Halococcus hamelinensis sp. nov., a novel halophilic archaeon isolated from stromatolites in Shark Bay, Australia

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Several halophilic archaea belonging to the genus *Halococcus* were isolated from stromatolites from Hamelin Pool, Shark Bay, Western Australia, collected during field trips in 1996 and 2002. This is the first incidence of halophilic archaea being isolated from this environment. Stromatolites are biosedimentary structures that have been formed throughout the earth's evolutionary history and have been preserved in the geological record for over 3 billion years. The stromatolites from Hamelin Pool, Western Australia, are the only known example of extant stromatolites forming in hypersaline coastal environments. Based on their 16S rRNA gene sequences and morphology, the isolates belong to the genus *Halococcus*. Strain 100NA1, isolated from stromatolites collected in 2002, was closely related to strain 100A6^T that was isolated from the stromatolites collected in 1996, with a DNA–DNA hybridization value of 94 ± 8 %. DNA–DNA hybridization values of strain 100A6^T with *Halococcus morrhuae* NRC 16008 and *Halococcus saccharolyticus* ATCC 49257^T were 17 ± 6 and 11 ± 7 %, respectively. The DNA G+C content of strain 100A6^T was 60·5 mol% (T_m). The main polar lipid was S-DGA-1, a sulphated glycolipid that has been detected in all strains of the genus *Halococcus*. Whole-cell protein profiles, enzyme composition and utilization of various carbon sources were distinct from those of all previously characterized *Halococcus* species. The recognition of this strain as representing a novel species within the genus *Halococcus* is justified, and the name *Halococcus hamelinensis* sp. nov. is proposed. The type strain is 100A6^T (=JCM 12892^T=ACM 5227^T).

The haloarchaea (order *Halobacteriales*) are very well adapted to hypersaline environments. They are capable of optimal growth in media containing 15–30 % (w/v) NaCl (Grant *et al.*, 2001). There are 20 different genera of halophilic archaea comprising 60 species (http://www.the-icsp.org/taxa/halobacterlist.htm). Members of the genus *Halococcus* have been shown to exhibit diverse phenotypes (Montero *et al.*, 1993) and to date four species (Grant *et al.*, 2001; Kamekura, 1998; Oren *et al.*, 1997) have been described: *Halococcus morrhuae* (Kocur & Hodgkiss, 1973), *Halococcus saccharolyticus* (Montero *et al.*, 1989), *Halococcus salifodinae* (Denner *et al.*, 1994) and *Halococcus dombrowskii* (Stan-Lotter *et al.*, 2002). These *Halococcus* species are more tolerant than other halobacteria under low salinity (Grant *et al.*, 2001) and are not lysed in distilled water or in *N*-lauroylsarcosine (Kamekura, 1998).

In a recent study, we characterized the microbial diversity of stromatolites in the hypersaline marine setting of Shark Bay, Western Australia (Burns *et al.*, 2004), and reported the presence of halophilic archaea using culture-independent approaches. Due to the restricted flow of seawater into Hamelin Pool and the high net evaporation rates, the salinity of the surface water in Hamelin Pool is twice that of normal seawater (Arp *et al.*, 2001). The living stromatolites are partially submerged in this hypersaline environment. Microbial communities present in these stromatolites must therefore be able to adapt to this hypersaline environment. Microfossils of ancient stromatolites over 3 billion years old

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Halococcus hamelinensis* sp. nov. 100A6^T is DQ017835.

are thought to be evidence of one of the earliest life forms on Earth (Byerly *et al.*, 1986; Walter *et al.*, 1980). The living stromatolites of Shark Bay represent analogues of these fossilized stromatolites, and are thus excellent natural laboratories for studying complex microbial communities that are involved in their formation. The 16S rRNA gene clone libraries constructed from the Shark Bay stromatolites consist of clones related to *Cenarchaeales* of the *Crenarchaeota*, and *Methanomicrobiales* and *Halobacteriales* of the *Euryarchaeota*. More specifically within the *Halobacteria*, clones clustering with the genera *Haloferax*, *Halogeometricum*, *Halobacterium*, *Halosimplex* and *Halococcus* were identified (Burns *et al.*, 2004). This revealed significant archaeal diversity associated with these stromatolites. However, the physiological role of archaea in stromatolite systems remains unexplored. In this study, we report the isolation and characterization of a novel halophilic archaeon from these stromatolites. The phenotypic characteristics of the isolates, including lipid profiles, as well as their phylogenetic assignment, based on their 16S rRNA gene sequences, DNA base compositions and DNA–DNA hybridization are described. The two characterized strains represent a novel species within the genus *Halococcus*.

Samples of columnar stromatolites were collected from the intertidal region of Hamelin Pool, Shark Bay, during two field trips in 1996 and 2002. Approximately 1 cm^2 of stromatolite was physically ground and suspended in 5 ml sterile 5 % (w/v) NaCl. The concentration of ions present in the seawater of Hamelin Pool is approximately twice that of seawater (Arp *et al.*, 2001); therefore, for the isolation of halophilic archaea from the stromatolites, solidified DSM97 medium (DasSarma *et al.*, 1995) was modified to mimic the salt ionic concentrations of Hamelin Pool. Media contained the following (g l^{-1}): Casamino acids, 7·50; yeast extract, 10·0; trisodium citrate, 3·00; NaCI, 250; KCI, 2·00; $MgCI_2.6H_2O$, 7·23; $MgSO_4.7H_2O$, 20·0; $FeSO_4.7H_2O$, 0·05; $MnSO_4.H_2O$, 0·20; $CaCI_2.2H_2O$, 2·70. One hundred millilitres was inoculated with 100 µl of stromatolite suspension in duplicate. Addition of three antibiotics (streptomycin, penicillin and ampicillin) at 100 µg ml^{-1} prevented the overgrowth of bacteria (Wais, 1988). When growth was observed by turbidity, cultures were spread or streaked on agar media. Petri dishes were incubated in sealed containers at 37 °C for 2–3 weeks and pure cultures were obtained by passaging single colonies on the same medium. Partial sequence analysis of the 16S rRNA gene suggested that random isolates were very similar to *Halobacterium* strain NCIMB 718. *Halobacterium* strain NCIMB 718 has yet to be studied taxonomically; however, the 16S rRNA gene sequences deposited suggest that the strain belongs to the species *Halococcus* (Kamekura *et al.*, 2004). Strain NCIMB 718 was also a Gram-negative coccus, further suggesting that it belongs to the genus *Halococcus*. In the present study, two isolates, 100NA1 and 100A6^T, were selected for further characterization. Strain 100NA1 was isolated from the stromatolites collected during a field trip in 2002, whereas 100A6^T was isolated from the stromatolites collected in 1996. Colonies on agar plates were approximately 1–2 mm in diameter and had bright-orange–pink pigmentation.

The two strains were non-motile cocci in liquid and solid cultures, as determined by both phase-contrast microscopy and electron microscopy (Fig. 1). The cells stained Gram-negative and occurred in pairs or tetrads in exponential phase, or as large clusters when the cells were in stationary phase. Thin sections revealed a thick cell envelope (Fig. 1) with an irregular outer layer. Scanning electron microscopy further supported the finding that the outer layer had an irregular consistency.

Physiological and biochemical characteristics of the strains are provided in the species description and are also summarized in Table 1. The tests were carried out according to the recommended minimal standard methods for the description of new taxa in the *Halobacteriales* (Oren *et al.*, 1997). The requirement for NaCl was determined in media containing 10–30 % NaCl (w/v). The requirement for magnesium was determined in media containing 0·5–50 mM $MgCl_2$ and $MgSO_4$, at the optimum NaCl concentration for growth. Oxidase activity was detected by spotting a loopful of culture on a paper strip containing a 1 % solution of tetramethyl-*p*-phenylenediamine dihydrochloride. In particular, the two isolates and strain NCIMB 718 were negative for oxidase activity, whereas *Hcc. morrhuae* NRC 16008, *Hcc. saccharolyticus* ATCC 49257^T, *Hcc salifodinae* DSM 8989^T and *Halobacterium salinarum* NRC-1 were all positive. API ZYM (bioMérieux) was also used for the identification of other enzymic activites (Humble *et al.*, 1977). Strips were inoculated with cells in the exponential phase of growth and were incubated at 37 °C for up to 24 h (Stan-Lotter *et al.*, 2002). The API ZYM strips revealed that the two isolates had similar enzyme profiles to strain NCIMB 718, except for leucine arylamidase and trypsin. The two isolates were positive for leucine arylamidase and negative for trypsin.

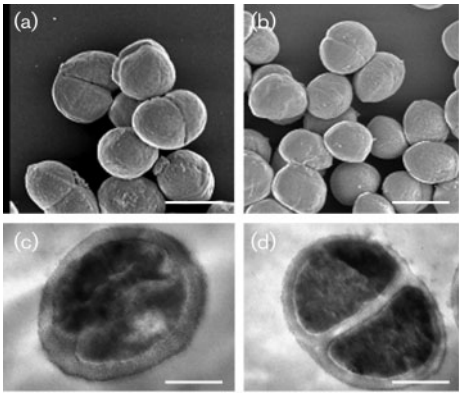

Fig. 1. Scanning electron micrographs of strains 100A6^T (a) and 100NA1 (b) grown in liquid culture (HP-DSM97; 15 % NaCl, w/v) and transmission electron micrographs of ultrathin sections of strains 100A6^T (c) and 100NA1 (d). Bars, 1 µm (a), 1·2 µm (b) and 200 nm (c, d).

Table 1. Comparison of phenotypic characteristics of previously characterized *Halococcus* species and strain 100A6^T

Taxa: 1, strain 100A6^T; 2, strain NCIMB 718 (data from this study); 3, *Hcc. morrhuae* DSM 1309 (Grant *et al.*, 2001; Kocur & Hodgkiss, 1973); 4, *Hcc. dombrowskii* NCIMB 13803^T (Stan-Lotter *et al.*, 2002); 5, *Hcc. saccharolyticus* ATCC 49257^T (Montero *et al.*, 1989); 6, *Hcc. salifodinae* DSM 8989^T (Denner *et al.*, 1994). Tests were performed at salinities of at least 15 % NaCl. All strains are catalase-positive and negative for urease. +, Positive reaction or growth; −, no reaction or growth; V, variable; ND, not determined.

Characteristic	1	2	3	4	5	6
Optimum NaCl concentration (%, w/v)	15	15	20–30	20–30	20–30	20–30
Growth at pH 9	+	ND	−	−	+	+
Optimum temperature (°C)	37	ND	37	37	37	40
$MgCl_2$ requirement (mM)	0·5–50	ND	5	2·5–60	60	<0·1
Oxidase	−	−	+	+	+	+
Starch hydrolysis	+	+	V	ND	−	ND
Indole production	−	+	+	ND	+	ND
Nitrate reduction	+/no gas	+/no gas	+/no gas	+	+	+
Sulphide reduction	−	+	+	ND	+	ND
Gelatin liquefaction	−	−	+	+	V	+
Utilization of carbohydrates for growth:						
D-Sorbitol	−	−	ND	ND	ND	ND
Fumarate	−	−	ND	ND	ND	ND
Galactose	+	−	−	−	ND	ND
Glucose	+	+	ND	−	ND	ND
Lactose	−	−	−	ND	+	ND
Maltose	+	+	ND	ND	+	ND
Trehalose	+	+	ND	ND	+	ND
Mannose	−	−	ND	ND	+	ND
Mannitol	+	−	ND	ND	+	ND
Raffinose	−	−	−	−	−	ND
Sucrose	+	−	ND	ND	−	ND
Xylose	+	−	−	+	−	+
L-Arginine	−	−	+	−	+	ND
L-Histidine	−	−	+	ND	+	ND
L-Lysine	−	−	ND	ND	+	ND
Acid production from glucose	+	+	−	−	+	+
Antibiotic sensitivity*:						
Rifampicin	S	ND	ND	S	ND	ND
Novobiocin	S	ND	S	S	R	S
Bacitracin	S	ND	S	S	S	S
Kanamycin	R	ND	ND	R	ND	ND
Streptomycin	R	ND	R	R	R	R
Tetracycline	R	ND	R	R	R	(S)
Penicillin	R	ND	R	R	R	R
Neomycin	R	ND	ND	ND	ND	ND

*R, Resistant; S, sensitive, (S), slightly sensitive.

Polar lipids were extracted with chloroform/methanol as described previously (Kamekura, 1993), except that the cells were broken before extraction by grinding with quartz sand in a mortar and pestle placed on ice. TLC was performed using Merck HPTLC silica gel 60 plates (art. 5641) in the solvent system chloroform/methanol/acetic acid/water (85 : 22·5 : 10 : 4, by vol.). One-dimensional TLC of polar lipids (Fig. 2) suggested that strain 100A6^T contained phosphatidylglycerol and phosphatidylglyceromethylphosphate and four glycolipids (marked with circles). The main glycolipid identified by R_F values and comparison with controls was S-DGA-1, a sulphated glycolipid that has been detected in other *Halococcus* species and in *Halobacterium* sp. NCIMB 718. The faster-moving and slowest-moving glycolipids have not been detected in *Halococcus* strains.

DNA was isolated and purified as described previously (Burns *et al.*, 2004). PCR was used for the amplification of the 16S rRNA gene as described by Burns *et al.* (2004), using the primers Arch 21F, Arch 958R and 1492Rc (DeLong,

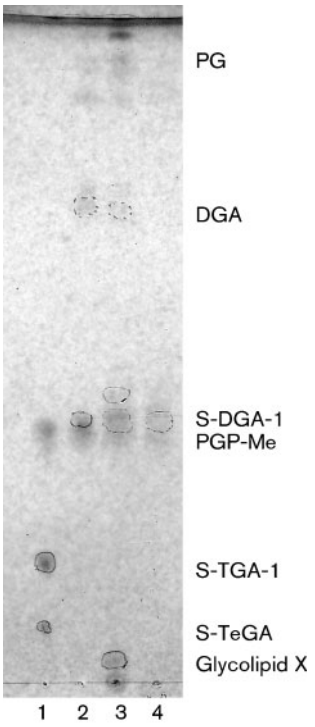

Fig. 2. One-dimensional TLC of polar lipids extracted from strain 100A6^T and three other halobacteria (plate was run from bottom to top). Lanes: 1, *Halobacterium salinarum* strain NRC-1; 2, *Haloferax volcanii* NCIMB 2012^T; 3, strain 100A6^T; 4, *Halobacterium* sp. NCIMB 718. Strain 100A6^T contained four glycolipids (circled), with the dominant one being S-DGA-1. PG, phosphatidylglycerol; PGP-Me, phosphatidylglycerolmethylphosphate.

1992). Automated sequencing was carried out as described previously (Neilan *et al.*, 2002). BLAST searches were used to identify similar sequences from GenBank. DNA sequences were aligned by using the multiple sequence alignment tool from CLUSTAL_X and phylogenetic tree reconstructions were performed as described previously (Neilan *et al.*, 2002). The G+C content was determined by using the thermal denaturation method and DNA–DNA reassociation was assessed as described previously (Bowman *et al.*, 1998). Sequences of the 16S rRNA genes of strains 100A6^T (1312 bases) and 100NA1 (1192 bases) were obtained. The highest identity match (98 %) of the two strains was with strain NCIMB 718, which has been suggested to belong to the genus *Halococcus* (Kamekura *et al.*, 2004).

Alignment of the 16S rRNA gene sequences with all published sequences of haloarchaea clearly showed that the strains belonged to the genus *Halococcus*, as they possessed 19 out of 21 signature bases of this genus (Kamekura *et al.*, 2004). The exceptions were bases at positions 116 and 772, where the signatures were C and T, respectively. The two strains isolated and strain NCIMB 718 possessed A at these positions, supporting the finding that the 16S rRNA genes of the two strains and strain NCIMB 718 showed the highest similarity (98 %). As discussed by Stan-Lotter *et al.* (2002), the genus *Halococcus* has two phylotypes. One phylotype contains *Hcc. salifodinae* and *Hcc. saccharolyticus*, whereas the other comprises *Hcc. morrhuae*, *Hcc. dombrowskii* and other coccoid strains. From the phylogenetic tree constructed, the two strains belonged to a separate lineage with strain NCIMB 718 (Fig. 3), within the genus *Halococcus*. However, between the two strains and NCIMB 718 there are sufficient levels of differences in their physiological and biochemical characteristics to render them as representing separate species.

The DNA base composition of strain 100A6^T was 60·0 mol% G+C and that of strain 100NA1 was 60·5 mol% G+C. These values are similar to that for *Hcc. morrhuae* NRC 16008 (61 mol%; Grant *et al.*, 2001) and were similar to values found for *Hcc. saccharolyticus* ATCC 49257^T (59·5 mol%) and strain NCIMB 718 (60·8 mol%). DNA–DNA hybridization between the two isolates showed that isolate 100A6^T had a relatedness value of 94±8 % to isolate 100NA1. Because of this high relatedness value to each other, the two strains were classed as representing the same species and hence further hybridization with other strains was only carried out for strain 100A6^T. Isolate 100A6^T had 74±10 % relatedness to strain NCIMB 718, 17±6 % relatedness to *Hcc. morrhuae* NRC 16008 and a lower relatedness value of 11±7 % to *Hcc. saccharolyticus* ATCC 49257^T. The relatedness value to *Halogeometricum borinquense* JCM 10706^T was 13±10 %. DNA–DNA hybridization of strain 100A6^T with *Hcc. salifodinae* DSM 8989^T was not carried out in this study. However, *Hcc. saccharolyticus* ATCC 49257^T was used as a representative of that cluster from the phylogenetic tree (Fig. 3). The DNA–DNA hybridization values revealed that strain 100A6^T was more closely related to strain NCIMB 718 than to any other *Halococcus* species. Strains are normally identified as representing different species when any two strains have less than 70 % DNA–DNA hybridization values (Wayne *et al.*, 1987) and the hybridization value between strain 100A6^T and strain NCIMB 718 was between 64 and 84 %. Therefore, based on DNA–DNA hybridization values alone, it is difficult to determine whether strain 100A6^T and strain NCIMB 718 represent two separate species. However, biochemical tests have shown distinct differences in properties between strains 100A6^T and NCIMB 718, indicating that strain 100A6^T does not belong to the same species as strain NCIMB 718 and that both strains are distantly related to the rest of the *Halococcus* species.

SDS gel electrophoresis was performed as described by 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). Strains 100A6^T and 100NA1 had protein profiles that were very similar to each other (Fig. 4). However, they did not resemble the profile of strain NCIMB 718. This result differed from the 16S rRNA gene analysis and DNA–DNA hybridization results, which indicated that

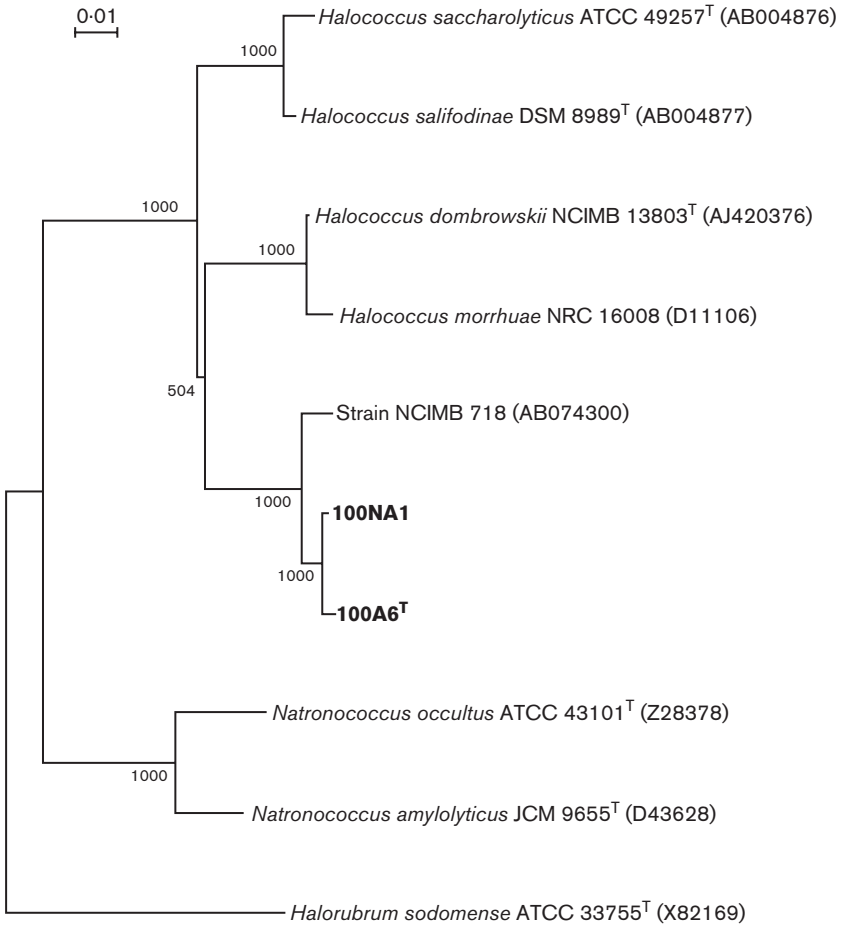

Fig. 3. Phylogenetic dendrogram of halobacteria based on 16S rRNA gene sequence data, indicating the position of the isolates 100NA1 and 100A6^T (GenBank accession no. DQ017835). The tree was constructed using the CLUSTAL_X program and *Halorubrum sodomense* ATCC 33755^T was used as an outgroup. Bootstrap values >500, based on 1000 resamplings, are indicated at nodes.

strains 100A6^T and 100NA1 were most similar to strain NCIMB 718.

Based on morphology, whole-cell protein pattern, physiological and biochemical characteristics, G+C content, DNA–DNA hybridization and polar lipid content, strains 100A6^T and 100NA1 were considered to represent the same species. They were similar to most halobacteria, being sensitive to rifampicin, novobiocin and bacitracin, but resistant to kanamycin, tetracycline, streptomycin, neomycin and penicillin. However, they differed from all known *Halococcus* species in terms of their physiological and biochemical characteristics (see Table 1). The optimum NaCl concentration for growth was 15 % (w/v), which contrasts with values of 20–30 % (w/v) for all of the other recognized *Halococcus* species. Of particular significance, strain 100A6^T was oxidase-negative, whereas all recognized *Halococcus* species are oxidase-positive. Most of the aerobic halophilic archaea are also oxidase-positive (Grant *et al.*, 2001). Strain 100A6^T was also indole-negative, whereas the other *Halococcus* species tested were indole-positive. In addition, strain 100A6^T was capable of hydrolysing starch, in contrast to the other *Halococcus* species. All *Halococcus* species tested were positive for sulphide reduction except strain 100A6^T. Strains 100A6^T and NCIMB 718 were negative for gelatin liquefaction, whereas the other *Halococcus* species were positive. Hence, the above differences, 16S rRNA gene sequences, DNA–DNA hybridization and protein profiles support our proposal of a novel species, *Halococcus hamelinensis* sp. nov. Lipid profiles of strain 100A6^T showed that the isolate had a similar lipid profile to those of all other *Halococcus* species; however, strain 100A6^T also contained an unknown glycolipid (X), which has not been identified in other *Halococcus* species.

Strains 100A6^T and 100NA1 were similar to many halobacteria that inhabit extreme conditions (Kamekura, 1998; Ochsenreiter *et al.*, 2002; Vreeland *et al.*, 2000, 2002; Wais, 1988); however, they were isolated from an environment where no archaea had been shown previously. As they were isolated from stromatolites collected several years apart, but were not identified in seawater surrounding the stromatolite (data not shown), we propose that this archaeon may be intrinsically associated with these stromatolites and thus may potentially play a role in some aspect of stromatolite formation. However, *in situ* studies such as fluorescence *in situ* hybridization need to be carried out to clarify the association of archaea with stromatolites. Mechanisms of

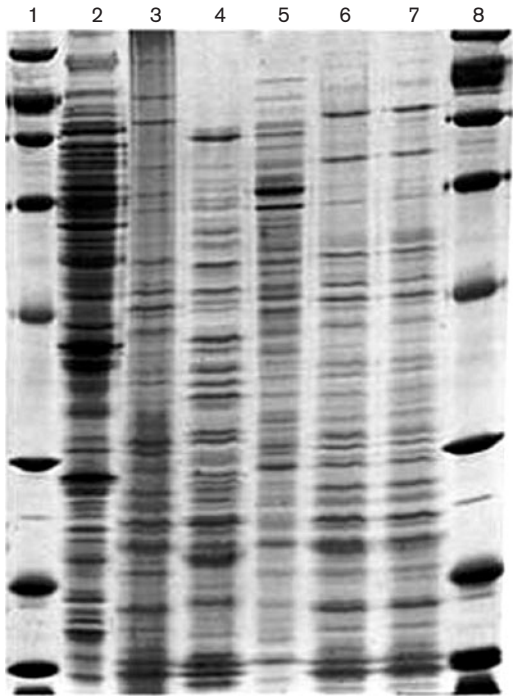

Fig. 4. Whole-cell proteins from various halophilic archaeal strains and isolates 100A6^T and 100NA1 following separation by SDS-PAGE. Approximately 20 μg protein per lane was applied, following lysis of cells. Proteins were stained with Coomassie blue. Lanes: 1 and 8, molecular mass markers; 2, *Halogeometricum borinquense* JCM 10706^T; 3, *Hcc. saccharolyticus* ATCC 49257^T; 4, *Hcc. morrhuae* NRC 16008; 5, strain NCIMB 718; 6, 100A6^T; 7, 100NA1.

osmotolerance by strain 100A6^T in this hypersaline environment, as well as interactions of this archaeon with other micro-organisms, are also currently under investigation.

Description of *Halococcus hamelinensis* sp. nov.

Halococcus hamelinensis (ha.me.li′nen.sis. N.L. masc. adj. *hamelinensis* pertaining to Hamelin Pool, where the type strain was isolated).

Cells are cocci with a diameter of 0·8–1·2 μm and occur singly, in pairs, tetrads or as irregular clusters. Gram-negative, non-motile and strictly aerobic. Small, bright-orange–pink, circular colonies are formed after 1 week incubation at 37 °C on agar media. The optimum NaCl concentration for growth is 15 % (w/v), with a doubling time of 13–15 h; capable of growth in 12·5–30 % (w/v) NaCl. The optimum temperature and pH range for growth are 37 °C and pH 4–9. 0·5–50 mM $MgCl_2$ is required for growth. Oxidase-negative and catalase-positive. Hydrolyses starch. Indole is not produced. Negative for sulphide reduction, urease and gelatin liquefaction. Utilizes glucose, sucrose, xylose, maltose, trehalose and glycerol as complex carbon sources in the presence of 0·1 % yeast extract; and glucose, mannitol, galactose, sucrose, xylose, maltose, trehalose, glycerol and ethanol as a single carbon source. Strong acidification occurs for sucrose, xylose, maltose and trehalose, whereas only slight acidification of glucose and glycerol occurs. Sensitive to rifampicin, novobiocin and bacitracin, but resistant to kanamycin, tetracycline, streptomycin, neomycin and penicillin (50 μg ml^{-1}). DNA G+C content is 60·0–60·5 mol% (T_m). Main polar lipid is S-DGA-1, a sulphated glycolipid detected in all strains of the genera *Haloferax* and *Halococcus*.

The type strain, 100A6^T (=JCM 12892^T=ACM 5227^T), was isolated from stromatolites of Shark Bay, Hamelin Pool, Western Australia.

Acknowledgements

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References

Arp, G., Reimer, A. & Reitner, J. (2001). Photosynthesis-induced biofilm calcification and calcium concentrations in Phanerozoic oceans. *Science* **292**, 1701–1704.

Bowman, J. P., McCammon, S. A., Brown, J. L. & McMeekin, T. A. (1998). *Glaciecola punicea* gen. nov., sp. nov. and *Glaciecola pallidula* gen. nov., sp. nov.: psychrophilic bacteria from Antarctic sea-ice habitats. *Int J Syst Bacteriol* **48**, 1213–1222.

Burns, B. P., Goh, F., Allen, M. & Neilan, B. A. (2004). Microbial diversity of extant stromatolites in the hypersaline marine environment of Shark Bay, Australia. *Environ Microbiol* **6**, 1096–1101.

Byerly, G. R., Lower, D. R. & Walsh, M. M. (1986). Stromatolites from the 3,300–3,500-Myr Swaziland supergroup, Barberton Mountain Land, South Africa. *Nature* **319**, 489–491.

DasSarma, S., Fleischmann, E. M. & Rodriguez-Valera, F. (1995). Media for halophiles. In *Archaea: a Laboratory Manual*, pp. 225–230. Edited by S. DasSarma & E. M. Fleischmann. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

DeLong, E. F. (1992). Archaea in coastal marine environments. *Proc Natl Acad Sci U S A* **89**, 5685–5689.

Denner, E. B. M., McGenity, T. J., Busse, H.-J., Grant, W. D., Wanner, G. & Stan-Lotter, H. (1994). *Halococcus salifodinae* sp. nov., an archaeal isolate from an Austrian salt mine. *Int J Syst Bacteriol* **44**, 774–780.

Grant, W. D., Kamekura, M., McGenity, T. J. & Ventosa, A. (2001). Class III. *Halobacteria* class. nov. In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 1, pp. 294–334. Edited by D. R. Boone, R. W. Castenholz & G. M. Garrity. New York: Springer.

Humble, M. W., King, A. & Phillips, I. (1977). API ZYM: a simple rapid system for the detection of bacterial enzymes. *J Clin Pathol* **30**, 275–277.

Jackman, P. J. H. (1987). Microbial systematics based on electrophoretic whole-cell protein patterns. *Methods Microbiol* **19**, 209–225.

Kamekura, M. (1993). Lipids of extreme halophiles. In *The Biology of Halophilic Bacteria*, pp. 135–161. Edited by R. H. Vreeland & L. I. Hochstein. Boca Raton, FL: CRC Press.

Kamekura, M. (1998). Diversity of extremely halophilic bacteria. *Extremophiles* **2**, 289–295.

Kamekura, M., Mizuki, T., Usami, R., Yoshida, Y., Horikoshi, K. & Vreeland, R. H. (2004). The potential use of signature bases from 16S

rRNA gene sequences to aid the assignment of microbial strains to genera of halobacteria. In *Halophilic Microorganisms*, pp. 77–100. Edited by A. Ventosa. Berlin: Springer.

Kocur, M. & Hodgkiss, W. (1973). Taxonomic status of the genus *Halococcus* Schoop. *Int J Syst Bacteriol* **23**, 151–156.

Montero, C. G., Ventosa, A., Rodriguez-Valera, F., Kates, M., Moldoveanu, N. & Ruiz-Berraquero, F. (1989). *Halococcus saccharolyticus* sp. nov., a new species of extremely halophilic non-alkaliphilic cocci. *Syst Appl Microbiol* **12**, 167–171.

Montero, C. G., Klenk, H. P., Nieto, J. J. & Ventosa, A. (1993). DNA-rRNA hybridization studies on *Halococcus saccharolyticus* and other halobacteria. *FEMS Microbiol Lett* **111**, 69–72.

Neilan, B. A., Burns, B. P., Relman, D. & Lowe, D. R. (2002). Molecular identification of cyanobacteria associated with stromatolites from distinct geographical locations. *Astrobiology* **2**, 271–280.

Ochsenreiter, T., Pfeifer, F. & Schleper, C. (2002). Diversity of Archaea in hypersaline environments characterized by molecular-phylogenetic and cultivation studies. *Extremophiles* **6**, 267–274.

Oren, A., Ventosa, A. & Grant, W. D. (1997). Proposed minimal standards for description of new taxa in the order *Halobacteriales*. *Int J Syst Bacteriol* **47**, 233–238.

Stan-Lotter, H., Pfaffenhuemer, M., Legat, A., Busse, H.-J., Radax, C. & Gruber, C. (2002). *Halococcus dombrowskii* sp. nov., an archaeal isolate from a Permian alpine salt deposit. *Int J Syst Evol Microbiol* **52**, 1807–1814.

Vreeland, R. H., Rozenzweig, W. D. & Powers, D. W. (2000). Isolation of a 250 million-year-old halotolerant bacterium from a primary salt crystal. *Nature* **407**, 897–900.

Vreeland, R. H., Straight, S., Krammes, J., Dougherty, K., Rosenzweig, W. D. & Kamekura, M. (2002). *Halosimplex carlsbadense* gen. nov., sp. nov., a unique halophilic archaeon, with three 16S rRNA genes, that grows only in defined medium with glycerol and acetate or pyruvate. *Extremophiles* **6**, 445–452.

Wais, A. C. (1988). Recovery of halophilic archaebacteria from natural environments. *FEMS Microbiol Ecol* **53**, 211–216.

Walter, M. R., Buick, R. & Dunlop, J. S. R. (1980). Stromatolites 3,400–3,500 Myr old from the North Pole area, Western Australia. *Nature* **284**, 443–445.

Wayne, L. G., Brenner, D. J., Colwell, R. R. & 9 other authors (1987). International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.