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

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Three halophilic isolates, strains Halo-G*T, AUS-1 and Naxos II, were compared. Halo-G*T 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 C20C20 derivatives of diethers of phosphatidylglycerol, phosphatidylglyceromethylphosphate 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 19316T =NCIMB 14426T =ATCC BAA-1602T).

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 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.,...
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 halarchaea. 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; MgCl₂, 6H₂O, 20; CaCl₂, 2H₂O, 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; MgSO₄, 10; CaCl₂, 2H₂O, 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; MgCl₂, 6H₂O, 20; CaCl₂, 2H₂O, 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 MgCl₂ were determined using the growth medium containing various concentrations of NaCl (0.9–5.2 M) and MgCl₂ (0–0.5 M). Phenotypic tests were performed according to the proposed minimal standards for the description of new taxa in the order Halobacterales (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; MgCl₂, 6H₂O, 20; CaCl₂, 2H₂O, 0.2; NH₄Cl, 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 H₂SO₄, 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 & Tijigat, 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 phosphatidylglycerol-sulfate derived from C20C20 glycerol diethers. A sulfated tgdylglyceromethylphosphate and phosphatidylglycerolsulfate were 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. corense* Ch2T (98.8%), *Hrr. trapanicum* NRC 34021T (98.8%), *Hrr. xinjiangense* BD-1T (98.7%), *Hrr. sodomense* ATCC 33755T (98.7%), *Hrr. ejinorense* EJ-32T (98.2%), *Hrr. distributum* JCM 9100T (97.9%) and *Hrr. ezzemoulense* 5.1T (97.3%). The signature sequences A, B and C for the genus *Halorubrum* (Grant et al., 2001) were present in all three strains without mismatches; *Hrr. xinjiangense* BD-1T had two mismatches in sequence C and *Hrr. ezzemoulense* 5.1T had two insertions 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 7099T, 35% with *Hrr. ejinorense* EJ-32T, 32% with *Hrr. litoreum* JCM 13561T, 31% with *Hrr. corense* JC 9275T, 28% with *Hrr. distributum* JCM 10118T, 25% with *Hrr. californensis* SF3-213T, 23% with *Hrr. tebenquichense* JCM 12290T, 21% with *Hrr. xinjiangense* JCM 12388T, 20% with *Hrr. arcis* JCM 13916T, 19% with *Hrr. terestre* VKM B-739T, 5% with *Hrr. sodomense* JCM 8880T, 3% with *Hrr. saccharovorum* ATCC 29252T and 2% with *Hrr. trapanicum* JCM 10477T. 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 7–10 days. Cells are non-motile and pigmented, 1.5–2 mm in diameter following incubation for 7–10 days. Growth is obtained within 24 h on plates containing 5% NaCl and supplemented with 1% glycerol, 0.5% sodium sulfite and 0.05% azide, which allows the selection of clones with desirable properties. Cells are pleomorphic, although most are rod-shaped (Supplementary Fig. S1).

**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 *Halococcus vallismortis* IFO 14741T and *Halococcus salifodinae* DSM 8989T were used as the outgroup. Bar, 0.01 expected changes per site.
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. \( \beta \)-galactosidase-positive; \( \alpha \)-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% (Tm). 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.

### Table 1. Differential characteristics of strains Halo-G\(^{+T} \), AUS-1 and Naxos II and type strains of related Halorubrum species

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<th>Feature</th>
<th>Halo-G(^{+T} )</th>
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<th>Naxos II</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<td>61.9</td>
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†LR, Long rod; P, pleomorphic; R, rod; SR, short rod.
§O, Orange; PO, pale orange; RD, red.
§Very slow growth, after about 4 weeks.
IIData from this study.
Acknowledgements

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References


