Halonotius pteroides gen. nov., sp. nov., an extremely halophilic archaean recovered from a saltern crystallizer

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Strains 1.15.5T, 2.27.5, 5.24.4 and 6.14.5 were isolated from a solar saltern. They have flattened, rod-shaped cells and are aerobic, extremely halophilic members of the domain Archaea and family Halobacteriaceae. Cells stained Gram-negative and grew optimally in media around neutral pH and containing 20–24 % (w/v) (strains 1.15.5T and 2.27.5) or 22–24 % (w/v) (5.24.4 and 6.14.5) salts. Mg2+ was not required. The DNA G+C contents of these isolates were all close to 58 mol%, and DNA–DNA cross-hybridization showed a mean relatedness of 77 %. Their 16S rRNA gene sequences differed by no more than 1.6 % from each other. Phylogenetic tree reconstructions with other recognized members of the Halobacteriaceae indicated that they formed a distinct clade, with the closest relative being Halorubrum saccharovorum (86.6–87.6 % 16S rRNA gene sequence similarity to the type strain). The only major polar lipid of all four isolates was the sulfated diglycosyl diether lipid S-DGD-1. By phase-contrast microscopy, the long, flattened cells of these strains often displayed a ’wing-like’ shape. The phenotypic and phylogenetic data support the placement of these isolates into a novel species in a new genus within the Halobacteriaceae, for which we propose the name Halonotius pteroides gen. nov., sp. nov. The type strain of Halonotius pteroides is 1.15.5T (=JCM 14355T =CECT 7525T =DSM 18729T), with the additional reference strains 2.27.5 (=JCM 14356 =DSM 18671), 5.24.4 (=JCM 14357 =DSM 18673) and 6.14.5 (=JCM 14358 =DSM 18692).

In a previous study, we described the cultivation and diversity of haloarchaea in an Australian saltern crystallizer (Burns et al., 2004). One of the dominant groups, representing 16 % of the population, formed a phylogenetically novel clade, and appeared to be specifically related to sequences previously recovered from Deep Lake (Bowman et al., 2000), a hypersaline lake in Antarctica. We referred to this clade, which consisted of 16S rRNA gene sequences from isolates and cloned genes, as the ‘Antarctic Deep Lake group’ (Burns et al., 2004). The characteristics of four isolates from that group, 1.15.5T, 2.27.5, 5.24.4 and 6.14.5, are presented here.

The minimal standards for the description of new taxa within the order Halobacteriales were followed (Oren et al., 1997), using previously described methods (Burns et al., 2007; Gutierrez et al., 2002; Torreblanca et al., 1986). For growth/activity tests, cells were inoculated into characterization medium CM (Burns et al., 2007), with or without the addition of a specific nutrient. Reference strains included Haloferax volcanii NCIMB 12012T, Halogeometricum borinquense JCM 10706T, Haloterrigena turkmenica JCM 9101T, Halomicrobium mukhoataei JCM 9738T and Halobiforma haloterrestris JCM 11627T. All cultures were incubated unshaken at 37 °C, unless stated otherwise.
Colonies took 4–8 weeks to grow on solid media, optimally on DBCM2 medium, with pyruvate as a carbon/energy source (Burns & Dyall Smith, 2006), and with agar (1.5%, w/v; Difco Bacto) or agarose as the gelling agent. After 8 weeks, colonies were small (0.5–1.0 mm diameter), convex, round, with an entire edge and an intense red colour. Liquid cultures were also red or pink in colour, depending upon the cell density. Under optimal growth conditions, cells displayed a range of flattened, angular or rounded shapes, but many were flat rods, often with rounded ends, resembling the wings of small insects (Fig. 1).

All strains were Gram-negative. By phase-contrast light microscopy, about 1–5% of cells exhibited weak motility, and transmission electron microscopy revealed that the cells possessed polar flagella. Growth was tested using a wide range of substrates (see species description) but, under the characterization conditions used, cells were able to grow only on glucose, glycerol or pyruvate. Cells were oxidase- and catalase-negative using conventional testing. All strains were unable to use nitrate (with or without L-arginine) or DMSO as alternative electron acceptors under anaerobic conditions.

The optimum temperature for growth was 37–40 °C, depending on the strain, and no growth was observed at 4 or 55 °C. Growth occurred over a wide range of NaCl concentration, from a minimum of 16% up to saturation. The optimum salinity was 20–24% (w/v) for strains 1.15.5T and 2.27.5 and 22% (w/v) for strains 5.24.4 and 6.14.5. All strains had no minimal magnesium ion requirement, but poor growth was observed in the absence of Mg2+ ions, and low to no growth occurred above 1.25 M Mg2+. Optimum growth varied with respect to both Mg2+-associated anion and isolate. Strain 1.15.5T had equal optima of 0.4 M for MgCl2 and MgSO4, while strains 2.27.5 and 5.24.4 shared a MgSO4 optimum of 0.4 M but had respective MgCl2 optima of 0.6 and 0.2–0.4 M. Isolate 6.14.5 had the broadest optima of all strains, demonstrating a MgSO4 optimum of 0.2–0.6 M and a MgCl2 optimum of 0.4–0.8 M.

All isolates had similar responses to pH, exhibiting growth over the range pH 5.5–8.5. Optimum growth was at pH 7.0–7.5 for isolates 1.15.5T and 2.27.5 and pH 7.0 for 5.24.4 and 6.14.5. The antibiotic sensitivities of the isolates were typical of halobacteria (see species description).

The polar lipids of the four isolates were identical by TLC (Fig. 2, lanes 1–4). In addition to phosphatidylglycerol and phosphatidylglycerophosphate methyl ester, the isolates possessed a glycolipid with the same mobility as the sulfated diglycosyl diether lipid S-DGD-1 of *Halobium mukohataei* JCM 9738T (Oren et al., 2002).

The DNA G+C contents of the strains, determined by the HPLC method of Tamaoka (1994), were 58.4 mol% (1.15.5T and 5.24.4) and 58.7 mol% (2.27.5 and 6.14.5). 16S rRNA gene sequences (Burns et al., 2004) were identical for strains

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**Fig. 1.** Phase-contrast image of cells of isolate 1.15.5T grown under optimum conditions. Cells display some pleomorphism, with flattened rods and other flattened shapes. Many of these have shapes that resemble the wings of insects. The other *Halonotius* isolates (2.27.5, 5.24.4 and 6.14.5) exhibited similar morphologies (not shown). Bar, 10 μm.

**Fig. 2.** TLC of polar lipids extracted using methods described by Kamekura (1993). Lanes: 1, strain 1.15.5T; 2, strain 2.27.5; 3, strain 5.24.4; 4, strain 6.14.5; 5, *Halobium mukohataei* JCM 9738T; 6, *Halobiforma haloterrestris* JCM 11627T; 7, *Halogeometricum borinquense* JCM 10706T. PG, Phosphatidylglycerol; PGP-Me, phosphatidylglycerophosphate methyl ester; S-DGD-1, sulfated diglycosyl diether lipid. Glycolipids were detected as purple spots, which are circled.
Between these strains, with isolate 1.15.5T showing 72–87 % sequence similarity, Haloferax volcanii (86.3–86.8 %) and Halogeometricum borinquense (85.5–87 %). DNA–DNA cross-hybridization, using the method of Ezaki et al. (1989), confirmed the close relationship between these strains, with isolate 1.15.5T showing 72–93 % relatedness to the other three strains. Together, these data indicate that the four isolates belong to the same species. Phylogenetic tree reconstructions using 16S rRNA gene sequences placed these isolates as a separate clade within the family Halobacteriaceae (Fig. 3).

The phenotypic characterization and phylogenetic data support the placement of these isolates in a novel species and a new genus within the Halobacteriaceae, for which we propose the name Halonotius pteroides gen. nov., sp. nov. Table 1 summarizes the distinguishing characteristics of the proposed genus from the most closely related genera.

**Description of Halonotius gen. nov.**

Halonotius (Ha.lo.no’ti.us. Gr. masc. n. hals, halos salt; L. masc. adj. notius southern; N.L. masc. n. Halonotius a salty southern one).

**Fig. 3.** Phylogenetic tree reconstruction showing relative placement of the novel strains. The tree is based on complete or nearly complete (>1300 nt) 16S rRNA gene sequences from organisms with validly published names. The tree shown was derived by maximum-likelihood, using the ARB package (Ludwig et al., 2004). Distance matrix and parsimony methods gave similar topologies (not shown). Bar, 0.10 expected nucleotide substitutions per site. Bootstrap values (using distance matrix methods) were derived from 1000 replicates and significant nodes (>75 %) are indicated by filled circles at branch points. The node indicated by an open circle was less well supported (<50 % but >75 %). The outgroup sequences (not shown) consisted of sequences representing most of the other genera of the Halobacteriaceae as well as that of Methanoseta concilii strain Opifikon (GenBank accession no. X16932.1).

Halonotius pteroides sp. nov.

Halonotius pteroides [p.te.ro.i’des. Gr. n. pteron wing; Gr. adj. (pte.ro.i’des) like; N.L. masc. adj. pteroides wing-like, named after the shape of many of the cells, which are flattened rods with rounded ends that appear similar to the wings of small insects].

Displays the following properties in addition to those given for the genus. Cells are flattened and display some variability in morphology, but many are flat rods, 0.7–1.5 μm wide and 2–6 μm long (Fig. 1). The ends of the rods are often rounded. Weakly motile with polar flagella. Colonies on agar medium are red with entire edges. Strictly aerobic; only oxygen is used as the final electron acceptor. Cannot utilize nitrate or DMSO as alternative electron acceptors. Growth occurs at pH 5.5–8.5, 25–45 °C and 16–36 % (w/v) NaCl. Cells lyse immediately in distilled water. Optimal growth occurs under neutrophilic conditions, above 18 % (w/v) salinity. Capable of growing in defined media, but very restricted in the substrates utilized. Grows best on pyruvate, but also capable of utilizing glucose or glycerol as sole carbon sources. Does not grow with any of the following substrates as the sole carbon and energy source (all at 10 mM): acetate, alanine, arabinose, arginine, aspartate, benzoate, betaine, butanol, butyrate, cellobiose, citrate, ethanol, formate, fructose, fumarate, galactose, galacturonate, glucononurate, glycine, glycolate, lactate, lactose, leucine, lysine, malate, malonate, mannitol, mannosone, methanol, propanol, propionate, ribose, serine, succinate, succrose, tartrate, threonine, urea, valine and xylose. Does not grow on cellulose, chitin or starch (each at 1.0 %, w/v). Acid is not produced from carbohydrate utilization. Negative for β-galactosidase activity and indole production. Sensitive to anisomycin, novobiocin, rifampicin and simvastatin, and resistant to ampicillin, bacitracin, chloramphenicol, cycloheximide, erythromycin, kanamycin, mycostatin, neomycin, streptomycin and tetracycline at 50 μg ml⁻¹. All four known strains were isolated from solar saltern crystallizer ponds.

The type strain is 1.15.5T (=JCM 14355T =CECT 7525T =DSM 18729T), isolated from Cheetham Salt Works, Geelong, Australia, with additional reference strains 2.27.5 (=JCM 14356 =DSM 18671), 5.24.4 (=JCM 14357 =DSM 18673) and 6.14.5 (=JCM 14358 =DSM 18692).

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Table 1. Phenotypic and other characteristics that distinguish strain 1.15.5T from closely related genera

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Strain 1.15.5T</th>
<th>Halorubrum</th>
<th>Halobaculum</th>
<th>Haloferax</th>
<th>Haloquadratum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphology</td>
<td>Flat rods/pleomorphic</td>
<td>Rods/pleomorphic rods</td>
<td>Rods</td>
<td>Pleomorphic, flat/rods</td>
<td>Flat squares</td>
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<tr>
<td>Cell size (μm)</td>
<td>0.7–1.5 × 2–6</td>
<td>0.12–1 × 0.5–7</td>
<td>0.5–1 × 5–10</td>
<td>0.4–3 × 0.5–3</td>
<td>2 × 2</td>
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<td>Motility</td>
<td>+</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
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<tr>
<td>NaCl optimum (M)</td>
<td>3.4–4.1</td>
<td>1.7–4.5/ND</td>
<td>1.5–2.5</td>
<td>1.7–5.2</td>
<td>3</td>
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<tr>
<td>NaCl range (M)</td>
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<td>1–5.2</td>
<td>2.4–6</td>
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<td>pH optimum</td>
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<td>6.5–7</td>
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<td>Anaerobic growth on nitrate</td>
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<td>Acid from carbohydrates</td>
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<td>+</td>
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<td>±</td>
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<tr>
<td>Pigmentation</td>
<td>Red</td>
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<td>Red/pink</td>
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<td>Major glycolipid</td>
<td>S-DGD-1</td>
<td>S-DGD-3</td>
<td>S-DGD-1</td>
<td>S-DGD-1</td>
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<tr>
<td>DNA G + C content (mol%)</td>
<td>58</td>
<td>62.7–72.1</td>
<td>70</td>
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References


