**Halorubrum persicum** sp. nov., an extremely halophilic archaeon isolated from sediment of a hypersaline lake

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An extremely halophilic archaeon belonging to the genus *Halorubrum*, strain C49T, was isolated from sediment of the hypersaline lake Aran-Bidgol in Iran. Phylogenetic analysis based on 16S rRNA gene sequence similarities showed that strain C49T was closely related to *Halorubrum saccharovorum* JCM 8865T (99.5%) and other species of the genus *Halorubrum*. Studies based on multilocus sequence analysis revealed that strain C49T is placed among the species of *Halorubrum*; the strain constituted a defined branch in comparison with the type strains of species of *Halorubrum*, while the 16S rRNA gene sequence divergence could not define the status of the newly isolated strain. For optimum growth, strain C49T required 20% (w/v) salts at pH 7.0 and 37°C under aerobic conditions. Mg2+ was not required. The cells were pleomorphic rods, motile and stained Gram-variable. Colonies of the strain were pink. Hypotonic treatment with <12% NaCl provoked cell lysis. The polar lipid pattern of strain C49T consisted of phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester derived from both C20C20 and C20C25 archaeol, phosphatidylglycerol sulfate and sulfated mannosyl glucosyl diether. The DNA G+C content was 64.2 mol%. DNA–DNA hybridization studies and average nucleotide identity confirmed that strain C49T constitutes a distinct genospecies. Data obtained in this study show that strain C49T represents a novel species, for which the name *Halorubrum persicum* sp. nov. is proposed. The type strain is C49T (=IBRC-M 10232T = JCM 30541T).

The genus *Halorubrum* was established by McGenity & Grant (1995) and constitutes a large group of extremely halophilic aerobes belonging to the family Halobacteriaceae, within the order Halobacteriales (domain Archaea). Members of the genus are widely distributed in diverse natural and artificial hypersaline environments such as marine salterns, salt lakes, coastal sabkhas, soda lakes, saline soils and salt-fermented and salt-preserved food products. At the time of writing, the genus contained 27 species with validly published names (Parte, 2014). Members of the genus are aerobic, chemo-organotrophic and obligatorily halophilic, with growth occurring in media containing 1.0–5.2 M NaCl. They use many substrates, including sugars, as sources of carbon and energy and produce colonies from pink to red in colour due to the presence of bacterioruberin carotenoid pigments. According to their pH requirements for growth, the genus includes neutrophilic and alkaliphilic species. The major polar lipids are C20C20 and sometimes C20C25 glycerol diether derivatives of phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester derived from both C20C20 and C20C25 archaeol, phosphatidylglycerol sulfate and a sulfated mannosyl glucosyl diether. Alkaliphilic species lack phosphatidylglycerol sulfate and glycolipids. The known DNA G+C content range is between 60.2 and 71.2 mol%. The type species is *Halorubrum saccharovorum* (McGenity & Grant, 2001; Oren et al., 2009).

For prokaryotes, the universal phylogenetic and taxonomic marker is the 16S rRNA gene, and its sequence is therefore

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**Abbreviations:** ANI, average nucleotide identity; DDH, DNA–DNA hybridization; MLSA, multilocus sequence analysis.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, atpB, ef-2, glnA and rpoB9 gene sequences of strain C49T are HG421000, KJ152247, KJ152276, KJ152362 and KJ152361, respectively.

Two supplementary figures are available with the online Supplementary Material.
considered essential in taxonomic studies. However, the haloarchaea are known for their frequent recombination across great genetic distances (Williams et al., 2012; Nelson-Sathi et al., 2012), its highly conserved nature does not allow relevant discrimination among closely related species with high 16S rRNA gene sequence similarity [for example, 99.4% sequence similarity between the type strains of *Halorubrum californiense* and *Halorubrum chaoviator* (Pesenti et al., 2008; Mancinelli et al., 2009)], their rRNA operons undergo intragenic recombination (Boucher et al., 2004) and it is the most frequently transferred gene among otherwise distinct lineages (Papke et al., 2004), making phylogeny and taxonomy based on 16S rRNA gene sequence comparison difficult and notoriously unreliable. The large number of strains isolated frequently as members of *Halorubrum* and the inconvenience of reliance on a single genetic marker (the 16S rRNA gene) for classification can lead to erroneous phylogenies and therefore to taxonomic misplacement. Analysis of protein-encoding housekeeping genes has been used to overcome some of these limitations; therefore, a multilocus sequence analysis (MLSA) approach has been used as an alternative phylogenetic marker within the family *Halobacteriaceae*, since it resolves and defines with sensitivity the phylogeny of closely related species in the haloarchaea. MLSA is a reliable method that differentiates individual strains from each other, groups of strains into species, and species into genera, and also identifies potential novel species as well as family-like relationships within the *Halobacteriales* (Papke et al., 2011).

Aran-Bidgol hypersaline lake, also called Masileh or Namak lake, is a natural ecosystem located in the central desert area of Iran, formed in the Pliocene period. The pH of the brine in the lake is neutral, the predominant salts are NaCl, Na2SO4, MgCl2, and MgSO4 with trace of carbonates, and it can be considered as a thalassohaline lake (Makhdoumi-Kakhki et al., 2012a). In previous studies of the haloarchaean population in this lake, representatives of several novel genera and species of this group of micro-organisms have been isolated and characterized (Amoozegar et al., 2012, 2013, 2014a, b, 2015; Makhdoumi-Kakhki et al., 2012b, c).

In the present study, we describe the properties of an extremely halophilic archaeon, strain C49T, isolated from sediment of Aran-Bidgol hypersaline lake (34°18′34″–45°51′33″–52°10′ E), on the basis of standard taxonomic methods following the minimal standards recommended by Oren et al. (1997) for describing novel taxa of the order *Halobacteriales*. Additionally, we carried out an MLSA approach and average nucleotide identity (ANI) analysis, which has been suggested to complement and advance the understanding of prokaryotic taxonomy and has been studied in other taxa (Konstantinidis & Tiedje, 2005) in order to examine and correlate the results of MLSA and DNA–DNA hybridization (DDH).

Isolation of haloarchaea from sediment was performed by plating directly 100 μl aliquots of brine, as well as using serial dilutions up to 10^{-6} from sediment samples, on solid Hv-YPC medium (Allers et al., 2004). The plates were incubated aerobically at 37°C in sealed plastic bags for up to 4 weeks. Representative colonies were then transferred to the same medium. A pure culture isolate was obtained after successive streaking. The isolate was designated strain C49T; colonies of the isolate showed pink pigmentation. For culturing in the laboratory, we used M1 20% medium (Rodriguez-Valera et al., 1980), prepared from SW 30% stock solution (Subow, 1931), which consists of (per litre): 234 g NaCl, 39 g MgCl2, 6H2O, 61 g MgSO4, 7H2O, 1 g CaCl2, 6 g KCl, 0.2 g NaHCO3 and 0.7 g NaBr. This solution was supplemented with 0.2% (w/v) yeast extract, 0.1% (w/v) Casamino acids and 0.005% (w/v) pyruvic acid sodium salt. The medium was adjusted to pH 7.0–7.2. For solid media, 2.0% (w/v) agar was used when necessary.

The strain was maintained on the same medium in slant tubes, and long-term preservation in cryotubes were prepared for freezing at −80°C by adding 250 μl glycerol/SW 30 (80:20, v/v) to 750 μl fresh culture (OD600 0.8–1.0) (Dyall-Smith, 2009).

Cell morphology and motility were examined in liquid medium after 7 days of growth by optical and phase-contrast microscopy (BX41; Olympus). Strain C49T showed long motile rods and pleomorphic cells, 5.0–10 μm in length and 1.2 μm in width, and cells were observed singly without grouping (Fig. S1, available in the online Supplementary Material).

Genomic DNA of strain C49T was obtained by the method of Marmur (1961). The 16S rRNA gene of strain C49T was amplified by PCR (Sambrook & Russell, 2001) using universal primers as described previously (DeLong, 1992; Aral et al., 1996). The PCR product was sequenced by StabVida (Oeiras, Portugal) using the Sanger method. Sequencing reactions were carried out using a BigDye terminator kit version 3.1 (Applied Biosystems). Sequencing products were purified by gel filtration and resolved in an ABI 3730XL sequencer. The nucleotide sequence of the 16S rRNA gene of strain C49T (1403 bp) was assembled with ChromasPro software version 1.7.6 and assembled with the ARB 5.5 software package (Ludwig et al., 2004). Sequence similarity was analysed by comparing the 16S rRNA gene sequence of strain C49T with known sequences from the EzTaxon-e database (http://www.ezbiocloud.net/eztaxon; Kim et al., 2012). Phylogenetic analysis based on almost complete 16S rRNA gene sequence (1403 bp) showed that strain C49T was closely related to *Halorubrum sacharovorum* JCM 8865T (99.5% similarity), *Hrr. lipolyticum* 9-3T (98.8%), *Hrr. halophilum* B8T (98.7%), *Hrr. kocurii* BG-1T (98.6%), *Hrr. lacusprofundi* ATCC 49239T (98.2%), *Hrr. arcis* AJ201T (97.7%) and *Hrr. aidentigenae* 31-hongT (97.1%). Lower similarities were obtained with the type strains of species of other haloarchaeal genera.

For phylogenetic analysis based on an MLSA approach, five housekeeping genes, *atpB*, *ef-2*, *glnA*, *ppsA* and *rpoB*, were amplified by PCR using primers designed for each locus
Phylogenetic study based on 16S rRNA gene sequence comparison was performed by reconstructing phylogenetic trees using the neighbour-joining, maximum-parsimony and maximum-likelihood clustering algorithms (Saitou & Nei, 1987) with the ARB program package version 5.5 (Ludwig et al., 2004). Maximum-likelihood analysis was performed with RAxML 7.0.4 using the general time reversible (GTR+G) model of nucleotide substitution (Stamatakis et al., 2005). Base-frequency filters were applied in the sequence-comparison analysis, and the effects on the results were evaluated. To evaluate the robustness of the phylogenetic tree, a bootstrap analysis (1000 replications) was performed (Felsenstein, 1985). The phylogenetic tree (Fig. 1) reconstructed by maximum-likelihood confirmed that strain C49T is related to *Hrr. saccharovorum* JCM 8865^T^ and is within the cluster constituted by species of the genus *Halorubrum* excluding *Hrr. halophilum* DSM 3754^T^ (AJ493654).

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**Fig. 1.** Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequence comparison, showing relationships between strain C49^T^ and 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 (%) based on 1000 replicates are shown for branches with more than 70% bootstrap support. Filled circles indicate that the corresponding nodes were also obtained in trees reconstructed with the maximum-parsimony and neighbour-joining algorithms. Bar, 2% substitution per nucleotide position.
Halorubrum. Topologies of phylogenetic trees inferred using
the neighbour-joining and maximum-parsimony algo-
rithms were highly similar to that of the tree reconstructed
by maximum-likelihood (Fig. 1).

Phylogeny based on housekeeping genes was carried out by
recovering each MLSA target gene sequence (atpB, ef-2,
ghnA, ppsA and rpoB’) from the GenBank database. In some
cases, sequences were obtained from the genomes of type
strain recovered from the NCBI ftp repository using the
top scoring BLASTN hits. Multiple-sequence alignments
were created taking into account the corresponding amino
cid alignments for protein-coding genes generated by
translating the genes to protein sequences in 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). Individual gene alignments
were trimmed manually down to the lengths of the PCR
amplicons and were then concatenated in the order
was determined by calculating the Akaike information crite-
ron with correction for small sample size (AICc). The
AICc

Fig. 2. Maximum-likelihood tree based on the five-gene (atpB, ef-2,
ghnA, ppsA and rpoB’) concatenated sequence showing phylogenetic relationships between members of the genus
Halorubrum and strain C49T. Bootstrap values >70% are
indicated. Halobacterium salinarum was used as an outgroup.
Bar, 10% substitution per nucleotide position.
or CAPS (pH 10.0–11.0) at 50 mM to the isolation
medium (Scorpio, 2000). The temperature range for
growth was determined by incubating at 4, 10, 20, 30, 37
and 45 °C in M1 medium with optimal NaCl and
MgCl2 concentrations. Gram staining was performed using
acetate acid-fixed samples, as described by Dussault (1955).
Anaerobic growth was tested in the presence of nitrate and
L-arginine by adding 3 % KNO3 or 4 % L-arginine to the
medium in filled stoppered tubes, as well as plates of
cultures incubated for 10 days at 37 °C in an anaerobic jar
(Oren et al., 1997). Catalase activity was determined by
adding 1 % (w/v) H2O2 solution to colonies on solid
medium. The oxidase test was performed using a DrySlide
assay (Difco). Tests for hydrolysis of starch, gelatin, casein
and Tween 80 were carried out as described by Barrow &
Feltman (2003). Tests for indole production from trypto-
phan and urea were performed as described by Gerhardt
et al. (1994). The methyl red, Voges–Proskauer and
Simmons’ citrate tests were performed as described by
Oren et al. (1997). The production of acid from different
carbohydrates was tested in a medium with 0.5 % (w/v)
yeast extract and supplemented with 1 % (w/v) of the
carbohydrate tested (Oren et al., 1997). Reduction of
nitrate was detected by using sulfanilic acid and
x-naphthylamine reagents (Smibert & Krieg, 1981). H2S

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formation was determined by monitoring the production of a black sulfide precipitate in solid M1 medium containing 0.5 % (w/v) sodium thiosulfate. To determine the utilization of different organic substrates such as carbohydrates, alcohols, amino acids and organic acids as carbon, nitrogen and energy sources, a medium containing 0.05 % (w/v) yeast extract and supplemented with 1 % (w/v) of the tested substrate (sterilized separately) was used as described by Ventosa et al. (1982). All phenotypic tests were carried out by growing the strains in the routine medium prepared at 20 % (w/v) total salts, pH 7.0 and at 37 °C.

Antimicrobial compound sensitivity tests were performed by spreading the culture suspension on M1 20 % solid medium plates and applying discs impregnated with antimicrobial compounds (Difco and Becton Dickinson, except discs containing anisomycin, which were prepared in our laboratory) following the Bauer–Kirby technique (Bauer et al., 1966) as described by Ventosa et al. (1982). The results were scored according to the manufacturers' instructions. Strain C49 T was sensitive to anisomycin (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).

Strain C49 T was capable of growing over a wide range of NaCl concentrations, from 15 to 30 % (w/v). Mg 2+ was not required for growth. Strain C49 T was catalase- and oxidase-positive. Phenotypic characteristics, range and optimum temperature, pH and NaCl concentrations for growth, hydrolysis of different compounds and utilization of several substrates are detailed in the species description. There were no discrepancies in phenotypic tests determined in our laboratory and those reported for closely related species. Differential features of strain C49 T from the type strains of the phylogenetically most closely related species of the genus Halorubrum. The default settings for both BLAST and NUCmer (NUCLEotide MUMmer) algorithms were applied for genome comparisons. ANI analysis showed that strain C49 T had low relatedness to Hrr. saccharovorum DSM 1137 T (91.1 %), Hrr. lipolyticum DSM 21995 T (91.0 %), Hrr. halophilum B8 T (91.0 %), Hrr. kocurii JCM 14978 T (90.7 %), Hrr. lacusprofundi ATCC 49239 T (90.3 %), Hrr. arcis JCM 13916 T (88.3 %) and Hrr. aidingense JCM 13560 T (88.3 %). The ANI values of strain C49 T with type strains of related species were lower than the 95–96 % cut-off limit for species delineation (Goris et al., 2007), showing that strain C49 T does not belong to a previously described species. Therefore, the DDH and ANI data are in agreement, endorsing the conclusion that this strain is genotypically distinct and should be assigned to a different species.

Cell culture of strain C49 T for chemotaxonomic analyses was obtained after 10 days of aerobic incubation in M1 liquid medium under optimal conditions (20 % NaCl, 37 °C and pH 7.0). The strains used for comparisons were cultured according to the original descriptions for each species and standardized 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 previously by Corelli & Lobasso (2006). The extracts were dried carefully using a SpeedVac Thermo Savan SPD1111V before weighing and then dissolved in chloroform to obtain a concentration of 10 mg lipid ml⁻¹. Total lipid extracts were analysed by high-performance TLC using HPTLC silica gel 60 plates crystal back (10 × 20 cm; Merck art. 5626); the plates were eluted in the solvent system chloroform/methanol/90 % 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 brief heating at 160 °C (Kates, 1986). Glycolipids appear as purple spots, and the remaining polar lipids as brown spots after prolonged heating; alternatively, plates were treated by spraying with a solution of primuline and detecting the lipids upon excitation by UV light (336 nm) (Fuchs et al., 2007). Furthermore, the following stains were used in order to identify the chemical nature of the lipids present in the TLC bands: molybdenum blue spray reagent (Sigma) for phospholipids (Kates, 1986), azure A/sulfuric acid for sulfatides and sulfoglycolipids (Kean, 1968) and ninhydrin in aceton/ lutidine (9 : 1) for free amino groups.
Table 1. Differential features that distinguish strain C49T from type strains of other closely related species of the genus Halorubrum

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*Data taken from: a, McGinity & Grant (1995); b, Cui et al. (2006); c, Yim et al. (2014); d, Gutierrez et al. (2008); e, Xu et al. (2007).

The thin-layer chromatogram of the polar lipids (Fig. S2) reveals that strain C49T possessed as major lipids phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester derived from both C20C20 and C20C25 archaeol, phosphatidylglycerol sulfate and one glycolipid chromatographically identical to sulfated mannosyl glucosyl diether. Diphosphatidylglycerol was identified as a minor phospholipid component, revealed above phosphatidylglycerol phosphate methyl ester. While the lipid profile of strain C49T possesses all the major lipids described for neutrophilic species of the genus Halorubrum (McGenity & Grant, 1995), the existence of a minor component not observed in the closely related strain Hrr. saccharovorum JCM 8865T (Fig. S2) provides further evidence of this strain’s status as a member of a unique species.

A polyphasic approach including phylogenetic analyses using 16S rRNA and MLSA gene sequence comparisons, polar lipid profiles, DDH and ANI values and detailed phenotypic characterization indicates clearly that strain C49T belongs to the genus Halorubrum and represents a novel species, for which the name Halorubrum persicum sp. nov. is proposed.

**Description of Halorubrum persicum sp. nov.**

*Halorubrum persicum* (per’si.cum. L. neut. adj. persicum of Persia).

Cells are long, motile, pleomorphic rods, 5.0–10 × 1.0–1.2 μm, and appear 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. Colonies on solid medium after incubation at 37 °C for 10 days are circular with regular edges, smooth, convex and 1–2 mm in diameter. During the first 5 days, colonies appear pink, and the colour gradually intensifies upon incubation for the next few days. Hypotonic treatment with less than 12 % NaCl provokes cell lysis. Gas vesicles inside the cells are not observed. Neutrophilic, growing at pH 7.0–8.0 (optimum pH 7.0–7.2), requires 15–30 % (w/v) NaCl for growth (optimum 20 %, w/v) and grows at 20–45 °C (optimum 37 °C) under aerobic conditions. Mg²⁺ is not required for growth. Chemo-organotrophic and strictly aerobic. Oxidase and catalase activities are positive. 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. Citrate is not utilized. Tween 80, starch, DNA, gelatin and casein are not hydrolysed. Tests for urease and phosphatase activities are negative. The following substrates are utilized for growth as sole sources of carbon and energy: D-glucose, maltose, sucrose, D-mannose, raffinose, trehalose, glycerol, sorbitol, D-mannitol, acetate, glutamate, lactate, malate, pyruvate, succinate and propionate. D-Galactose, D-fructose, lactose, sorbose, l-arabinose and rhamnose are not used as sole sources of carbon and energy. The following compounds are used as sole sources of carbon, nitrogen and energy: l-serine, threonine, glycine, asparagine and l-lysine. Isoleucine is not used as sole source of carbon, nitrogen and energy. The polar lipid pattern consists of phosphatidyglycerol, phosphatidylglycerol phosphate methyl ester derived from both C20C20 and C20C25 archaeol, phosphatidylglycerol sulfate and sulfated mannosyl glycosyl diether as major lipids. Diphosphatidylglycerol and a minor phospholipidic component are weakly present.

The type strain is C49T (=IBRC-M 10232T = JCM 30541T), isolated from sediment of the hypersaline lake Aran-Bidgol in Iran. The DNA G+C content of the type strain is 64.2 mol% (Tm).

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