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

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Strains 1.15.5^T, 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 1.15.5^T 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 saccharovororum* (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 1.15.5^T (=JCM 14355^T =CECT 7525^T =DSM 18729^T), 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, 1.15.5^T, 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; Torrealba *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 2012^T, *Halogeometricum borinquense* JCM 10706^T, *Haloterrigena turkmenica* JCM 9101^T, *Halomicrobium mukohataei* JCM 9738^T and *Halobiforma haloterrestris* JCM 11627^T. All cultures were incubated unshaken at 37 °C, unless stated otherwise.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of isolates 1.15.5^T, 2.27.5, 5.24.4 and 6.14.5 are AY498641, AY498646, EF090605 and AY498648, respectively.

Colonies took 4–8 weeks to grow on solid media, optimally on DBCM2 medium, with pyruvate as a carbon/energy source (Burns & Dyal 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 1.15.5^T 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 1.15.5^T 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 1.15.5^T 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 *Halomicrobium mukohataei* JCM 9738^T (Oren *et al.*, 2002).

The DNA G + C contents of the strains, determined by the HPLC method of Tamaoka (1994), were 58.4 mol% (1.15.5^T 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

Fig. 1. Phase-contrast image of cells of isolate 1.15.5^T 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 1.15.5^T; 2, strain 2.27.5; 3, strain 5.24.4; 4, strain 6.14.5; 5, *Halomicrobium mukohataei* JCM 9738^T; 6, *Halobiforma haloterrestris* JCM 11627^T; 7, *Halogeometricum borinquense* JCM 10706^T. PG, Phosphatidylglycerol; PGP-Me, phosphatidylglycerophosphate methyl ester; S-DGD-1, sulfated diglycosyl diether lipid. Glycolipids were detected as purple spots, which are circled.

2.27.5 and 5.24.4, while the sequences of the other two strains differed from each other by less than 1.7%. Among recognized members of the *Halobacteriaceae*, the closest relatives were the type strains of *Halorubrum saccharovorum* (86.6–87.6% 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 1.15.5^T 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 *Methanosaeta concilii* strain Opfikon (GenBank accession no. X16932.1).

Gram-negative, flat rods, often with rounded ends. Aerobic heterotrophs. Oxidase and catalase tests are negative. The genus belongs phylogenetically to the family *Halobacteriaceae*. Known habitats are salt lakes and saltern crystallizer ponds. The type species is *Halonotius pteroides*. Following the published guidelines (Oren & Ventosa, 2000), we propose the three-letter genus abbreviation *Hns*.

Description of *Halonotius pteroides* sp. nov.

Halonotius pteroides [pte.ro.i'des. Gr. n. *pteron* wing; Gr. adj. (*e*)*ides* 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, gluconuronate, glycine, glycolate, lactate, lactose, leucine, lysine, malate, malonate, mannitol, mannose, methanol, propanol, propionate, ribose, serine, succinate, sucrose, tartrate, threonine, urea, valine and xylose. Does not grow on cellulose, chitin or starch (each at 0.1%, 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 1.15.5^T (=JCM 14355^T =CECT 7525^T =DSM 18729^T), 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 1.15.5^T from closely related genera

Data for related genera were taken from Oren (2006) and Cui *et al.* (2007). +, Positive; −, negative; ±, variable; ND, no data available.

Characteristic	Strain 1.15.5 ^T	<i>Halorubrum</i>	<i>Halobaculum</i>	<i>Haloferax</i>	<i>Haloquadratum</i>
Morphology	Flat rods/pleomorphic	Rods/pleomorphic rods	Rods	Pleomorphic, flat/rods	Flat squares
Cell size (µm)	0.7–1.5 × 2–6	0.12–1 × 0.5–7	0.5–1 × 5–10	0.4–3 × 0.5–3	2 × 2
Motility	+	±	±	±	−
NaCl optimum (M)	3.4–4.1	1.7–4.5/ND	1.5–2.5	1.7–5.2	3
NaCl range (M)	2.7–6	0.5–4.5	1–2.5	1–5.2	2.4–6
pH optimum	Neutral	Neutral–9.5	6–7	5.5–10	6.5–7
Temperature optimum (°C)	45	31–50	40	32–53	45
Nitrite from nitrate	−	+	+	+	−
Anaerobic growth on nitrate	−	−	−	+ / − / ND	−
Acid from carbohydrates	−	+ / ND	+	±	−
Starch hydrolysis	−	±	+	±	−
Indole production	−	±	−	±	−
Pigmentation	Red	Red/purple	Red	Red/pink	Red
Major glycolipid	S-DGD-1	S-DGD-3	S-DGD-1	S-DGD-1	S-DGD-1
DNA G + C content (mol%)	58	62.7–72.1	70	60–64	47

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