Halorubrum halodurans sp. nov., an extremely halophilic archaeon isolated from a hypersaline lake

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Two extremely halophilic archaea, strains Cb34 T and C170, belonging to the genus Halorubrum, were isolated from the brine of the hypersaline lake Aran-Bidgol in Iran. Cells of the two strains were motile, pleomorphic rods, stained Gram-variable and produced red-pigmented colonies. Strains Cb34 T and C170 required 25 % (w/v) salts, pH 7.0 and 37 °C for optimal growth under aerobic conditions; 0.3 M Mg2+ was required. Cells of both isolates were lysed in distilled water and hypotonic treatment with <10 % NaCl provoked cell lysis. Phylogenetic analysis based on 16S rRNA gene sequence similarities showed that these two strains were closely related to Halorubrum cibi B31 T (98.8 %) and other members of the genus Halorubrum. In addition, studies based on the rpoB9 gene revealed that strains Cb34 T and C170 are placed among the species of Halorubrum and are closely related to Halorubrum cibi B31 T, with rpoB9 gene sequence similarity less than or equal to 95.7 %. The polar lipid patterns of both strains consisted of phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, phosphatidylglycerol sulfate and sulfated mannosyl glucosyl diether. The DNA G+C content was 62.1–62.4 mol%. DNA–DNA hybridization studies confirmed that strains Cb34 T and C170 constitute a distinct species. Data obtained in this study show that the two strains represent a novel species, for which the name Halorubrum halodurans sp. nov. is proposed. The type strain is Cb34 T (=CECT 8745 T =IBRC-M 10233 T).

The haloarchaea are a group of extremely halophilic, aerobic archaea included in the class Halobacteria (Grant et al., 2001) that comprises 48 genera including the genus Halorubrum, established by McGenity & Grant (1995). Currently, it is the largest haloarchaeal genus, with 31 validly published species names (Parte, 2015). Species of the genus Halorubrum have been isolated from diverse natural and artificial hypersaline environments such as salterns, salt lakes, coastal sabkhas, soda lakes, saline soils and salt-fermented and salt-preserved food. It is often reported as the dominant genus present in many hypersaline environments; their ubiquity and adaptability to nearly all possible hypersaline conditions has been demonstrated by cultivation and culture-independent methodologies (Ghai et al., 2011; Makhdoumi-Kakhki et al., 2012a; Fernández et al., 2014a, b). Species of this genus are aerobic, chemo-organotrophic and require high concentrations of NaCl in media (e.g. 1.0–5.2 M) for growth. The genus is phenotypically highly variable: its members use many carbon substrates including a wide range of sugars as sources of energy. However, some species use only single carbon sources for growth. They produce bacterioruberin carotenoid pigments that colour colonies pink to red. Most described species are neutrophilic, but some are alkaliophilic and grow optimally at pH 9.5. The major polar lipids are C20C20 and sometimes C20C25.
The pH of the lake brine is neutral, the predominant salts the central desert area of Iran, formed in the Pliocene period. Aran-Bidgol hypersaline lake is a natural ecosystem located in the region. Advantages of the 16S rRNA gene marker (Walsh et al., 2011). Among the single copy protein-encoding genes investigated previously for evolutionary and taxonomic studies in haloarchaea, the rpoB' gene has been reported to be very successful in overcoming the above disadvantages of the 16S rRNA gene marker (Walsh et al., 2004; Enache et al., 2007; Minegishi et al., 2010; Papke et al., 2011; Fullmer et al., 2014; Ram-Mohan et al., 2014). Aran-Bidgol hypersaline lake is a natural ecosystem located in the central desert area of Iran, formed in the Pliocene period. The pH of the lake brine is neutral, the predominant salts are NaCl, Na2SO4, MgCl2 and MgSO4 with trace amounts of carbonates, and it is considered a thalassohaline lake (Makhdoumi-Kakhki et al., 2012a). In previous studies of the haloarchaeal population in this lake, several novel genera and species of this group of micro-organisms were isolated and characterized (Amoozegar et al., 2012, 2013, 2014a, b, 2015; Makhdoumi-Kakhki et al., 2012b, c). In the present study, the properties of two extremely halophilic archaea, strains Cb34 and C170, are described on the basis of standard taxonomic methods following the proposed minimal standards recommended by Oren et al. (1997) for describing novel taxa of the order Halobacteriales. Additionally, we have included an rpoB' gene phylogeny to complement and reinforce our results.

Strains Cb34 and C170 were isolated from brine of lake Aran-Bidgol (34°18′–34°45′ N 51°33′–52°10′ E). Isolation of haloarchaea was performed by plating 100 μl brine, as well as using serial dilutions from sediment samples up to 10⁻⁶, directly on Hv-YPC solid medium (Allers et al., 2004). The plates were incubated aerobically at 37°C in sealed plastic bags for up to 4 weeks. The growth and optimum requirements for NaCl, Mg²⁺, pH and temperature were determined in the routine medium M1I, changing the recipe for testing growth at different concentrations of salts. The range of NaCl (5–30%, w/v) was tested at intervals of 5%. Strains Cb34 and C170 grew in the presence of 20–30% (w/v) total salts (optimum 25%, w/v). Cells of both isolates were lysed in distilled water as well as after hypotonic treatment with less than 10% (w/v) NaCl. The magnesium range was tested using MgCl₂ (0–10%, w/v) at intervals of 1% (w/v). Routine cultivation was performed at 37°C and pH 7.2. Strains Cb34 and C170 have a minimal magnesium ion requirement of 0.3 M Mg²⁺. The pH range for growth was assayed at pH 5.5–10.0 at intervals of 0.5
Halorubrum cibi

Differential features of strains Cb34T and C170 from the type strains of closely related species are shown in Table 1.

Antimicrobial compound sensitivity tests were performed by spreading a culture suspension on plates of solid medium M1 20 % and applying discs (BD) soaked with antimicrobial compounds following the Bauer–Kirby technique (Bauer et al., 1966) as described by Ventosa et al. (1999). Discs containing amikacin (50 μg) were prepared in our laboratory and applied in the same way. Strains Cb34T and C170 were sensitive to amikacin (50 μg), bacitracin (10 IU), novobiocin (30 μg), rifampicin (5 μg), erythromycin (15 μg), streptomycin (10 μg) and trimethoprim/sulfamethoxazole (1.25/23.75 μg) and resistant to ampicillin (10 μg), chloramphenicol (30 μg), nalidixic acid (30 μg), neomycin (30 IU), gentamicin (10 IU), kanamycin (30 μg), penicillin G (10 IU) and tetracycline (30 μg).

Genomic DNA of strains Cb34T and C170 was obtained by the method of Marmur (1961). The 16S rRNA gene was amplified by PCR (Sambrook & Russell, 2001) using universal primers as described previously (DeLong, 1992; Arahal et al., 1996). The complete rpoB (RNA polymerase subunit B') gene sequence of strain Cb34T was obtained from the genome sequence (Fullmer et al., 2014). The rpoB gene of strain C170 was amplified by PCR, using the primers designed by Fullmer et al. (2014) and Ram Mohan et al. (2014). The sequencing primer M13 (18 bp) was added to the 5' end of the degenerate primers to sequence PCR products more efficiently (Fullmer et al., 2014; Ram-Mohan et al., 2014). The PCR products were separated by gel electrophoresis with agarose (1 % w/v) and gels were stained with ethidium bromide. An exACTGene mid-range plus DNA ladder (Fisher Scientific International Inc.) was used to estimate the size of the amplicons.

Amplicons were purified using the Wizard SV Gel and PCR Cleanup system (Promega). The purified 16S rRNA gene PCR product was sequenced by StabVida (Oeiras, Portugal) and the product of the rpoB gene by Genewiz Inc. (Boston, USA) using Sanger sequencing technology. Sequencing reactions were carried out in both directions by the dideoxynucleotide chain-termination method using the BigDye terminator kit version 3.1 from Applied Biosystems. Sequencing products were purified by gel filtration and resolved in an ABI 3130XL or ABI 3730XL DNA Analyzer sequencer, according to the manufacturer’s instructions. The nucleotide sequences of the 16S rRNA genes of strains Cb34T and C170 were aligned with ChromasPro software version 1.7.6 and ARB 5.5 software package (Ludwig et al., 2004). Sequence similarity was analysed by comparing the 16S rRNA gene sequences of strains Cb34T and C170 with known sequences from the EzTaxon-e database (http://www.ezbiocloud.net/eztaxon; Kim et al., 2012). The similarity analysis based on complete 16S rRNA gene sequences showed that the sequences of strains Cb34T (1402 bp) and C170 (1379 bp) were
7.0.4 using the general time-reversible (GTR) model of maximum-likelihood analysis was performed with RAxML

Table 1. Features that distinguish strains Cb34 and C170 from type strains of closely related species of the genus Halorubrum

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<td>62.1</td>
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*Data from: a, Roh & Bae (2009); b, Gutierrez et al. (2011); c, Gutierrez et al. (2008); d, Feng et al. (2005); e, Fan et al. (2004); f, Cui et al. (2006).
SeaView (Gouy et al., 2010), aligning the proteins using MUSCLE (version 3.8.31) (Edgar, 2004) with its refine function and then reverting back to the nucleotide sequences. Alignments were edited using Mesquite version 2.75 (Maddison & Maddison, 2011). The best model of evolution was determined by calculating the Akaike information criterion with correction for small sample size (AICc). The jModelTest 2.1.4 (Darriba et al., 2012) program was used to compute likelihoods from the nucleotide alignment and to perform the AICc test (Akaike, 1974). The AICc reported the best-fitting model to be TVM + Gamma estimation + invariable site estimation. A maximum-likelihood phylogeny was generated from the multiple-sequence alignment using the PhyML version 3.0.360-500 (Guindon et al., 2010) with 1000 bootstrap replicates. The model and parameters used in PhyML corresponded to the one favoured by the jModel test. The number of nucleotide differences in pairwise comparisons was determined using MEGA 5 (Tamura et al., 2011).

The rpoB phylogenetic tree showed that strains Cb34T and C170 clustered with the species of Halorubrum but formed an independent and defined branch, clearly separated from the other species that constitute this genus (Fig. 2).

The G+C content of the genomic DNA was determined by thermal denaturation using the melting midpoint (Tm) (Marmur & Doty, 1962) using the equation of

\[ T_m = 94.1^\circ C \left( \frac{2(A+T) - (G+C)}{2(A+T) + (G+C)} \right) + 16.6^\circ C \text{ (Marmur & Doty, 1962)} \]

**Fig. 1.** Neighbour-joining phylogenetic tree based on 16S rRNA gene sequence comparison, showing the relationships between strains Cb34T and C170 and other members of the genus Halorubrum and other related haloarchaea. The sequence data used were obtained from the GenBank database (accession numbers in parentheses). Bootstrap values (%) are based on 1000 replicates and are shown for branches with more than 70 % bootstrap support. Filled circles indicate that the corresponding nodes were also obtained in the trees generated with the maximum-parsimony and maximum-likelihood algorithms. Bar, 2 % substitution.
Owen & Pitcher (1985). The DNA G + C content of strains Cb34\(^T\) and C170 was 62.1–62.4 mol\%, within the range reported for the genus *Halorubrum*, 60.2–71.2 mol\% (McGenity & Grant 2001; Roh & Bae 2009).

DNA–DNA hybridization (DDH) studies between strains Cb34\(^T\) and C170 and type strains of the phylogenetically most closely related species of the genus *Halorubrum* were performed in triplicate by the competition procedure of the membrane filter method of Johnson (1994). DDH between strains Cb34\(^T\) and C170 was 100 \%, and that between strain Cb34\(^T\) and the most closely related strains *Hrr. cibi* JCM 15757\(^T\), *Hrr. aquaticum* EN-2\(^T\), *Hrr. kocurii* CECT 7322\(^T\), *Hrr. alkaliphilum* JCM 12358\(^T\), *Hrr. tibetense* JCM 11889\(^T\) and *Hrr. lipolyticum* JCM 13559\(^T\) was 42, 59, 57, 51, 56 and 50 \%, respectively. These values are below the threshold of 70 \% recommended for definition of prokaryote species (Wayne et al., 1987; Stackebrandt & Goebel, 1994; Stackebrandt et al., 2002), indicating that these levels of DDH were low enough for these strains to be assigned to a genotype distinct species within the genus *Halorubrum*. Therefore, the DDH and *rpoB* sequence data obtained for this study are in agreement, endorsing the conclusion that the two strains are genotypically distinct and should be assigned to a different species.

Cell biomass of strains Cb34\(^T\) and C170 for chemotaxonomic analyses was obtained after 10 days of aerobic incubation in M1 liquid medium under optimal conditions: 25 \% (w/v) NaCl, 37 °C and pH 7.0. The strains used for comparisons were cultured according to the authors’ description for each strain and standardizing to the same incubation conditions. Polar lipids were extracted with chloroform/methanol following the method for extraction of membrane polar lipids of halophilic archaea described by Corcelli & Lobasso (2006); the extracts were dried carefully using a SpeedVac Thermo Savan SPD111V before weighing and then dissolved in chloroform to obtain a concentration of 10 mg lipid ml\(^{-1}\). Total lipid extracts were analysed by one-dimensional HPTLC on Merck silica-gel plates crystal back (Merck art. 5626; 10 x 20 cm); the plates were eluted in the solvent system chloroform/90 % methanol/acetic acid (65 : 4 : 35, by vol.) (Angelini et al., 2012; Corral et al., 2013). To detect all polar lipids, the plate was sprayed with sulfuric acid, 5 \% in water, and charred by heating at 160 °C (Kates, 1986). The glycolipids
Description of *Halorubrum halodurans* sp. nov.


Cells are motile, pleomorphic rods, 5.0–10 × 1.0–4.0 μm, and occur singly without grouping. Gram-stain-variable; in young cultures, most cells are Gram-stain-negative, while a few cells are observed as Gram-stain-positive. Gas vesicles are not observed inside cells. Colonies on solid medium after incubation at 37 °C for 10 days are circular, with regular edges, smooth, convex and 0.5–1 mm in diameter. During the first 5 days, colonies appear pink, and the colour gradually intensifies to red on incubation over subsequent days. Cells are lysed in distilled water; hypotonic treatment with less than 10 % (w/v) NaCl induces cell lysis. Has a minimal magnesium ion requirement of 0.3 M Mg²⁺. The species is neutrophilic, growing at pH 6.0–8.0 with an optimum at pH 7.0–7.2. Requirements for growth on NaCl are 20–30 % (w/v) with an optimum of 25 % (w/v). Grows at 20–45 °C (optimum 37 °C). Chemo-organotrophic and strictly aerobic. Oxidase and catalase activities are detected. Anaerobic growth does not occur with nitrate or L-arginine. Arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase are not produced. H₂S is produced from sodium thiosulfate or cysteine; indole is not produced from tryptophan. Nitrate is reduced to nitrite, but nitrite is not reduced further and no gas is formed. Methyl red and Voges–Proskauer tests are negative, and citrate is not utilized. Extracellular hydrolysis of Tween 80, starch, DNA, gelatin and casein is not observed. Tests for urease and phosphatase activities are negative. The following substrates are utilized for growth as sole sources of carbon and energy: D-glucose, sucrose, D-mannose, trehalose, glycerol, D-mannitol, acetate, glutamate, lactate, malate, pyruvate, succinate and propionate. D-Galactose, D-fructose, D-ribose, D-xylose, lactose, maltose, sorbose, sorbitol, raffinose, L-arabinose, rhamnose, fumarate and citrate are not used as sole sources of carbon and energy. The following amino acids are used as sole sources of carbon, nitrogen and energy: L-serine, threonine, glycine, asparagine and L-lysine. Isoleucine, alanine, arginine and ornithine are not used as sole sources of carbon, nitrogen and energy. The polar lipid pattern consists of PG, PGP-Me, PGS and S-DGD as major lipids. Diphosphatidylglycerol and minor phospholipidic components are present at low levels. The DNA G+C content is 62.1–62.4 mol% (*Tₘ*).

The type strain is Cb34ᵀ (= CECT 8745ᵀ = IBRC-M 10233ᵀ), isolated from sediment of the hypersaline lake Aran-Bidgol in Iran. The DNA G+C content of the type strain is 62.1 mol% (*Tₘ*).

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References


