Halopenitus persicus gen. nov., sp. nov., an archaeon from an inland salt lake

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A novel pale pink-pigmented halophilic archaeon, strain DC30^T, was isolated from Aran-Bidgol salt lake, a hypersaline playa in Iran. Cells of strain DC30^T were non-motile and pleomorphic, from rods to triangular or disc-shaped. Strain DC30^T required at least 1.7 M NaCl and 0.05 M MgCl₂ for growth (optimum, 3 M NaCl and 0.1 M MgCl₂). The optimum pH and temperature for growth of strain DC30^T were pH 7.5 and 40 °C, respectively, although it was capable of growth over pH and temperature ranges of 6.5–8.5 and 25–50 °C, respectively. Analysis of the 16S rRNA gene sequence showed that strain DC30^T was a member of the family *Halobacteriaceae*. However, it had low 16S rRNA gene sequence similarities of 92.4 %, 89.4 % and 89.1 % to the most closely related haloarchaeal taxa, the type species of the genera *Halorubrum*, *Halogramum* and *Haloplanus*, respectively. The DNA G + C content was 66.0 mol%. Phosphatidylglycerol and phosphatidylglycerol phosphate methyl ester, common phospholipids found in haloarchaea, were present. Three minor phospholipids and one unidentified glycolipid were also observed. The only quinone present was MK-8(II-H₂). The physiological, biochemical and phylogenetic differences between strain DC30^T and other previously described genera of extremely halophilic archaea suggest that strain DC30^T represents a novel species in a new genus within the family *Halobacteriaceae*, for which the name *Halopenitus persicus* gen. nov., sp. nov. is proposed. The type strain of *Halopenitus persicus* is DC30^T (=IBRC 10041^T=KCTC 4046^T).

The red–pink colour is a common feature of hypersaline aquatic environments and is due to the very high cell densities ($\geq 10^7$ cells ml⁻¹) of extremely halophilic microorganisms of the family *Halobacteriaceae* that impart a red colour (Oren, 2002). Other red-pigmented bacteria, such as species of the genus *Salinibacter*, may also be present, but are usually less abundant than haloarchaea (Antón *et al.*, 2008). The family *Halobacteriaceae* (order *Halobacteriales*, class *Halobacteria*, domain *Archaea*) includes aerobic microorganisms which need at least 1.5 M NaCl for growth (Grant *et al.*, 2001). Except for some members of the genus

Natrialba, which are non-pigmented, all members of the family *Halobacteriaceae* are pink to red-pigmented due to the presence of carotenoids (Kamekura & Dyal-Smith, 1995; Hezayen *et al.*, 2001; Grant *et al.*, 2001). In this study, we describe a pale pink-pigmented, extremely halophilic archaeon isolated from Lake Aran-Bidgol, an inland hypersaline playa in Iran, and we propose that it represents a novel species in a new genus within the family *Halobacteriaceae*.

Strain DC30^T was isolated from a brine sample collected in November 2007 from the saline Lake Aran-Bidgol (34° 26' N, 51° 48' E). The water of the lake had a temperature of 38 °C at the time of sampling, a pH of 7.1 and a salinity of 31 %. This playa can be considered as thalassohaline, with NaCl as the major salt. For strain isolation, modified growth medium (MGM) was used with 23 % total salt concentration, as described in the Halohandbook (Dyal-Smith, 2006). This medium contained a 23 % salt mixture, prepared from a 30 % stock solution, consisting of (l⁻¹): 240 g

Abbreviations: MGM, modified growth medium; PGS, phosphatidylglycerol sulfate.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain DC30^T is JF979130.

Three supplementary figures are available with the online version of this paper.

NaCl, 35 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 30 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 7 g KCl and 1 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, supplemented with 1 % (w/v) peptone (Merck) and 0.2 % (w/v) yeast extract (Merck); 1.5 % (w/v) agar was used for solidified media if necessary. The pH of the medium was adjusted to 7.2–7.4 with 2 M Tris-base (Merck). Brine samples were cultured in 23 % MGM solid medium after preparing the appropriate dilutions at the laboratory. Inoculated plates were incubated at 40 °C for up to two months. After successive cultivation, a pure isolate designated strain DC30^T was obtained. The characterization of this strain was achieved by following the minimal standards recommended by Oren *et al.* (1997) for describing novel taxa of the order *Halobacteriales*. The type strains *Haloferax volcanii* DSM 3754^T and *Halorubrum saccharovorum* DSM 1137^T were used as reference strains in positive and negative testing.

The genomic DNA of strain DC30^T was extracted as described by Lam in section 4.2.2 of the *Halohandbook* version 7 (Dyall-Smith, 2006) and the 16S rRNA gene was amplified using the archaeal universal primers: 21F (DeLong, 1992) and 1492R (Lane *et al.*, 1985). PCR products were purified with the DNA purification kit (Roche) according to the manufacturer's instructions, and electrophoresed on a 1 % agarose gel to check quality. Ligation of the PCR products with the pGEM-T vector, transformation of *Escherichia coli* DH5 α , and selection of the transformants were carried out with a pGEM-T TA cloning kit (Promega) according to the manufacturer's instructions. Multiple clones were randomly selected and then sequenced by Macrogen (South Korea) to determine whether the strain possessed multiple distinct 16S rRNA gene sequences. Phylogenetic analysis was performed using the software package MEGA version 4 (Tamura *et al.*, 2004) after obtaining multiple alignments of data available from public databases using CLUSTAL_X (Thompson *et al.*, 1997). Clustering was performed using the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and minimum-evolution (Rzhetsky & Nei, 1992) methods. Bootstrap analysis was used to evaluate the tree topology of the neighbour-joining data by performing 1000 resamplings (Felsenstein, 1985).

Ten almost complete 16S rRNA gene sequences (1440 nt) of strain DC30^T were obtained. Sequence comparisons indicated that they had identical sequences. The 16S rRNA gene sequence analysis showed that strain DC30^T was a member of the family *Halobacteriaceae*, but there were low 16S rRNA gene sequence similarities to type strains of other members of this family. The closely related haloarchaeal taxa were: *Halorubrum luteum* CGSA15^T (93.1 % 16S rRNA gene sequence similarity), *Halogramum amylolyticum* TNN58^T (89.4 %) and *Haloplanus vescus* RO5-8^T (89.1 %); while the 16S rRNA gene sequence similarity with the type species of the genera *Halorubrum*, *Halogramum* and *Haloplanus* was 92.4 %, 88.7 % and 88.1 %, respectively. Phylogenetic analysis using the neighbour-joining algorithm revealed that strain DC30^T was in a separate clade to these genera (Fig. 1). The phylogenetic position was also confirmed by trees

generated using the minimum-evolution and maximum-parsimony algorithms (see Figs S1 and S2, available in IJSEM Online).

Cell morphology and motility were examined using a microscope (BX41; Olympus) equipped with phase-contrast optics. For photography, drops of exponentially growing liquid cultures were used directly without fixing. Colony morphology was observed on agar medium under optimal growth conditions after incubation at 40 °C for 14 days. The Gram reaction was determined by following the method outlined by Dussault (1955). Strain DC30^T was non-motile, pleomorphic, from rods to triangular or disc-shaped, and stained Gram-negative. Colonies on agar plates were small (approximately 1.0 mm diameter), convex, round, with an entire edge and pale pink-pigmented.

Physiological tests were conducted using liquid or solid (1.5 % agar) MGM as mentioned above, unless stated otherwise. Liquid cultures were incubated at 40 °C on a shaking incubator at 200 r.p.m. Growth rates were determined by monitoring the increase in OD₆₀₀. Growth temperature range was examined in liquid MGM at temperatures from 20 °C to 55 °C with 5 °C intervals. For growth experiments at different pH values a range of 5.0–9.0 was tested; the buffers MES (pH 5–6.5), HEPES (pH 7–8) and CHES (pH 8.5–9) were added at a concentration of 50 mM. The requirements for NaCl and MgCl_2 for growth were determined in media containing 0–5 M NaCl (in 0.5 M increments) or 0–1 M MgCl_2 (in 0.05 M increments), respectively.

Strain DC30^T grew over a temperature range of 25–50 °C (optimum, 40 °C) and a pH range of 6.5–8.5 (optimum, pH 7.5). Routine cultivation was conducted at 40 °C and pH 7.5. Strain DC30^T was capable of growing over a wide range of NaCl concentrations, from 1.7 M (10 %) to 4.3 M (25 %) [optimum, 3.0 M (17 %)]. At least 0.05 M MgCl_2 was required for growth (optimum, 0.1 M MgCl_2).

Acid production from carbohydrates was tested in unbuffered MGM and was determined by measuring the initial and final pH of the medium. The culture was considered positive for acid production if the pH decreased by at least 1 pH unit. To test for carbon source utilization, peptone was omitted from MGM and yeast extract concentration was reduced to 0.1 g l⁻¹ (Oren *et al.*, 1997). The ability of strain DC30^T to grow anaerobically in the presence of DMSO (5.0 g l⁻¹) and to ferment arginine (5.0 g l⁻¹) was tested in MGM prepared anaerobically in serum tubes according to the procedures described by Bryant (1972) and Balch & Wolfe (1976). Growth and gas formation with nitrate as the electron acceptor were tested in 10 ml stoppered tubes, completely filled with liquid growth medium to which NaNO_3 (5 g l⁻¹) had been added, and containing an inverted Durham tube (Oren *et al.*, 1997). Tween hydrolysis activity was detected as described by Gutiérrez & González (1972). Casein, gelatin and starch hydrolysis were determined as described by Oren *et al.* (1997). Tests for catalase and

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship between strain DC30^T and its close relatives within the family *Halobacteriaceae*. GenBank accession numbers are given in parentheses. The sequence of the methanogenic archaeon *Methanospirillum hungatei* JF-1^T was used as an outgroup. Bootstrap values (%) are based on 1000 replicates. Only values >70 % are shown. Bar, 0.01 substitutions per nucleotide position.

oxidase activities were performed as described by González *et al.* (1978). Production of H₂S was tested by growing strain DC30^T in liquid MGM supplemented with 0.5 % (w/v) Na₂S₂O₃ (Oren *et al.*, 1997). Tryptone water medium supplemented with 23 % total salts was used to test indole production (Smibert & Krieg, 1994). Susceptibility to antimicrobials was determined by the disc diffusion method after spreading the strain on solid MGM (Oren *et al.*, 1997).

Strain DC30^T was catalase- and oxidase-positive. It could hydrolyse DNA, Tweens 20 and 40, but not Tweens 60 and 80, casein, gelatin or starch. Strain DC30^T utilized D-fructose, D-galactose, D-glucose and maltose. Acid was produced from D-glucose and maltose, but not from D-galactose, sucrose, D-fructose, D-xylose, D-ribose, D-mannitol, D-mannose, trehalose, L-arabinose or lactose. The detailed physiological and biochemical characteristics of strain DC30^T are listed in Table 1 and in the genus and species descriptions.

The DNA G + C content of strain DC30^T was determined by the HPLC method (Mesbah *et al.*, 1989) and was 66.0 mol%, which is within the range described for the family *Halobacteriaceae* (Grant *et al.*, 2001). This value is similar to that reported for the genus *Haloplanus* but very different from those of the genera *Halorubrum* and *Halogranum* (Table 1).

Polar lipid composition and respiratory quinones were determined by the services of the DSMZ and Dr Brian Tindall (Braunschweig, Germany). Polar lipids were separated by 2D silica gel TLC. Methods, including the solvents in each direction and detection reagents, were described by Hezayen *et al.* (2001). Phosphatidylglycerol and phosphatidylglycerol phosphate methyl ester were present but phosphatidylglycerol sulfate (PGS) was not observed. One unidentified glycolipid and three minor phospholipids were also observed (Fig. S3). The absence of PGS in this novel archaeon differentiates this taxon from phylogenetically

Table 1. Differential characteristics between strain DC30^T and closely related genera within the order *Halobacteriales*

Taxa: 1, DC30^T; 2, *Halorubrum*; 3, *Halogramum*; 4, *Haloplanus*; 5, *Halobellus*; 6, *Halobacterium*. Data are from this study and the type species of the genus *Halorubrum* (*Halorubrum saccharovorum*; Grant *et al.*, 2001); *Halogramum* (*Halogramum rubrum*; Cui *et al.*, 2010); *Haloplanus* (*Haloplanus natans*; Bardavid *et al.*, 2007); *Halobellus* (*Halobellus clavatus*; Cui *et al.*, 2011) and *Halobacterium* (*Halobacterium salinarum*; Grant *et al.*, 2001). +, Positive; –, negative; ND, no data available.

Characteristic	1	2	3	4	5	6
Cell shape	Pleomorphic	Rods	Pleomorphic	Pleomorphic	Rods	Small rods
Cell size (µm)	0.6–1.5 × 4.9–8.7	0.6–1.2 × 2.5	1.0–2.0	2.5–8.0	0.5–1.0 × 1.0–6.0	0.5–1.0 × 1.0–6.0
Motility	–	+	+	–	+	+
Pigmentation	Pale pink	Orange–red	Red	Pink	Red	Red
Optimum NaCl (M)	3.0	2.5–4.5	3.9	3.0	2.6	3.4–4.3
Mg ²⁺ requirement	+	+	+	+	–	+
Lysis in distilled water	+	+	+	+	+	+
Optimum temperature (°C)	40	50	37	40	37	50
H ₂ S production	–	+	+	ND	–	+
Reduction of nitrate to nitrite	–	–	–	+	+	+
Production of acid from D-glucose	+	+	+	–	+	–
Hydrolysis of gelatin	–	–	+	–	–	+
Indole production	–	–	–	+	+	+
Presence of PGS	–	+	–	+	+	+
DNA G + C content (mol%)	66.0	71.2	55.7–57.6	66.2	61.5	67.1
16S rRNA gene similarity to DC30 ^T (%)	100	93.1	89.4	89.1	88.4	86.9

related members of the family *Halobacteriaceae*: *Halorubrum*, *Haloplanus* and *Halobellus*, which contain PGS (Grant *et al.*, 2001; Bardavid *et al.*, 2007; Cui *et al.*, 2011). Furthermore, the minor phospholipids associated with strain DC30^T were not detected in the genus *Halogramum*, another closely phylogenetically related taxon (Cui *et al.*, 2010). Respiratory lipoquinones were determined as described by Wainø *et al.* (2000). MK-8(II-H₂) was the only respiratory lipoquinone present in strain DC30^T.

In conclusion, the results of the morphological and physiological properties of the novel isolate, the low 16S rRNA gene sequence similarities with other genera of the family *Halobacteriaceae* and the distinctive components of the polar lipid profile suggest that strain DC30^T represents a novel species of a new genus in the family *Halobacteriaceae*, for which the name *Halopenitus persicus* gen. nov., sp. nov. is proposed.

Description of *Halopenitus* gen. nov.

Halopenitus (Ha.lo.pe'ni.tus. Gr. n. *hals halos* salt; L. masc. adj. *penitus* inner, interior; N.L. masc. n. *Halopenitus* intended to mean an archaeon isolated from an inland salt lake).

Cells are non-motile and pleomorphic, from rods to triangular or disc-shaped. Gram-staining-negative. Colonies are pale pink-pigmented. Strictly aerobic; oxygen is used as the terminal electron acceptor. Growth occurs at pH 6.5–8.5, at 25–50 °C and at 1.7–4.3 M (10–25 %) NaCl. At least 0.05 M MgCl₂ is required for growth. Optimal growth occurs at pH 7.5, 40 °C, 3.0 M (17 %) NaCl and 0.1 M

MgCl₂. Extremely halophilic. Polar lipids include phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, one unidentified glycolipid and three minor phospholipids. MK-8(II-H₂) is the only respiratory lipoquinone present. The DNA G + C content of the type strain of the only species in the genus is 66.0 mol% (HPLC method). Phylogenetically affiliated to the family *Halobacteriaceae*. The type species is *Halopenitus persicus*. Recommended three-letter abbreviation of the genus: *Hpt*.

Description of *Halopenitus persicus* sp. nov.

Halopenitus persicus (per'si.cus. L. masc. adj. *persicus* of Persia, where the type strain was isolated).

Exhibits the following properties in addition to those given in the genus description. Cell size of 0.6–1.5 × 4.9–8.7 µm when grown in liquid MGM under optimum conditions. Colonies are small (approximately 1.0 mm diameter). Cells lyse in water. Catalase- and oxidase-positive. Does not grow under anaerobic conditions with nitrate, arginine or DMSO. Nitrate reduction to nitrite, and gas formation from nitrate are not observed. Hydrolyses DNA, Tweens 20 and 40 but not Tweens 60 and 80, casein, gelatin or starch. Indole is not produced from tryptophan. Acid is produced from D-glucose and D-maltose, but not from L-arabinose, D-fructose, sucrose, D-mannitol, D-galactose, lactose or D-ribose. H₂S is not produced from thiosulfate. Does not produce arginine dihydrolase, lysine decarboxylase or ornithine decarboxylase. Methyl red and Voges–Proskauer tests are negative. Utilizes D-fructose, D-galactose, D-glucose, maltose, L-asparagine, L-glycine, L-proline, and L-valine as

sole sources of carbon and energy, but not L-arabinose, sucrose, D-mannitol, D-ribose, L-arginine, L-methionine, L-aspartic acid, L-cysteine, L-phenylalanine or L-tyrosine. Susceptible to the following antimicrobial compounds (μ g per disc unless otherwise stated): anisomycin (35), bacitracin (10 U), nitrofurantoin (300) and novobiocin (5). Resistant to amoxicillin (25), ampicillin (10), cephalotin (30), carbenicillin (100), cefoxitin (30), chloramphenicol (30), erythromycin (5), gentamicin (10), kanamycin (5), nalidixic acid (30), neomycin (30), penicillin G (10 U), polymyxin B (100 U), rifampicin (5), streptomycin (10), tetracycline (30) and tobramycin (10).

The type strain is DC30^T (=IBRC 10041^T=KCTC 4046^T), and was isolated from Aran-Bidgol salt lake, Iran. The DNA G+C content of the type strain is 66.0 mol% (HPLC).

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