia and Naxos, Greece

Rocco L. Mancinelli[1], **Ragnhild Landheim**[1], **Cristina Sanchez-Porro**[2], **Marion Dornmayr-Pfaffenhuemer**[3], **Claudia Gruber**[3], **Andrea Legat**[3], **Antonio Ventosa**[2], **Christian Radax**[4], **Kunio Ihara**[5], **Melisa R. White**[1], and **Helga Stan-Lotter**[3]

[1]SETI Institute, 515 N. Whisman Road, Mountain View, CA 94043, USA

[2]Department of Microbiology and Parasitology, Faculty of Pharmacy, University of Sevilla, 41012 Sevilla, Spain

[3]Division of Molecular Biology, Department of Microbiology, University of Salzburg, Billrothstraße 11, A-5020 Salzburg, Austria

[4]Dr.-Petter-Str. 20, A-5020 Salzburg, Austria

[5]Center for Gene Research, Nagoya University, Nagoya 464-8602, Japan

Abstract

Three halophilic isolates, strains Halo-G*^{T}, AUS-1 and Naxos II, were compared. Halo-G* was isolated from an evaporitic salt crystal from Baja California, Mexico, whereas AUS-1 and Naxos II were isolated from salt pools in Western Australia and the Greek island of Naxos, respectively. Halo-G*^{T} had been exposed previously to conditions of outer space and survived 2 weeks on the Biopan facility. Chemotaxonomic and molecular comparisons suggested high similarity between the three strains. Phylogenetic analysis based on the 16S rRNA gene sequences revealed that the strains clustered with *Halorubrum* species, showing sequence similarities of 99.2–97.1 %. The DNA–DNA hybridization values of strain Halo-G*^{T} and strains AUS-1 and Naxos II are 73 and 75 %, respectively, indicating that they constitute a single species. The DNA relatedness between strain Halo-G*^{T} and the type strains of 13 closely related species of the genus *Halorubrum* ranged from 39 to 2 %, suggesting that the three isolates constitute a different genospecies. The G+C content of the DNA of the three strains was 65.5–66.5 mol%. All three strains contained $C_{20}C_{20}$ derivatives of diethers of phosphatidylglycerol, phosphatidylglycerolmethylphosphate and phosphatidylglycerolsulfate, together with a sulfated glycolipid. On the basis of these results, a novel species that includes the three strains is proposed, with the name *Halorubrum chaoviator* sp. nov. The type strain is strain Halo-G*^{T} (=DSM 19316^T =NCIMB 14426^T =ATCC BAA-1602^T).

The current classification of halophilic archaea is based on phenotypic characteristics, chemical data (polar lipid composition) and genetic data (16S rRNA gene sequence information and DNA–DNA hybridization) (Oren *et al.*, 1997; Grant *et al.*, 2001). Strains of the genus *Halorubrum* are known to use carbohydrates as sources of carbon and energy, as

Correspondence Helga Stan-Lotter helga.stan-lotter@sbg.ac.at.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains Halo-G*^{T}, AUS-1 and Naxos II are AM048786, D32081 and AJ400624.

Phase-contrast micrographs of cells of strains Halo-G*^{T} and Naxos II and 2D TLC of the polar lipids of strains Halo-G*^{T}, AUS-1 and Naxos II are available as supplementary material with the online version of this paper.

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was first described for *Halorubrum saccharovorum* (Tomlinson & Hochstein, 1976), the type species of the genus (McGenity & Grant, 2001). At the time of writing, the genus *Halorubrum* contains 19 species with validly published names: *Hrr. saccharovorum* (Tomlinson & Hochstein, 1976), *Hrr. sodomense* (Oren, 1983), *Hrr. lacusprofundi* (Franzmann *et al.*, 1988), *Hrr. trapanicum* (McGenity & Grant, 1995), *Hrr. coriense* and *Hrr. distributum* (Kamekura & Dyall-Smith, 1995), *Hrr. vacuolatum* (Kamekura *et al.*, 1997; Grant & Larsen, 1989), *Hrr. tebenquichense* (Lizama *et al.*, 2002), *Hrr. terrestre* (Ventosa *et al.*, 2004), *Hrr. tibetense* (Fan *et al.*, 2004), *Hrr. xinjiangense* (Feng *et al.*, 2004), *Hrr. alkaliphilum* (Feng *et al.*, 2005), *Hrr. lipolyticum* and *Hrr. aidingense* (Cui *et al.*, 2006), *Hrr. orientale* (Castillo *et al.*, 2006), *Hrr. ezzemoulense* (Kharroub *et al.*, 2006), *Hrr. arcis* (Xu *et al.*, 2007), *Hrr. litoreum* (Cui *et al.*, 2007) and *Hrr. ejinorense* (Castillo *et al.*, 2007).

We describe here three halophilic archaeal strains that were isolated from a marine intertidal area along the coast of Baja California, Mexico (strain Halo-G*^{T}; 28° N 114° W), natural salt-water pools on the Western Australian coast (strain AUS-1) and from a salt lake on the island of Naxos, Greece (strain Naxos II; 37° 04′ 35.77″ N 25° 20′ 52.21″ E). The three strains belong to the genus *Halorubrum* and proved to be very similar in their properties, suggesting a wide distribution of these haloarchaea. In addition, strain Halo-G*^{T} is of special significance, because it had been dried onto quartz disks and flown on the Biopan facility, a small retrievable capsule developed by the European Space Agency for exposure of biological samples in low Earth orbit (ESA, 2005), and survived exposure to conditions of outer space for 2 weeks (Mancinelli *et al.*, 1998).

All strains were isolated by enrichment in liquid medium and repeated streaking on agar medium as follows. For strain Halo-G*^{T}, the medium contained (g l^{-1}) casein hydrolysate (HyCase; Sigma), 5; yeast extract (Difco), 5; NaCl, 200; KCl, 2; $MgCl_2$.$6H_2O$, 20; $CaCl_2$. $2H_2O$, 0.2 (adjusted to pH 7.4). For strain AUS-1, the medium contained (g l^{-1}) polypeptone (Daigo Eiyo), 3.3; trisodium citrate, 3; NaCl, 250; KCl, 2; $MgSO_4$, 10; $CaCl_2$.$2H_2O$, 0.2 (adjusted to pH 7.2 with NaOH). For strain Naxos II, M2 medium was used, containing (g l^{-1}) HyCase, 5; yeast extract (Difco), 5; Tris, 12.1; NaCl, 200; KCl, 2; $MgCl_2$.$6H_2O$, 20; $CaCl_2$.$2H_2O$, 0.2 (adjusted to pH 7.4 with HCl). For solidification, 20 g agar l^{-1} was added to each medium. Routine cultivation was in M2 medium at 40 °C and pH 7.4. Growth ranges and optima of NaCl and $MgCl_2$ were determined using the growth medium containing various concentrations of NaCl (0.9–5.2 M) and $MgCl_2$ (0–0.5 M). Phenotypic tests were performed according to the proposed minimal standards for the description of new taxa in the order *Halobacteriales* (Oren *et al.*, 1997). The methods used were described previously (Stan-Lotter *et al.*, 2002; Gruber *et al.*, 2004). All tests were performed at least in triplicate with the exception of utilization of amino acids, which was tested in duplicate. Unless otherwise indicated, tests were done in M2 medium at pH 7.2–7.4 with incubation at 37 °C. The utilization of carbohydrates or amino acids was tested in a semi-defined medium which contained (g l^{-1}) yeast extract, 0.2; Tris, 6.05; NaCl, 233; KCl, 2; $MgCl_2$.$6H_2O$, 20; $CaCl_2$.$2H_2O$, 0.2 ; NH_4Cl, 0.053; trace element solution (Malik, 1983), 0.1 ml; adjusted to pH 7.4 with HCl. Incubation was done in test tubes without shaking for 7 weeks and utilization of substrates was judged by cellular growth (Stan-Lotter *et al.*, 2002). Susceptibility to antibiotics was tested by spreading cell suspensions on culture plates and applying discs impregnated with the following amounts of antibiotic: ampicillin (10 μg), anisomycin (10 μg), bacitracin (10 μg), chloramphenicol (10 μg), erythromycin (10 μg), kanamycin (10 μg), neomycin (10 μg), novobiocin (5 μg), rifampicin (10 μg) and tetracycline (10 μg).

Cell motility and morphology were observed under a phase-contrast light microscope and in dark field (Leica DM E). Gram staining of cells was performed according to Dussault

(1955). Colony morphology was observed on agar medium under optimal growth conditions after incubation for 30 days.

Polar lipids were extracted with chloroform/methanol as described previously (Stan-Lotter *et al.*, 2002). One- and two-dimensional TLC was performed with silica gel 60 plates (10×10 cm), using the solvent systems of Kamekura & Dyall-Smith (1995) and Stan-Lotter *et al.* (2002), respectively. Detection of phospholipids and functional groups was done as described previously (Stan-Lotter *et al.*, 2002); in addition, sulfated lipids were detected by spraying with 0.016 % azure A (Sigma) in 1 mM H_2SO_4, according to Sprott *et al.* (2003).

The 16S rRNA genes of strains Halo-G*^{T} and Naxos II were amplified by PCR using the primers Archae21F and 1525R, as described previously (Gruber *et al.*, 2004). The nearly full-length nucleotide sequence (approx. 1400 bp) was determined for each strain. The 16S rRNA gene sequence of strain AUS-1 had been determined and deposited previously (Ihara *et al.*, 1999). The web-based software MEGA 3 (http://www.megasoftware.net; Kumar *et al.*, 2004) was used for sequence analysis and for construction of the phylogenetic tree. Comparison of the sequences with those of members of the family *Halobacteriaceae* was based on the neighbour-joining method (Saitou & Nei, 1987). In addition, maximum-parsimony and maximum-likelihood algorithms were used as described previously (Gruber *et al.*, 2004) .

Chromosomal DNA for hybridization experiments was isolated and purified according to the methods described by Wilson (1987) and Marmur (1961). Determination of the G+C content was performed by the DSMZ Identification Service, following cell disruption with a French press and purification on hydroxyapatite (Cashion *et al.*, 1977). Further details of the method have been described previously (Stan-Lotter *et al.*, 2002). DNA–DNA hybridization studies were performed by the competition procedure of the membrane method (Johnson, 1994), described in detail by Arahal *et al.* (2001a, b). The hybridization temperature was 57.1 °C, which is within the limit of validity for the filter method (De Ley & Tijtgat, 1970), and the percentage of hybridization was calculated according to Johnson (1994). The experiments were carried out in triplicate. A few DNA–DNA hybridization experiments were performed by the DSMZ Identification Service (Stan-Lotter *et al.*, 2002), using the thermal renaturation method of De Ley *et al.* (1970) with modifications by Huß *et al.* (1983).

The organisms are rods, 2–5 μm long. Liquid 96-h cultures of strains Halo-G*^{T}, AUS-1 and Naxos II were motile and pleomorphic, although rod-shaped cells were most common (see Supplementary Fig. S1, available in IJSEM Online). All three strains were capable of growing over a range of NaCl concentrations from 2.0 M (12 %) to 5 M (30 %). They grew optimally in the presence of 4.3 M (25 %) NaCl, as has been shown for most extremely halophilic archaea. More details on phenotypic characteristics and results from nutritional tests are given in the species description.

TLC of polar lipids (Supplementary Fig. S2) suggested that all three strains contained phosphatidylglycerol, phosphatidylglyceromethylphosphate and phosphatidylglycerolsulfate derived from $C_{20}C_{20}$ glycerol diethers. A sulfated glycolipid S-DGD was also detected. This profile is similar to those reported for the neutrophilic species of *Halorubrum* (McGenity & Grant, 2001).

The 16S rRNA gene sequence of strain Halo-G*^{T} was very similar to those of AUS-1 and Naxos II (99.8 % similarity to both); it was closely related to those of *Hrr. coriense* Ch2^T (98.8 %), *Hrr. trapanicum* NRC 34021^T (98.8 %), *Hrr. xinjiangense* BD-1^T (98.8 %), *Hrr. litoreum* Fa-1^T (98.7 %), *Hrr. sodomense* ATCC 33755^T (98.7 %), *Hrr. ejinorense* EJ-32^T (98.2 %), *Hrr. distributum* JCM 9100^T (97.9 %) and *Hrr. ezzemoulense* 5.1^T (97.3 %). Fig. 1 shows a phylogenetic tree that delineates the relationship of the three strains to each other

and to the *Halorubrum* species. Similar topologies were obtained when other treeing methods were used (maximum-parsimony and maximum-likelihood; not shown). The signature sequences A, B and C for the genus *Halorubrum* (Grant *et al.*, 2001) were present in all three strains without mismatches; *Hrr. xingjiangense* BD-1^T had two mismatches in sequence C and *Hrr. ezzemoulense* 5.1^T had two inserts in sequence B. In summary, it was concluded that strains Halo-G*^{T}, AUS-1 and Naxos II formed a new distinct phylogenetic branch within the genus *Halorubrum*.

The DNA–DNA relatedness between strain Halo-G*^{T} and strains Naxos II and AUS-1 was 75 and 73 %, respectively (determined in triplicate). In addition, the 13 *Halorubrum* type strains that showed 16S rRNA gene sequence similarities higher than 97 % with strain Halo-G*^{T} [determined by using the FASTA search and/or the EzTaxon 2.0 program; http://www.eztaxon.org (Chun *et al.*, 2007)] were included in DNA–DNA hybridization experiments. The level of DNA–DNA relatedness between strain Halo-G*^{T} and related *Halorubrum* species was as follows (three experiments each): 39 % with *Hrr. ezzemoulense* CECT 7099^T, 35 % with *Hrr. ejinorense* EJ-32^T, 32 % with *Hrr. litoreum* JCM 13561^T, 31 % with *Hrr. coriense* JCM 9275^T, 28 % with *Hrr. distributum* JCM 10118^T, 25 % with *Hrr. californensis* SF3-213^T, 23 % with *Hrr. tebenquichense* JCM 12290^T, 21 % with *Hrr. xinjiangense* JCM 12388^T, 20 % with *Hrr. arcis* JCM 13916^T, 19 % with *Hrr. terrestre* VKM B-739^T, 5 % with *Hrr. sodomense* JCM 8880^T, 3 % with *Hrr. saccharovorum* ATCC 29252^T and 2 % with *Hrr. trapanicum* JCM 10477^T. These data indicated that strain Halo-G*^{T} does not belong to any of these 13 other species, since DNA relatedness values <70 % have been suggested to justify designation to different species (Wayne *et al.*, 1987); on the other hand, they showed that strains Halo-G*^{T}, Naxos II and AUS-1 are members of the same species.

The phenotypic features, DNA–DNA hybridization values and phylogenetic data based on the 16S rRNA gene sequence comparison clearly supported the placement of strains Halo-G*^{T}, AUS-1 and Naxos II in a novel species of *Halorubrum*, for which we propose the name *Halorubrum chaoviator* sp. nov. Table 1 shows features of all three strains that permit differentiation of the novel species from other related *Halorubrum* species.

Description of *Halorubrum chaoviator* sp. nov.

Halorubrum chaoviator [cha.o.vi.a'tor. Gr. n. *chaos* empty space, the void; L. n. *viator* traveller; N.L. n. *chaoviator* (nominative in apposition) the traveller of the void, referring to the exposure of the type strain to conditions of outer space in the Biopan facility].

Cells stain Gram-negative. Cells are pleomorphic, although most are rod-shaped (Supplementary Fig. S1). Cells are approx. 2.0–5.0×0.5–0.8 μm. Colonies are circular and red pigmented, 1.5–2 mm in diameter following incubation for 30 days at 37 °C. Growth occurs at pH 7.0–8.5, 28–50 °C and NaCl concentrations of 2.0–5.0 M (12–30 %). No growth at 10 °C. Optimal growth occurs at pH 7.4, 37 °C and 4.3 M (25 %) NaCl. Extremely halophilic; cells lyse in water. The requirement for magnesium is variable among strains. Chemo-organotrophic, aerobic and oxidase- and catalase-positive. β-Galactosidase-positive; α-galactosidase activity is variable among strains. Anaerobic growth with nitrate or L-arginine does not occur. Tween 80, aesculin and gelatin are not hydrolysed. Starch hydrolysis and nitrate reduction to nitrite are variable among strains. Indole is not formed. Acid is produced from (+)-D-glucose, (+)-D-galactose, lactose and maltose, but not from (−)-D-fructose or sucrose. The following substrates are utilized as sole carbon and energy sources: (+)-D-galactose, (+)-D-glucose, (−)-D-fructose, maltose and lactose. No growth occurs on sucrose, L-arginine, L-glutamic acid or DL-phenylalanine. Polar lipids include phosphatidylglycerol, phosphatidylglyceromethylphosphate and phosphatidylglycerolsulfate derived from $C_{20}C_{20}$ glycerol diethers and the sulfated glycolipid S-DGD. Susceptible to

bacitracin and novobiocin; not susceptible to ampicillin, anisomycin, chloramphenicol, erythromycin, kanamycin, neomycin, rifampicin or tetracycline. The DNA G+C content of the three known strains is 65.5–66.5 mol% (T_m).

The type strain is strain Halo-G*^{T} (=DSM 19316^T =NCIMB 14426^T =ATCC BAA-1602^T), which was isolated from an evaporitic salt crystal from Baja California, Mexico. Its G+C content is 65.5 mol%. Strains AUS-1 (=JCM 9573) and Naxos II, reference strains of the species, were isolated from Western Australia and the Greek island of Naxos, respectively.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by the Austrian Science Funds (FWF), project P16260 (to H. S.-L.), and the NASA Astrobiology Institute Cooperative Agreement NNA04CC05A (to R. L. M.). We thank Michael Sulzner, Austrian Ministry of Health, for help with the isolation of strain Naxos II.

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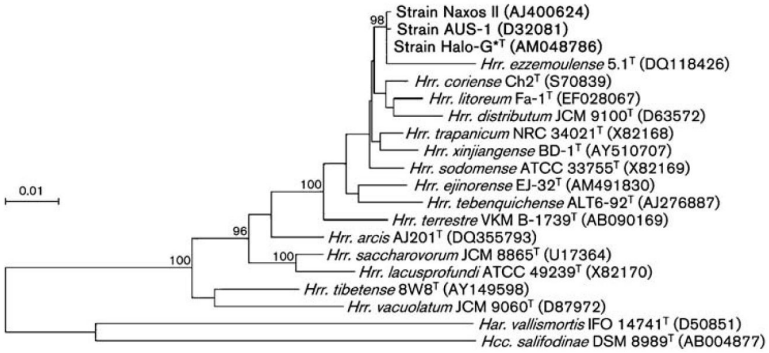

Fig. 1.

Phylogenetic tree based on the neighbour-joining algorithm, showing the relationships of strains Halo-G*^{T}, AUS-1 and Naxos II and several *Halorubrum* type strains. The tree is based on an alignment of 16S rRNA gene sequences. Sequence accession numbers are given in parentheses. Bootstrap values higher than 80 out of 100 subreplicates are indicated at the respective bifurcations. The sequences of *Haloarcula vallismortis* IFO 14741^{T} and *Halococcus salifodinae* DSM 8989^{T} were used as the outgroup. Bar, 0.01 expected changes per site.

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Int J Syst Evol Microbiol. Author manuscript; available in PMC 2011 September 29.

Table 1

Differential characteristics of strains Halo-G*^{T}, AUS-1 and Naxos II and type strains of related *Halorubrum* species

Strains used for comparison: 1, *Hrr. coriense* DSM 10284^{T}; 2, *Hrr. trapanicum* JCM 10477^{T}; 3, *Hrr. xinjiangense* JCM 12388^{T}; 4, *Hrr. litoreum* JCM 13561^{T}, 5, *Hrr. sodomense* ATCC 33755^{T}; 6, *Hrr. ejinorense* CECT 7194^{T}; 7, *Hrr. ezzemoulense* CECT 7099^{T}. Unless indicated, data for reference strains were taken from McGenity & Grant (2001), Feng *et al.* (2004), Kharroub *et al.* (2006), Castillo *et al.* (2007) and Cui *et al.* (2007). +, Positive; −, negative; PGS, phosphatidylglycerolsulfate; ND, no data available.

Feature	Halo-G*^{T}	AUS-1	Naxos II	1	2	3	4	5	6	7
Morphology†	R/P	R/P	R/P	LR/P	R/P	SR	R	R/P	R	R/P
Cell width (μm)	0.8	0.8	0.5–0.8	0.5	0.7–1.0	0.8–1.2	0.3–0.5	0.5	1.0–1.5	0.6
Cell length (μm)	2.0–4.0	2.0–4.0	2.0–5.0	5.0	1.5–3.0	1.8–2.6	2.0–5.0	2.5–5.0	5.0–8.0	1.5–3.0
Pigmentation‡	RD	RD	RD	RD–O	PO	RD	RD	O–RD	RD	RD
Motility	+	+	+	+	−	+	+	+	−	+
Growth at 2 M NaCl	−/+§	−/+§	−/+§	−/+//§	−	+	+	+	−	−
Temperature optimum (°C)	37	37	37	50	37	40	37–42	40	37	37–40
Growth at 10 °C	−	−	−	−	−	+	ND	−	ND	ND
Mg^{2+} requirement	50 mM	None	20 mM	5 mM	ND	None	30 mM	5 mM	None	Required
Nitrite from nitrate	−	+	−	−	ND	−	+	ND	+	+
Hydrolysis of:										
Starch	+	−	+	+//	−	−	−	+	−	−
Gelatin	−	−	−	−//	−	ND	−	−	−	−
Utilization of:										
(+)-D-Glucose	+	+	+	+	+	+	+	+	−	+
(−)-D-Fructose	+	+	+	+	+	+	−	+	−	−
(+)-D-Galactose	+	+	+	+//	+	−	+	+	−	−
Sucrose	−	−	−	+	+	+	+	−	−	+
Lactose	+	+	+	+//	−	−	−	+	−	−
Maltose	+	+	+	+//	+	+	+	+	−	+
Presence of:										
PGS	+	+	+	+	+	+	+	+	−	+
S-DGD	+	+	+	+	+	+	+	+	−	+

Feature	Halo-G*^{T}	AUS-1	Naxos II	1	2	3	4	5	6	7
G+C content (mol%)	65.5	65.5	66.5	67.3[//]	64.3	68.0	64.9	67.4	61.0	61.9

[†] $_{LR}$, Long rod; $_{P}$, pleomorphic; $_{R}$, rod; $_{SR}$, short rod.

[‡] $_{O}$, Orange; $_{PO}$, pale orange; $_{PRD}$, pale red; $_{RD}$, red.

[§] Very slow growth, after about 4 weeks.

[//] Data from this study.

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