Natronomonas moolapensis sp. nov., non-alkaliphilic isolates recovered from a solar saltern crystallizer pond, and emended description of the genus Natronomonas

David G. Burns,1 Peter H. Janssen,2 Takashi Itoh,3 Hiroaki Minegishi,4 Ron Usami,4 Masahiro Kamekura5 and Mike L. Dyall-Smith6

Correspondence
Mike L. Dyall-Smith
mike.dyallsmith@gmail.com

1Microbiological Diagnostic Unit, University of Melbourne, Parkville 3052, Australia
2Grasslands Research Centre, Tennent Drive, Private Bag 11008, Palmerston North 4442, New Zealand
3Japan Collection of Microorganisms, RIKEN BioResource Center, Saitama 351-0198, Japan
4Bio-Nano Electronics Research Centre, Toyo University, Kawagoe 350-8585, Japan
5Halophiles Research Institute, 677-1 Shimizu, Noda-shi, Chiba 278-0043, Japan
6Max Planck Institute of Biochemistry, Department of Membrane Biochemistry, Am Klopferspitz 18, D-82152 Martinsried, Germany

Two isolates of non-alkaliphilic, extremely halophilic archaea, with very similar characteristics, were recovered from a marine solar saltern crystallizer. Cells were pleomorphic, motile and Gram-stain-negative and grew on a limited range of carbon sources, with pyruvate being the best substrate. Optimum growth occurred at 18–20% (w/v) NaCl, pH 6.0–8.5 and 37–40°C. Both isolates possessed typical archaeal lipids, and their 16S rRNA gene sequences were 99.8% identical. Phylogenetic tree reconstructions indicated that they were most closely related to the haloalkaliphile Natronomonas pharaonis (97.5% similarity to the type strain), but the different phenotypic properties and low DNA–DNA hybridization values between Nmn. pharaonis DSM 2160T and the two isolates suggested that they represent a novel species within the genus Natronomonas. The name Natronomonas moolapensis sp. nov. is proposed for these isolates, with the type strain being 8.8.11T (JCM 14361T = CECT 7526T = DSM 18674T). An emended description of the genus Natronomonas is also provided.

Strains 8.8.11T and 4.03.5 were isolated from a crystallizer pond at the Cheetham marine solar saltern at Moolap, Victoria, Australia (38°09.841′S 144°25.274′E). Details of the site, water chemistry and methods of isolation have been described previously (Burns et al., 2004a, b). Strain 4.03.5 was isolated on solid medium (Burns et al., 2004a), while strain 8.8.11T was isolated using extinction culturing (Burns et al., 2004b). On solid media (23% MGM or 25% DBCM), the isolates took 2–4 weeks to form small colonies (0.5–1.0 mm diameter) that were convex and round with an entire edge and pink or red in colour. Liquid cultures were also pink to red in colour, depending upon the cell density, but old cultures (>4 weeks) gradually faded to off-white. Under optimal growth conditions, cells exhibited a range of morphologies, including short rods, flat tetragonal shapes and cocci, with dimensions ranging from 0.7 to 1.7 μm (Fig. 1). Both strains were motile and Gram-stain-negative. Gas vesicles were observed in a small proportion of cells of strain 8.8.11T. The isolates were characterized following the recommended minimum standards (Oren et al., 1997).

Under the characterization conditions, cells were able to grow only on acetate, butanol, butyrate, ethanol, glycerol, lactate, propanol, propionate and pyruvate, with pyruvate providing the best growth. Cells were oxidase- and catalase-negative, even when these tests were incubated for up to 10 min, while Haloferax volcanii NCMB 2012T was positive within this time. They did not hydrolyse starch or casein, and did not show β-galactosidase activity. Indole was not produced in tryptophan-containing media. Strain 8.8.11T was unable to grow under anaerobic conditions using either nitrate (with or without L-arginine) or DMSO as alternative electron acceptors, but 4.03.5 reduced nitrate.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of isolates 8.8.11T and 4.03.5 are AY498645 and EF127644, respectively.
to nitrite. The optimum growth temperature for strain 4.03.5 was 37–40 °C, compared with 45 °C for strain 8.8.11T. The minimum growth temperature in the tested range was 25 °C; no growth was observed at 4 or 55 °C.

Growth occurred over a wide range of NaCl concentrations, from a minimum of 12–14 % (isolate 8.8.11T) and 14 % (isolate 4.03.5) (w/v) up to saturation (both). The optimum NaCl concentration was 18–20 % (w/v) for both strains. Neither strain had a minimal magnesium requirement, but poor growth was observed in the absence of Mg\(^{2+}\) ions and low to no growth was found above 1.25 M Mg\(^{2+}\). Isolate 4.03.5 had a MgCl\(_2\) optimum of 0.2 M and MgSO\(_4\) optimum of 0.4 M, while 8.8.11T had comparatively elevated preferences for 0.4 M MgCl\(_2\) and 0.4–0.6 M MgSO\(_4\). Their antibiotic sensitivities were similar to those of other haloarchaea (see species description). Both isolates grew well at pH 6–8.5, while the closest relative of these organisms (see below), *Natronomonas pharaonis*, grows optimally at pH 8.5 and not at all at pH 6.0 (Falb et al., 2005; Soliman & Trüper, 1982).

The G+C content of the total DNA was 63.8 mol% for strain 4.03.5 and 63.4 mol% for strain 8.8.11T. The 16S rRNA gene sequences of these isolates showed 99.8 % identity, and clustered tightly together within the family *Halobacteriaceae* in phylogenetic tree reconstructions (Fig. 2). The closest relative was *Nmn. pharaonis* DSM 2160\(^T\) (sequence identity 97.5 %), with which the isolate sequences formed a strongly supported clade, while the type strains of species of other genera were significantly more distant, such as *Haloarcula vallismortis* (87.4 %), *Halomicrobium mukohataei* (87.6 %), Hfx. volcanii (89.2 %), *Halorubrum saccharovorum* (88.5 %) and *Halogeometricum borinquense* (88 %). DNA–DNA hybridization between the DNAs of strains 4.03.5 and 8.8.11T was above 85 %, indicating they belonged to the same species while, between the DNA of either strain and that of the closest relative, *Nmn. pharaonis* DSM 2160\(^T\), the relatedness was 2–5 %.

The mobilities of the lipids of the two isolates were identical by TLC (Fig. 3). In addition to phosphatidylglycerol and phosphatidylglycerophosphatidylmethylester, the isolates possessed an unknown glycolipid with greater mobility than the sulfated triglycosyl diether (S-TGD) of *Halobacterium salinarum*. The isolates also possessed a lipid that co-chromatographed with the phosphatidylglycerol sulfate (PGS) spot seen in the profile of *Hbt. salinarum* (just below the spot of PGP-Me). In this chromatogram, phosphatidylglycerosulfate displayed a mobility close to that of the glycolipid S-DGD-1 of *Haloferax sulfurifontis*, but the two lipids are readily distinguished on the basis of carbohydrate staining, as indicated in Fig. 3.

The phylogenetic affiliation described above indicates the two isolates should be included in the genus *Fig. 1. Phase-contrast micrograph of cells of isolate 8.8.11\(^T\) grown in DBCM2 medium. Bar, 10 \(\mu\)m.*

*Fig. 2. Phylogenetic tree reconstruction based on complete or nearly complete 16S rRNA gene sequences (accession numbers in parentheses). The tree was derived by maximum-likelihood, using the ARB package on Mac OSX (Ludwig et al., 2004). Bar, 0.1 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 name Halonotius pteroides is proposed by Burns et al. (2010) in this issue. Outgroup sequences were from representatives of most other genera of the *Halobacteriaceae*, as well as that of *Methanoseta concilii* strain Opfikon (GenBank accession no. X16932.1) (not shown).*
Natronomonas. However, the two isolates differed from the only known species, \textit{Nmn. pharaonis}, by their distinct phenotypic properties, such as cell morphology, pH for growth, pattern of carbon sources utilized and lipid composition, and the low DNA–DNA hybridization (2–5\%). On the other hand, the two isolates had similar phenotypic properties and high DNA–DNA relatedness (85\%), indicating they should be classified within the same species. We propose a novel species, \textit{Natronomonas moolapensis} sp. nov., to accommodate the two isolates. We also propose that the genus description be emended.

\textbf{Emended description of the genus \textit{Natronomonas} Kamekura et al. 1997}

Gram-negative rods or pleomorphic shapes (including short rods, flats, tetragons and cocci). Colonies are pigmented red. Chemo-organotrophic and aerobic. Halophilic, requiring at least 2 M NaCl. Alkaliphilic or non-alkaliphilic. Alkaliphilic strains grow between pH 7 and 10, with optimum growth at pH 8.5, while non-alkaliphilic strains grow at pH 5.5–8.5, with optimum growth at pH 7–7.5. The DNA G+C content is 63–64 mol\%. Polar lipids are glycerol-diether analogues of phosphatidylglycerol, phosphatidylglycerophosphate methyl ester and phosphatidic acid. Unknown phospholipids or glycolipid may exist. Phytanyl-sterterpanyl moieties (C\textsubscript{20}C\textsubscript{25}) are present. The type species is \textit{Natronomonas pharaonis}.

\textbf{Description of \textit{Natronomonas moolapensis} sp. nov.}

\textit{Natronomonas moolapensis} (moo.la.pen’sis. N.L. fem. adj. \textit{moolapensis} from Moolap, the place where the saltern crystallizer is situated from which the first isolates were recovered).

Cells stain Gram-negative and have varying morphologies (short rods, flat tetragonal shapes or cocci) with dimensions ranging from 0.7 to 1.7 $\mu$m. Colonies on agar medium are red with entire edges. Aerobic. Motile. Cells are oxidase- and catalase-negative using conventional testing. Strain 4.03.5 can reduce nitrate to nitrite, but cannot utilize DMSO as an alternative electron acceptor, while strain 8.8.11\textsuperscript{T} cannot utilize either nitrate or DMSO as alternative electron acceptors. Growth occurs at pH 5.5–8.5, 25–45 °C and 14–36\% (w/v) NaCl. Cells lyse immediately in distilled water, and a minimum ~14\% salts is required for growth. Optimal growth occurs at around pH 7–7.5 and at 18–20\% NaCl. Capable of growing in defined media but can use relatively few substrates. Grows best on pyruvate, and can use acetate, butanol, butyrate, ethanol, glucose (strain 4.03.5 only), glycerol, lactate, propanol and propionate as sole carbon sources. Incapable of utilizing alanine, arabinose, arginine, aspartate, benzoate, betaine, cellobiose, citrate, formate, fructose, fumarate, galactose, galacturonate, glucuronate, glucose (strain 8.8.11\textsuperscript{T} only), glycine, glycolate, lactose, leucine, lysine, malate, malonate, mannitol, mannoside, methanol, ribose, serine, succinate, sucrose, tartrate, threonine, uracil, valine and xylose (all at 10 mM) and cellulose, chitin and starch (0.1% w/v) as sole carbon and energy sources. Acid is not produced from carbohydrate utilization. Negative for $\beta$-galactosidase activity and indole production. Gas vesicles in some cells. Sensitive to mycostatin, novobiocin, rifampicin and simvastatin and resistant to ampicillin, anisomycin, bacitracin, chloramphenicol, cycloheximide, erythromycin, kanamycin, neomycin, streptomycin and tetracycline at 50 \mu g ml$^{-1}$. Phylogenetically affiliated to the \textit{Halobacteriaceae}.

The type strain is 8.8.11\textsuperscript{T} (=JCM 14361\textsuperscript{T} =CECT 7526\textsuperscript{T} =DSM 18674\textsuperscript{T}). An additional reference strain is 4.03.5 (=JCM 14360 =DSM 18672). Both strains were isolated from Cheetham Salt Works, Geelong, Australia.

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{Fig_3.png}
\caption{TLC of polar lipids extracted from strains 8.8.11\textsuperscript{T} (lane 5) and 4.03.5 (replicates in lanes 2–4) in comparison to the lipids of \textit{Hbt. salinarum} JCM 8978\textsuperscript{T} (lane 1) and \textit{Hfx. sulfurifontis} JCM 12327\textsuperscript{T} (lane 6), using methods described previously (Kamekura, 1993). The origin is at the bottom. PG, Phosphatidylglycerol; PGP-Me, phosphatidylglycerophosphate methyl ester; PGS, phosphatidylglycerosulfate; S-DGD-1, sulfated diglycosyl diether lipid. Glycolipids were detected as purple spots, which are marked with asterisks.}
\end{figure}

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