Haloferax elongans sp. nov. and *Haloferax mucosum* sp. nov., isolated from microbial mats from Hamelin Pool, Shark Bay, Australia

Michelle A. Allen,[1] Falicia Goh,[1] Stefan Leuko,[2] Akinobu Echigo,[3] Toru Mizuki,[3] Ron Usami,[3] Masahiro Kamekura,[4] Brett A. Neilan[1,2] and Brendan P. Burns[1,2]

Correspondence
Brett A. Neilan
b.neilan@unsw.edu.au

[1]School of Biotechnology and Biomolecular Sciences, University of New South Wales, Sydney, NSW 2052, Australia

[2]Australian Center for Astrobiology, Macquarie University, Building E8C 153, Sydney, NSW 2109, Australia

[3]Department of Applied Chemistry, Faculty of Engineering, and Bio-Nano Electronics Research Center, Toyo University, 2100 Kujirai, Kawagoe, Saitama 350-8585, Japan

[4]Halophiles Research Institute, 677-1 Shimizu, Noda, Chiba 278-0043, Japan

Extremely halophilic archaea were cultivated from smooth and pustular microbial mats collected from Hamelin Pool, Shark Bay, Western Australia. On the basis of morphology, two phenotypes were present and 16S rRNA gene sequence analysis indicated that all strains were most closely related to members of the genus *Haloferax* (98.1–99.4 % similarity). One representative strain from each phenotype was selected for further taxonomic characterization. Strain SA5^T, isolated from the smooth mat, formed small (~1 mm diameter), red, translucent colonies on agar medium and strain PA12^T, isolated from the pustular mat, formed large (3–5 mm diameter), pink, mucoid, domed colonies. Both strains grew in media with 1.7–5.1 M NaCl, required at least 0.2 M Mg^{2+} for growth and had pH optima of 7.4. The 16S rRNA gene similarity between strains SA5^T and PA12^T was 97.1 %. Physiological properties, G+C content and polar lipid composition supported placement of both strains in the genus *Haloferax*. Phenotypic analysis indicated that the two strains were distinct from each other and from all other members of the genus. This was confirmed by the low DNA–DNA relatedness between strains SA5^T and PA12^T (18–30 %) and between both strains and all other recognized *Haloferax* species. Two novel species of the genus *Haloferax* are proposed to accommodate these novel isolates, *Haloferax elongans* sp. nov. (type strain SA5^T=JCM 14791^T=ATCC BAA-1513^T=UNSW 104100^T) and *Haloferax mucosum* sp. nov. (type strain PA12^T=JCM 14792^T=ATCC BAA-1512^T=UNSW 104200^T).

Hamelin Pool, Shark Bay, Australia, is a hypersaline embayment in which the mean salt concentration is roughly double that of seawater (0.852 M Na^+, 0.090 M Mg^{2+}, 0.018 M Ca^{2+}, 1.01 M Cl^-; Arp *et al.*, 2001), with morphologically diverse microbial mats and stromatolites (lithified mats) forming in the intertidal regions. These microbial communities are exposed to salt, desiccation and UV stress, suggesting that they may be habitats for novel biodiversity, and are important for our understanding of stromatolite formation processes. During our surveys of the microbial diversity of the microbial mats and stromatolites of Hamelin Pool (Burns *et al.*, 2004; Allen, 2006; Leuko *et al.*, 2007), 13 archaeal strains were isolated from the smooth and pustular mats of the pool. The smooth mat has an even, light brown/green-pigmented surface and displays defined laminations corresponding to distinct green (cyanobacterial), pink (unknown) and black (sulfate-reducing) layers, whereas the pustular mat has an irregular, dark purple/brown-pigmented surface comprised predominantly of cyanobacteria and with little observable lamination (Logan *et al.*, 1974; Allen, 2006). The isolates formed two clusters

Abbreviations: DGA-1, diglycosyl archaeol-1; PG, phosphatidylglycerol; PGP-Me, phosphatidylglycerophosphate methyl ester; PGS, phosphatidylglycerosulfate; S-DGA-1, sulfated diglycosyl archaeol-1.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *Haloferax elongans* sp. nov. SA5^T and *Haloferax mucosum* sp. nov. PA12^T are DQ860977 and DQ860980, respectively.

Phase-contrast micrographs of late-exponential-phase cells of SA5^T and PA12^T (Fig. S1), SDS-PAGE patterns of whole-cell proteins (Fig. S2) and TLC analysis of polar lipids (Fig. S3) of SA5^T and PA12^T and reference taxa are available with the online version of this paper.

(or groups) within the genus *Haloferax* on the basis of cell morphology and 16S rRNA gene sequence analysis.

Currently, there are nine recognized species of *Haloferax*: *Haloferax volcanii* (Mullakhanbhai & Larsen, 1975), *Haloferax mediterranei* (Rodriguez-Valera *et al.*, 1983), *Haloferax denitrificans* (Tomlinson *et al.*, 1986), *Haloferax gibbonsii* (Juez *et al.*, 1986), *Haloferax alexandrinus* (Asker & Ohta, 2002), *Haloferax lucentense* (Gutierrez *et al.*, 2002), *Haloferax sulfurifontis* (Elshahed *et al.*, 2004), *Haloferax prahovense* (Enache *et al.*, 2007) and *Haloferax larsenii* (Xu *et al.*, 2007). In this paper, taxonomic characterization of a representative isolate from each of the clusters described above is presented and two novel species are proposed.

A halophilic archaeal medium [containing ($g\,l^{-1}$): Casamino acids, 7.5; yeast extract, 10.0; trisodium citrate, 3.00; NaCl, 150; KCl, 2.00; $MgSO_4.7H_2O$, 20.0; $MgCl_2.6H_2O$, 7.23; $CaCl_2.2H_2O$, 2.70; $FeSO_4.7H_2O$, 0.05; $MnSO_4.H_2O$, 0.0002; adjusted to pH 7.4; Goh *et al.*, 2006] was inoculated with portions of smooth or pustular mat collected from Hamelin Pool in April 2004. Plates were incubated aerobically at 37 °C in the dark and colonies with pink or red morphology appeared after 2 weeks. The strains were purified by repeated streaking for single colonies and purity was confirmed by uniform cell and colony morphology and by 16S rRNA gene amplification and sequencing. DNA extraction and 16S rRNA gene amplification methods have been described previously (Allen, 2006).

On the basis of 16S rRNA gene sequences and colony and cell morphologies, the 13 isolates formed two groups. Seven strains from the smooth mat, designated strains SA1–SA7, and one strain from the pustular mat, designated strain PA15, formed small (1 mm), red, translucent, convex colonies on agar medium. This group of strains all formed pleomorphic rods (see supplementary Fig. S1 available with the online version of this paper) and analysis of their partial 16S rRNA gene sequences indicated they were most closely related to *Hfx. larsenii* (99.0–99.4 % similarity). Strain SA5^T was selected as a representative strain for further taxonomic characterization.

Five strains, designated PA12–14, PA16 and PA17, isolated from the pustular mat formed pink–red, entire, opaque, domed, mucoid colonies, 3–5 mm in diameter and exhibited pleomorphic cell morphology (see supplementary Fig. S1 available with the online version of this paper). Analysis of their partial 16S rRNA gene sequences indicated they were most closely related to *Hfx. mediterranei* (98.1–98.8 % similarity). Strain PA12^T was selected as a representative strain for further taxonomic characterization.

Characterization of strains SA5^T and PA12^T was carried out according to the proposed minimal standards for the description of novel taxa in the order *Halobacteriales* (Oren *et al.*, 1997). Morphology, nutritional requirements, growth characteristics, miscellaneous biochemical activities and sensitivity to antimicrobial agents were determined as outlined previously (Goh *et al.*, 2006; Allen, 2006). *Hfx. volcanii*, *Hfx. mediterranei* and *Hfx. lucentense* were used as controls for the biochemical tests. Generation times for SA5^T and PA12^T were determined by the method of Robinson *et al.* (2005) at 53 and 48 °C, respectively. The NaCl, pH and temperature ranges for growth, sensitivity to antibiotics and carbon source utilization results are presented in the species descriptions. Selected characteristics that distinguish strains SA5^T and PA12^T from other *Haloferax* species are shown in Table 1.

Of note, strain PA12^T was oxidase-negative and strain SA5^T exhibited an oxidase-variable response (Table 1). The first oxidase-negative halophilic archaeon to be identified (*Halococcus hamelinensis*) was isolated recently from Hamelin Pool stromatolites (Goh *et al.*, 2006). This suggests that the oxidase-negative phenotype may be a selective advantage in the Hamelin Pool environment. Furthermore, both strains required higher salt levels for growth than are found in Hamelin Pool seawater. Survival and growth of these strains within the Hamelin Pool environment may be facilitated by the intertidal location of the smooth and pustular mats, as regular cycles of desiccation may result in concentrated salt pockets within the fabric of the microbial mats.

Whole-cell protein profiles were determined by SDS-PAGE (Stan-Lotter *et al.*, 2002). SDS-PAGE of whole-cell proteins can be used as a rapid method for distinguishing between bacterial species (Jackman, 1987) and both strains showed different patterns compared to known representatives of the same genus (see supplementary Fig. S2 available with the online version of this paper).

Polar lipids were extracted from SA5^T, PA12^T and reference strains by the method of Kamekura (1993). TLC and staining of the lipids was performed as outlined previously (Usami *et al.*, 2005). Both strains possessed sulfated diglycosyl archaeol-1 (S-DGA-1) and lacked phosphatidylglycerosulfate (PGS) (see supplementary Fig. S3 available with the online version of this paper), as is characteristic for species of the genus *Haloferax* (Kamekura *et al.*, 2004).

Phylogenetic trees were reconstructed from 16S rRNA gene sequences by the neighbour-joining method and the maximum-likelihood algorithm using ARB (Ludwig *et al.*, 2004). Both the neighbour-joining tree (Fig. 1) and the maximum-likelihood tree (not shown) indicated that strain SA5^T clustered most closely with *Hfx. larsenii* and strain PA12^T clustered most closely with *Hfx. mediterranei*. All signature bases of the genus *Haloferax* (Kamekura *et al.*, 2004) were conserved in the 16S rRNA genes of SA5^T and PA12^T and sequence similarities with *Halogeometricum borinquense*, their closest relative outside the genus *Haloferax*, were 91.8 and 92.9 %, respectively.

DNA G+C content was determined by the HPLC method of Tamaoka & Komagata (1984) and DNA–DNA hybridization was performed by the fluorometric method of Ezaki *et al.* (1989). The two isolates had low DNA–DNA relatedness to each other and to all recognized *Haloferax*

Table 1. Phenotypic and other characteristics that distinguish the Hamelin Pool isolates SA5^T and PA12^T from other *Haloferax* species

Taxa: 1, SA5^T (data from this study); 2, PA12^T (data from this study); 3, *Hfx. mediterranei* JCM 8866^T (Rodriguez-Valera *et al.*, 1983); 4, *Hfx. volcanii* JCM 8879^T (Mullakhanbhai & Larsen, 1975); 5, *Hfx. lucentense* JCM 9276^T (Gutierrez *et al.*, 2002); 6, *Hfx. denitrificans* JCM 8864^T (Tomlinson *et al.*, 1986); 7, *Hfx. gibbonsii* JCM 8863^T (Juez *et al.*, 1986); 8, *Hfx. alexandrinus* JCM 10717^T (Asker & Ohta, 2002); 9, *Hfx. sulfurifontis* JCM 12327^T (Elshahed *et al.*, 2004); 10, *Hfx. prahovense* JCM 13924^T (Enache *et al.*, 2007); 11, *Hfx. larsenii* JCM 13917^T (Xu *et al.*, 2007). Additional data are from Tindall (1992), Tindall *et al.* (1989), Ventosa (2001) and Robinson *et al.* (2005). +, Positive; −, negative; ±, variable; ND, not determined; w, weakly positive.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Pigmentation	Red	Pink–red	Pink	Red–orange	Pink	Orange–red	Orange–red	Red	Salmon pink	Beige–orange	Orange–red
Motility	Rotating	−	+	Rotating	+	−	+	−	−	−	+
NaCl range (M)	1.7–5.1	1.7–5.1	1.3–4.7	1.0–4.5	1.8–5.1	1.5–4.5	1.5–5.2	1.7–5.2	1.0–5.2	2.5–5.2	1.0–4.8
NaCl optimum (M)	2.6–3.4	2.6–3.4	2.9	1.7–2.5	4.3	2.0–3.0	2.5–4.3	4.3	2.1–2.6	3.5	2.2–3.4
Minimum Mg^{2+} (M)	0.2	0.2	0.02	0.02	ND	ND	0.2	0.33	0.001	ND	0.005
Temp. range (°C)	30–55	23–55	25–45	ND	10–40	30–55	25–55	20–55	18–50	23–51	25–55
Temp. optimum (°C)	53	42–53	35–37	45	37	50	35–40	37	32–37	38–48	42–45
pH range	7.0–9.0	6.0–10.0	ND	ND	5–9	6–8	5–8	5.5–7.5	4.5–9.0	6.0–8.5	6.0–8.5
Generation time (h)	0.53*	0.96†	1.20	1.83	ND	4	ND	ND	ND	ND	ND
Oxidase test	±	−	+	+	+	+	+	+	+	+	+
H_2S formation from thiosulfate	−	−	−	+	+	+	+	+	+	+	+
Hydrolysis of:											
Gelatin	+	+	+	−	−	+	+	+	+	−	+
Casein	+	+	+	−	−	−	+	−	−	−	−
Starch	+	−	+	−	−	−	−	−	−	+	+
Tween 80	+	−	+	−	+	−	+	+	+	+	+
Acid production from:											
Mannose	−	−	+	−	ND	ND	+	−	−	ND	−
Galactose	−	−	ND	+	−	ND	+	−	+	−	−
Xylose	−	−	+	+	+	ND	+	+	+	−	−
Sucrose	+	+	+	+	−	+	+	+	+	−	w
Resistance to:											
Rifampicin	+	+	−	−	ND	−	−	+	+	−	−
Bacitracin	+	+	−	−	ND	−	−	−	+	+	−
DNA G+C content (mol%)	61.4	60.8	60	63.4	64.5	65.3	61.8	59.5	60.5	63.7	62.2
DNA–DNA reassociation:‡											
With SA5^T (%)	100	18	20 (19)	29 (18)	20 (27)	28 (19)	25 (23)	24 (19)	25 (33)	20 (25)	22 (19)
With PA12^T (%)	30	100	14 (15)	29 (17)	22 (16)	23 (17)	30 (20)	24 (32)	19 (21)	21 (15)	24 (22)

*The generation time for strain SA5^T was determined at 53 °C.

†The generation time for strain PA12^T was determined at 48 °C.

‡DNA–DNA reassociation values were obtained between non-labelled DNA of reference strains (3–11) and labelled DNA of SA5^T and PA12^T, respectively. The values in parentheses were obtained between labelled DNA of reference strains and non-labelled DNA of SA5^T and PA12^T, respectively.

species (33 % or less; Table 1), suggesting that strains SA5^T and PA12^T both represent novel species.

Phylogenetic, phenotypic and chemotaxonomic data indicate that strains SA5^T and PA12^T are members of the genus *Haloferax*. The DNA–DNA hybridization data, whole-cell protein profiles and phenotypic characteristics (Table 1) justify the creation of two novel species within the genus *Haloferax* to accommodate these strains, for which the names *Haloferax elongans* sp. nov. and *Haloferax mucosum* sp. nov., respectively, are proposed.

Description of *Haloferax elongans* sp. nov.

Haloferax elongans (e.lon′gans. N.L. part. adj. *elongans* elongating/extending, referring to the capacity of the species to form very long rods).

Cells are pleomorphic, with irregular short rods and pleomorphic forms common during stationary phase and in suboptimal media. During exponential growth under optimum conditions, cells elongate to up to 12 μm long. Rotational motility is observed in elongated cells.

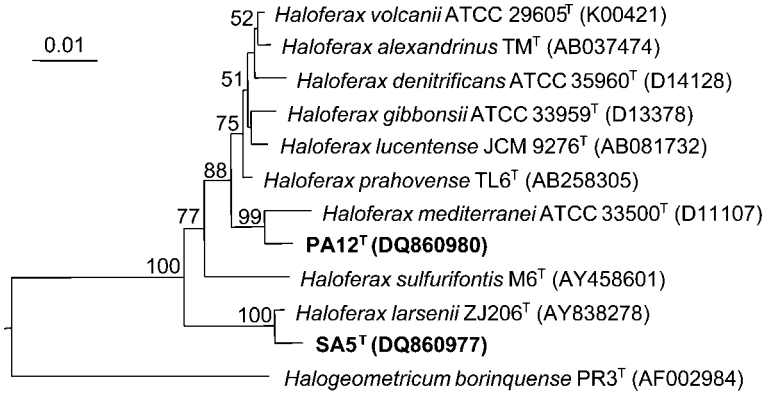

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships between strains SA5^T and PA12^T and related taxa. Bootstrap values >50 % (1000 resamplings) are shown. Bar, 0.01 substitutions per nucleotide position. *Halobacterium salinarum* DSM 671 was used as the outgroup (not shown). GenBank accession numbers are indicated for each strain.

Gram-negative. Colonies on complex agar medium are small (1–2 mm diameter), red, entire, translucent and convex. Growth occurs in 1.7–5.1 M NaCl, with optimum growth at 2.6–3.4 M NaCl. Growth occurs in media containing at least 0.2 M Mg^{2+} or more, with an optimum of 0.4 M. At least 0.04 M Mg^{2+} is required for maintenance of native cell morphology and cell lysis occurs in NaCl solutions of less than 0.85 M. Grows at pH 7.0–9.0 and 30–55 °C, with optimum growth at pH 7.4 and 53 °C. The generation time at 53 °C is 32±3 min. Chemo-organotrophic. Aerobic. Oxidase-variable and catalase-positive. Nitrate is not reduced to nitrite. Does not produce gas from nitrate. Indole is produced from tryptophan. H_2S is not produced from thiosulfate. Anaerobic growth is not observed with either nitrate, DMSO or arginine. Gelatin, casein, starch and Tween 80 are hydrolysed. The following substrates are used as carbon sources: glucose, glycerol, maltose, sucrose and trehalose. Acid is produced from glucose, glycerol, maltose, sucrose and trehalose. The following substrates are not used as carbon sources: ethanol, fumarate, galactose, glycine, lactose, mannitol, mannose, raffinose, xylose, L-arginine, L-histidine, L-lysine, L-ornithine and D-sorbitol. Sensitive to novobiocin (50 μg ml^{-1}). Resistant to ampicillin, bacitracin, chloramphenicol, cycloheximide, erythromycin, gentamicin, neomycin, penicillin, streptomycin and tetracycline (all at 50 μg ml^{-1}), ciprofloxacin (2.5 μg per disk), norfloxacin (10 μg per disk) and rifampicin (1 μg per disk). Lipids S-DGA-1, diglycosyl archaeol-1 (DGA-1), phosphatidylglycerophosphate methyl ester (PGP-Me) and phosphatidylglycerol (PG) are present, but PGS is not present.

The type strain is SA5^T (=JCM 14791^T=ATCC BAA-1513^T=UNSW 104100^T), isolated from a smooth mat, Hamelin Pool, Shark Bay, Western Australia. The DNA G+C content of the type strain is 61.4 mol%.

Description of *Haloferax mucosum* sp. nov.

Haloferax mucosum (mu.co′sum. L. neut. adj. *mucosum* slimy, a property of the colonies).

Cells are pleomorphic and non-motile. Gram-negative. Colonies on complex agar medium are 3–5 mm in diameter, pink–red, entire, opaque, mucoid and domed. Growth occurs in 1.7–5.1 M NaCl, with optimum growth at 2.6–3.4 M. Growth occurs in media containing at least 0.2 M Mg^{2+} or more. Greater than 0.04 M Mg^{2+} is required for maintenance of native cell morphology and cell lysis occurs in NaCl solutions of 0.85 M or less. Grows at pH 6.0–10.0 and 23–55 °C, with optimum growth at pH 7.4 and 42–53 °C. The generation time at 48 °C is 57±3 min. Chemo-organotrophic. Aerobic. Oxidase-negative and catalase-positive. Nitrate is not reduced to nitrite. Does not produce gas from nitrate. Indole is produced from tryptophan. H_2S is not produced from thiosulfate. Anaerobic growth with nitrate, DMSO or arginine does not occur. Gelatin and casein are hydrolysed, but starch and Tween 80 are not. The following substrates are used as carbon sources: glucose, glycerol, maltose, sucrose and trehalose. Acid is produced from glucose, glycerol, maltose, sucrose and trehalose. The following substrates are not used as carbon sources: ethanol, fumarate, galactose, glycine, lactose, mannitol, mannose, raffinose, xylose, L-arginine, L-histidine, L-lysine, L-ornithine and D-sorbitol. Sensitive to novobiocin (50 μg ml^{-1}). Resistant to ampicillin, bacitracin, chloramphenicol, cycloheximide, erythromycin, gentamicin, neomycin, penicillin, streptomycin and tetracycline (all at 50 μg ml^{-1}), ciprofloxacin (2.5 μg per disk), norfloxacin (10 μg per disk) and rifampicin (1 μg per disk). Lipids S-DGA-1, DGA-1, PGP-Me and PG are present, but PGS is not present.

The type strain is PA12^T (=JCM 14792^T=ATCC BAA-1512^T=UNSW 104200^T), isolated from a pustular mat, Hamelin Pool, Shark Bay, Western Australia. The DNA G+C content of the type strain is 60.8 mol%.

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