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Diversity of *Haloquadratum* and other haloarchaea in three, geographically distant, Australian saltern crystallizer ponds

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Abstract *Haloquadratum walsbyi* is frequently a dominant member of the microbial communities in hypersaline waters. 16S rRNA gene sequences indicate that divergence within this species is very low but relatively few sites have been examined, particularly in the southern hemisphere. The diversity of *Haloquadratum* was examined in three coastal, but geographically distant saltern crystallizer ponds in Australia, using both culture-independent and culture-dependent methods. Two 97%-OTU, comprising *Haloquadratum*- and *Halorubrum*-related sequences, were shared by all three sites, with the former OTU representing about 40% of the sequences recovered at each site. Sequences 99.5% identical to that of *Hqr. walsbyi* C23^T were present at all three sites and, overall, 98% of the *Haloquadratum*-related sequences displayed ≤2% divergence from that of the type strain. While haloarchaeal diversity at each site was relatively low (9–16 OTUs), seven phylogroups (clones and/or isolates) and 4 different clones showed ≤90% sequence identity to classified taxa, and appear to represent novel genera. Six of these branched together in phylogenetic tree reconstructions, forming a clade (MSP8-clade) whose members were only distantly related to classified taxa. Such sequences have only rarely been previously detected but were found at all three Australian crystallizers.

Keywords Archaea · Halobacteria · Halobacteriaceae · Hypersaline · Cultivation · Biodiversity

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Introduction

The microbial flora of thalassohaline, hypersaline waters, with salinities close to saturation, have been studied for many years, but only relatively recently has it been possible to identify and to culture many of the dominant groups (Antón et al. 2002; Burns et al. 2004a, b). The majority are extremely halophilic Archaea belonging to the family *Halobacteriaceae* (Oren 2002), although halophilic Bacteria, such as *Salinibacter*, may also be present (Antón et al. 2002). A peculiar organism which often dominates these microbial communities is the square haloarchaeon of Walsby (Walsby 1980), an extremely thin, square-shaped organism that was first cultivated in the laboratory in 2004 (Bolhuis et al. 2004; Burns et al. 2004b). Formally described in 2007, it has been named *Haloquadratum walsbyi*, with the type strain being C23^T (Burns et al. 2007).

The cells of *Hqr. walsbyi* are easily recognized under light microscopy not only by their shape, thin squares of about 2 μm per edge, but also because they contain phase-bright gas vesicles and phase-dark granules of PHB (Burns et al. 2007; Walsby 1980). They often make up 40–80% of total cells in neutral, thalassohaline, hypersaline waters, such as salt lakes, sabkhas and solar saltern crystalliser ponds (Antón et al. 1999; Burns et al. 2004a). The genome sequence of a Spanish isolate (HBSQ001) has been determined (Bolhuis et al. 2006), and was later compared to environmental DNA sequences recovered from the same saltern crystalliser pond from which this isolate was recovered (Cuadros-Orellana et al. 2007). Compared to other haloarchaea, the genome of *Hqr. walsbyi* has an unusually low G+C% (=49); its gene density is much lower (mainly due to the presence of many pseudogenes); and it forms a remarkably coherent species, with 16S rRNA gene sequences from environmental DNA that do not diverge by more than 1.6% from that of the isolate HBSQ001 (Legault et al. 2006). Indeed, the two described members of this genus, one from Australia and the other from Spain, show only 2 nt differences in their 16S rRNA genes (strains $C23^T$ and HBSQ001, respectively) (Burns et al. 2007), yet they were isolated from ponds 16,950 km apart.

Clone library and FISH studies of individual saltern crystallizers in Israel, Spain, Tunisia and Turkey (Antón et al. 1999; Baati et al. 2008; Mutlu et al. 2008; Oren et al. 2006), also support the view that *Hqr. walsbyi* is widespread, often dominant within the microbial community, and forms a tightly coherent species. In hypersaline waters where *Haloquadratum* is not dominant (and perhaps not present), other haloarchaeal groups dominate, such as *Halorubrum*, *Haloarcula*, or *Halobacterium* (Bidle et al. 2005; Bowman et al. 2000; Pašić et al. 2007), but the reasons for this are unknown.

While the divergence of *Haloquadratum* appears to be low, other, common haloarchaea that co-exist in these and similar waters, such as *Halorubrum* and *Haloarcula*, display a more usual level of speciation. *Halorubrum* has 24 recognized species, and *Haloarcula* has seven (http://www.the-icsp.org/taxa/halobacterlist.htm). 16S rRNA gene sequence studies indicate that many more species exist in nature (Burns et al. 2004a; Ochsenreiter et al. 2002). Why is it that *Haloquadratum* appears to be far more homogeneous than similar organisms that compete with it for space and nutrients? To determine if this difference reflects the small number of sites that have so far been studied, mostly in the northern hemisphere, we examined the diversity of haloarchaea in the crystallizer ponds of three, widely separated, Australian solar salterns. Using cultivation-independent and culture-dependent methods, two major groups of haloarchaea were found to be present in similar proportions at the three locations (*Haloquadratum* and *Halorubrum*), but each site also possessed a significant fraction of unique organisms representing novel genera within the Halobacteriaceae. The great majority of *Haloquadratum*-related sequences were found to be very closely related showing ≤2% divergence, but there was some evidence of speciation.

Materials and methods

Sample sites, collection, and water analyses

The sites, water chemistry and microbial counts are summarized in Table 1 and supplementary Table 1. Water samples (0.8–1 L) were collected from each saltern crystallizer in a sterile glass Schott bottle submerged 25 cm beneath the surface. These were sent by overnight courier to the laboratory (at ambient temperature) and cell samples were removed and processed for DNA extraction the next day. The three Australian sites were the Cheetham Salt Ltd crystallizer ponds at: Dry Creek, crystallizer pond no. 7, Price, South Australia; Bajool crystallizer pond no. 4, Bajool, Queensland; and Lara storage pond no. 1, Corio, Victoria. Chemical analyses were performed at a private laboratory. Total cell counts were performed as described previously (Burns et al. 2004a; Dyall-Smith 2001).

Media and cultivation

DBCM2 medium and modified growth medium (MGM) were prepared as described previously (Dyall-Smith 2001). Solid media used 1.5% agar (Difco-Bacto). Cultivation from water samples used direct plating on MGM and by liquid extinction cultures using DBCM2 medium, following the protocols described previously (Burns et al. 2004a, b).

DNA extraction, clone libraries, sequence analysis and phylogenetic tree reconstructions

DNA extraction and the construction of clone libraries of 16S rRNA genes were performed as described previously (Burns et al. 2004a). Briefly, the cells from 10 ml of water sample were pelleted, broken open using a bead-beater, and genomic DNA extracted using chloroform/isoamyl alcohol extraction, then precipitated from the aqueous phase with 0.54 vol of isopropanol. For each DNA preparation, five replicate PCRs (each 50 μl in volume) were prepared, and 16S rRNA genes amplified using PCR primers that selectively amplified archaeal genes [archF1, ATTCCGGTT GATCCTGC (Ihara et al. 1997); 1492ra, ACGGHTACC TTGTTACGACTT (Grant et al. 1999)]. Replicate PCRs were pooled, separated on agarose gels, and the ~1.5 kb DNA band cloned using the Invitrogen TOPO TA cloning

Table 1 Water sample characteristics, microscopic and 16S rRNA gene diversity analyses

Sample sites	Coordinates	Rainfall (mm)[a]	Temperature (°C)[a]	pH	SG (g/ml)	Total salt (w/v) (%)	Cell count (cells/ml)	Sequences (*n*)	OTU (97%)	Diversity indices				
										H	E_H	1-*D*	Chao	ACE
Dry Creek, SA, crystalliser 7	34°48′48.03″S 138°35′1.09″E	445	11–21	7.24	1.23	34	$2.02 \pm 0.33 \times 10^7$	41	15	2.10	0.81	0.78	39	49
Bajool, Qld., crystalliser 4	23°37′13.72″S 150°43′11.29″E	681	17–28	7.15	1.23	34	$2.66 \pm 0.37 \times 10^7$	40	9	1.64	0.74	0.76	13	13
Lara, Vic., storage pond 1	38°4′10.61″S 144°26′3.81″E	446	9–20	7.07	1.24	34	$1.20 \pm 0.16 \times 10^7$	40	11	1.89	0.80	0.79	15	17

All indices were calculated using the MOTHUR package, version 1.4 (http://schloss.micro.umass.edu/mothur/Main_Page)

SA South Australia, *Qld.* Queensland, *Vic* Victoria, *SG* specific gravity, *H* Shannon–Weaver index of diversity, E_H evenness, *D* Simpson index of diversity, presented as 1-*D*, *Chao* Chao 1 richness estimate, *ACE* ACE richness estimate

[a] Average annual rainfall, and average annual maximum and minimum temperatures. Data from the Australian Bureau of Meteorology website (http://www.bom.gov.au)

kit. Clone sequences were screened for chimeric PCR products using the online program Bellerophon ver.3 (http://greengenes.lbl.gov/) (Huber et al. 2004). Sequences were then used to reconstruct phylogenetic trees using the ARB phylogeny software package by Ludwig et al. (1998) (http://www.arb-home.de). Sequences were imported into ARB, aligned with the full-length 16S rRNA gene sequences of the Greengenes database (http://greengenes.lbl.gov), checked for consistency and, where necessary, manually adjusted and added to the main backbone tree using the parsimony addition methods within ARB. The maximum likelihood algorithm was used for full tree reconstruction, as implemented within the ARB package (AXML). Bootstrap values were assigned based on 1000 replicate tree reconstructions using distance (NJ/Felsenstein correction) or parsimony (PHYLIP/DNAPARS) algorithms. Diversity indices, OTU clustering, and multiple sample statistics were computed using the MOTHUR suite of programs, version 1.4, available at http://schloss.micro.umass.edu/.

Sequences

The 16S rRNA gene sequences retrieved in this study have been deposited at Genbank (www.ncbi.nlm.nih.gov), and have accession numbers GQ374922–GQ375042.

Results

Sample sites, water chemistry and microscopy

Three coastal, solar salterns, at widely differing locations in Australia, were selected. These were in South Australia (Dry Creek), Victoria (Lara), and Queensland (Bajool). The Bajool site is about 1700 km from either the Lara or Dry Creek sites, and it is about 640 km between the latter two sites (Fig. 1). Crystallizer pond samples were collected prior to harvesting of salt in May 2007, and were at salt saturation (~34% w/v). Sample locations, climatic data, water analyses and cell counts are presented in Table 1, and the detailed water chemistry is given in supplementary Table 1. The salt compositions were similar to those reported for other marine solar salterns (Burns et al. 2004a), and all had total cell counts above 10^7 cells/ml, with high proportions (>40%) of cells displaying the typical morphological characteristics of *Hqr. walsbyi* (Burns et al. 2007; Walsby 1980).

Archaeal diversity

To assess the diversity of *Archaea* present in each water sample, cells were collected by centrifugation, their DNA

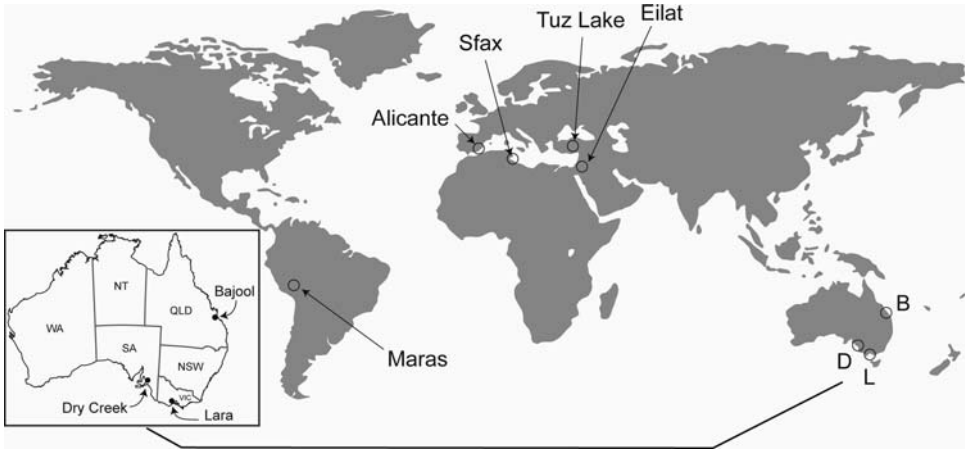


Fig. 1 Map showing the locations of sample sites in Australia that were used in this study (Bajool, Lara, Dry Creek). These are indicated as *black circles* on a world map, and on the enlargement of Australia shown in *left*, *lower box*. The state boundaries and names are indicated on the enlargement. The world map also indicates the sites where the *Hqr. walsbyi* 16S rRNA gene sequences presented in Table 2 were recovered. For the small map of Australia, the letters indicate *B* Bajool, *L* Lara, *D* Dry Creek

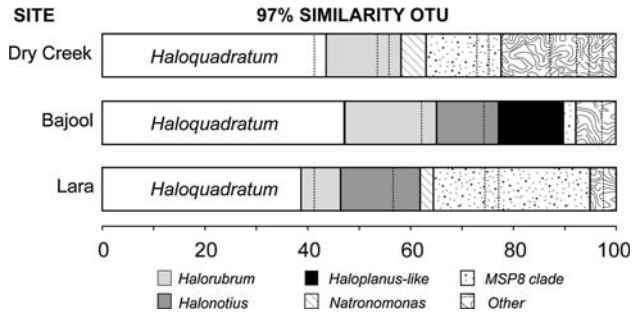


Fig. 2 Diversity of archaeal clone library sequences present in saltern crystalliser ponds at the three sites. Cloned 16S rRNA genes were sequenced, and the aligned sequences were collected into OTU at the 97% sequence similarity level (furthest neighbor, MOTHUR). The sample site is stated at the *left* of each bar. Some OTUs were closely related to validly described members of the family Halobacteriaceae (see Fig. 3) and, where appropriate, these have been clustered together with the same fill shade/pattern, and delimited with solid vertical lines. The key below indicates the taxon to which each shade/pattern cluster corresponds. *Dashed vertical lines* mark off individual OTUs within clusters, and the entire bar length for each site represents 100% of the sequences from that site, as indicated by the scale below. See the text and Fig. 3 for details about the MSP8 clade. Haloarchaeal sequences not closely related to the six designated groups in the figure are collected together under other

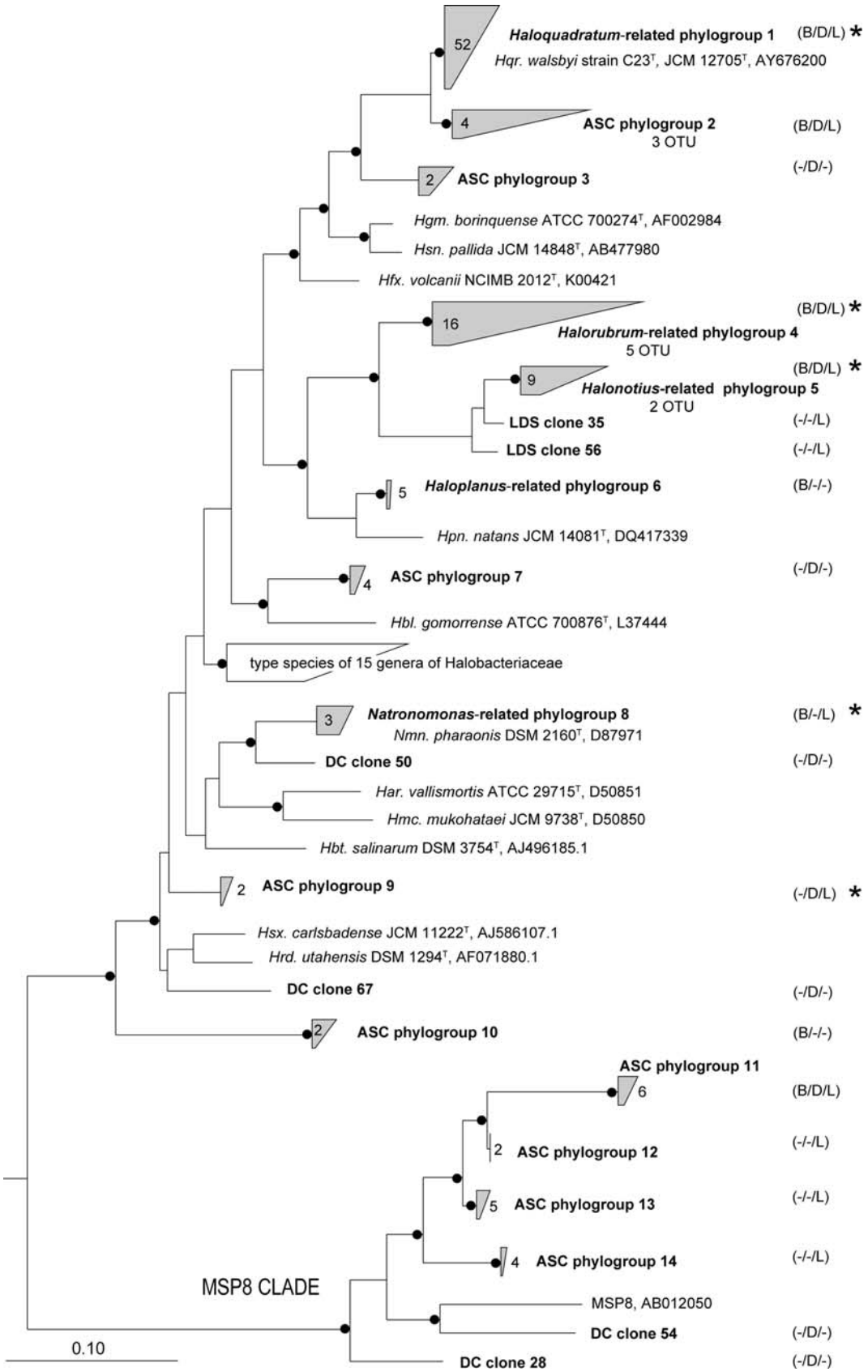
extracted, and 16S rRNA genes PCR amplified using haloarchaeal-specific primers. The products were cloned into TOPO plasmids and sequenced (see Methods), and about 40, near-complete 16S rRNA gene sequences (average of 1,440 nt) were determined for each sample. Table 1 summarizes the numbers of clones sequenced, the number of 97% sequence similarity OTUs, indices of community variation and estimates of species richness. Sequences were imported into the ARB phylogeny package, aligned to the Greengenes pre-aligned dataset (http://greengenes.lbl.gov), and compared by percentage similarity (Fig. 2) and phylogenetic tree reconstructions (Fig. 3).

As expected from the initial microscopy, sequences related to *Haloquadratum* dominated the clone libraries of

Fig. 3 Phylogenetic tree reconstruction using cloned 16S rRNA gene sequences derived from three saltern crystallizer ponds (see "Materials and methods" for details). Shown is a representative Maximum Likelihood tree (AxML, ARB phylogeny package). Bootstrap confidence values, derived from 1000 distance matrix replications, are indicated by *filled circles* (>75%) at branch points. Representatives of the currently recognized members of the family Halobacteriaceae were included, and a methanogen sequence was used as an outgroup (*Methanosaeta concilii*). Organism names are shown with culture collection numbers and sequence database accessions. The *Halonotius* reference sequence in phylogroup 5 was AY498641. For convenience, the branches of sequences belonging to single or closely related 97%-OTUs have been collapsed into numbered phylogroups, prefixed either with a name of a related taxon, or with the acronym ASC (Australian Saltern Crystallizer). Where phylogroups contain more than one OTU, this is indicated below the phylogroup name. Numbers in or next to the trapeziums representing collapsed branches are the number of clone sequences. Single clone sequences that formed isolated branches are named according to site of origin and clone or isolate number (e.g., DC clone 50, indicating a clone from Dry Creek). The sites of origin of sequences are indicated in *brackets* at the *right* of the tree by: *B* Bajool; *D* Dry Creek; *L* Lara; or −, absent; and a nearby *asterisk* indicating isolates belonging to this phylogroup was recovered

all three samples, representing just over 40% of the sequences at each site (Fig. 2). *Halorubrum*-related sequences were also significant at all sites (8–20%), while *Halonotius*-related sequences were present at two sites (10–15%). The latter group was identified in a previous study (Burns et al. 2004a), and has recently been formally described (Burns et al. 2009a). The number of 97%-similarity groups (97%-OTUs) varied between 9 and 16, a relatively low number compared to the microbial communities found in non-extreme environments (Janssen et al. 2002), but typical for crystallizer ponds (Benlloch et al. 2002; Pašić et al. 2007). Each site possessed sequences representing novel taxa, and these were highly variable between the sample sites, with many showing very low levels of similarity to classified taxa of the Halobacteriaceae.

The relationships between the archaeal sequences recovered from the three sites are more clearly seen when


52
Haloquadratum-related phylogroup 1
(B/D/L) *
Hqr. walsbyi strain C23T, JCM 12705T, AY676200
4
ASC phylogroup 2
3 OTU
(B/D/L)
2
ASC phylogroup 3
(-/D/-)
Hgm. borinquense ATCC 700274T, AF002984
Hsn. pallida JCM 14848T, AB477980
Hfx. volcanii NCIMB 2012T, K00421
16
Halorubrum-related phylogroup 4
5 OTU
(B/D/L) *
9
Halonotius-related phylogroup 5
2 OTU
(B/D/L) *
LDS clone 35
(-/-/L)
LDS clone 56
(-/-/L)
5
Haloplanus-related phylogroup 6
(B/-/-)
Hpn. natans JCM 14081T, DQ417339
4
ASC phylogroup 7
(-/D/-)
Hbl. gomorrense ATCC 700876T, L37444
type species of 15 genera of Halobacteriaceae
3
Natronomonas-related phylogroup 8
(B/-/L) *
Nmn. pharaonis DSM 2160T, D87971
DC clone 50
(-/D/-)
Har. vallismortis ATCC 29715T, D50851
Hmc. mukohataei JCM 9738T, D50850
Hbt. salinarum DSM 3754T, AJ496185.1
2
ASC phylogroup 9
(-/D/L) *
Hsx. carlsbadense JCM 11222T, AJ586107.1
Hrd. utahensis DSM 1294T, AF071880.1
DC clone 67
(-/D/-)
2
ASC phylogroup 10
(B/-/-)
ASC phylogroup 11
6
(B/D/L)
2
ASC phylogroup 12
(-/-/L)
5
ASC phylogroup 13
(-/-/L)
4
ASC phylogroup 14
(-/-/L)
MSP8 CLADE
MSP8, AB012050
DC clone 54
(-/D/-)
0.10
DC clone 28
(-/D/-)

presented as a phylogenetic tree reconstruction, as shown in Fig. 3, which includes all the clone sequences as well as some isolate sequences from this study (see below) along with representatives of 27 genera of the Halobacteriaceae. For convenience, closely branching sequences have been collapsed into numbered phylogroups, most of which consist of sequences belonging to the same 97%-OTU. Where a phylogroup contains more than one OTU, this is indicated under the name. Single sequences that branch separately from the others are named according to site and clone number (e.g., LDS clone 35). 51 clone sequences were closely similar to each other and to the rRNA gene sequence of *Hqr. walsbyi* C23^T (analyzed in more detail below), and they branched tightly together forming *Haloquadratum*-related phylogroup 1. By comparison, the next most dominant phylogroup, *Halorubrum*-related phylogroup 4, was considerably more divergent, as denoted in Fig. 3 by differences in their rightwards-extending peaks, and the number of 97%-OTUs. Several minor groups are scattered throughout the tree, and show varying affinities to currently classified taxa. A particularly deeply branching group of related sequences (bottom of Fig. 3) was designated the MSP-clade (discussed further below), and contained sequences from all three sites.

In parallel to the culture-independent assessment of archaeal diversity, cultivation was also used in order to confirm, and possibly extend, the diversity captured by the PCR-generated clone library sequences. Using the methods described in our previous studies, water samples were plated onto solid media (MGM), and also titrated in DBCM2 liquid medium (extinction cultures), and incubated for at least 12 weeks (plates) or 8 weeks (liquid). A total of 201 pure isolates of haloarchaea were recovered, and a random selection of 132 of these had near-complete 16S rRNA gene sequences determined. Due to the biases involved in cultivation, and that many isolates had nearly identical 16S rRNA gene sequences, only a few of these sequences were included in the tree reconstruction shown in Fig. 3 (see legend). Cultivation results are summarized at the right side of the tree, where asterisks indicate the phylogroups for which isolates were recovered. Representatives of five phylogroups were cultivated, including the major common clades related to *Haloquadratum*, *Halorubrum*, and *Halonotius*. The sequences of isolates closely matched (≥99% identity) clone library sequences of their corresponding phylogroup, excepting phylogroup 9, for which only isolates were recovered. The closest full-length matches in Genbank to the two phylotype 9 sequences showed only 95% identity, e.g., accession GQ282621 (isolate TNN28, saltern crystallizer, China). For many of the phylogroups and deeply branching clone sequences, isolates were not recovered, including members of the MSP8-clade. Seven phylogroups and four clones showed only ≤90% sequence identity to classified taxa, and so appear to represent novel genera.

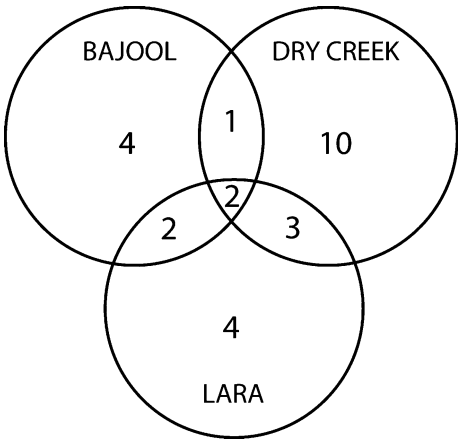


Fig. 4 Venn diagram showing the shared and unique 97%-OTUs between the three sample sites. The same clone and isolate sequences from this study that were included in Fig. 3 were also used in this figure. Analysis and diagram generation were performed using the MOTHUR v 1.4 suite of programs (http://schloss.micro.umass.edu/)

A Venn diagram shown in Fig. 4 summarizes the shared and site-specific diversity of 97% OTUs for the three sites, using all the sequence data from both clones and isolates. Apart from the two clades found at all sites, there are 4–10 97%-OTUs that are found only at a single site, and 1–3 that are shared between two of the three sites.

Looking more closely at the *Haloquadratum*-related sequences of phylogroup 1 (Fig. 3), the 52 sequences determined in this study differed from each other and from the type strain, *Hqr. walsbyi* C23^T, by ≤2%. A more closely related subgroup of these sequences that differed from each other by ≤0.5% included members originating from all three sites, while even more similar sequences (99.7% identity) were shared between the Bajool and Dry Creek sites, and between the Bajool and Lara sites. Since the *Hqr. walsbyi* genome contains two 16S rRNA genes that differ by 3 positions (0.2% divergence, strain C23^T, unpublished data), at least some of the divergence observed between very similar clone sequences could derive from differences in paralogous genes. Indeed, in an alignment of these clone sequences, the same 3 positions were hypervariable, and two displayed the same base variation as seen between the C23^T copies, i.e., C↔T at *E. coli* positions 198 and 845.

To compare the sequences derived from this study with those from other sites, *Hqr. walsbyi*-related 16S rRNA gene sequences were retrieved from Genbank by a BLASTN search using the C23^T sequence. 28 sequences of at least 1400 nt, of good quality and non-chimeric (Bellerophon checked), and that were deposited as part of a published study, were retained and aligned with C23^T

Table 2 *Haloquadratum* sequences reported by other published studies

Countries (site)	Sequence origin	Accessions	Similarity to C23^T (%)	References
Israel (Eilat)	1 PCR-clone/ArcA11	DQ103682	99.6	Sorensen et al. (2005)
Peru (Maras)	Clone 2A5[a]	AY987831	98.9	Maturrano et al. (2006)
Spain (Alicante)	Cosmid clone 7B05	85372643	99.6	Legault et al. (2006)
	Cosmid clone 2B08	85680316	99.7	
	Hqr. walsbyi HBSQ001	110666976	99.9	Bolhuis et al. (2006)
Tunisia (Sfax)	12 PCR-clones/SFE1D061	CU467224[b]	99.2–99.8	Baati et al. (2008)
	9 PCR-clones/SFE1A101	CU467209[c]	99.3–99.7	
Turkey (Tuz Lake)	DGGE[d] band 4A	–	99	Mutlu et al. (2008)
Turkey (coastal)	DGGE[d] band 8A	–	100	
Turkey (Tuz Lake)	PCR-clones/July	EF459726–9	99.3–99.7	

[a] The *Haloquadratum* sequences from this study contained ambiguous bases, so only one representative sequence was used here

[b] Representative clone of 12 clones, forming the dominant archaeal OTU in crystallizer pond S5

[c] Representative clone of nine clones, forming the third most dominant archaeal OTU in crystallizer pond S5

[d] *DGGE* Denaturing gradient gel electrophoresis. These were sequences obtained from PCR amplified environmental DNA that was separated by DGGE

sequence (Table 2). Within this group of sequences were members that differed from *Hqr. walsbyi* C23^T by only 2–5 nt, and had been recovered from saltern crystallizer ponds in Spain (Bolhuis et al. 2006) (Legault et al. 2006), Israel (Sorensen et al. 2005), Turkey (Mutlu et al. 2008), Tunisia (Baati et al. 2008) and Peru (Maturrano et al. 2006) (see Fig. 1 for locations). Except for the high-altitude Peruvian saltern, all of the *Hqr. walsbyi*-related sequences recovered at the other sites differed by less than 1% from that of the (Australian) type strain. Data from the studies cited in Table 2, as well as other, previous published studies, indicate that *Hqr. walsbyi* was numerically significant at all of these sites (e.g., Oren et al. 2006), and at most sites it was the dominant archaeal clade.

By comparison, the *Halorubrum*-related sequences (phylogroup 4) displayed 7% sequence divergence, and this value was unchanged if the sequences of the 78 cultured isolates that branched with this phylogroup were included (data not shown). Similar divergence values have been reported in previous studies of natural isolates of *Halorubrum* spp. (Legault et al. 2006), and even within the classified species of this genus, the 16S rRNA gene sequence divergence is 7–8%.

Phylogroup 2 is a tight cluster of 4 OTUs, each consisting of a single clone sequence. The sequences branched closely to each other and to phylogroup 1. Two sequences shared 97% sequence similarity to the 16S rRNA gene of *Hqr. walsbyi* C23^T, and may represent novel species of *Haloquadratum*. The other two sequences were 96.4% similar to each other but only 93% similar to *Hqr. walsbyi* C23^T, and so probably represent different species of a separate, as yet uncultivated, genus (Fry et al. 1991).

The closest matching sequences in Genbank to the other phylogroups and phylotypes are given in supplementary Table 2. Phylogroup 3 was represented by two clone sequences, and these were 97% identical to an isolate (CSW 2.24.4) recovered previously in this laboratory from the Moolap saltern crystallizer, 10 km from the Lara site (Burns et al. 2004a). Phylogroup 5 includes clones and isolates, and represents a new genus within the Halobacteriaceae, with the type species being *Halonotius pteroides* strain 1.15.5 (Burns et al. 2009a). The latter strain was also isolated from the Moolap site. Phylogroup 8 sequences are closely related to *Natronomonas pharaonis*, a haloalkaliphile isolated from an African soda lake with a pH around 10. *Nmn. pharaonis* has an optimum pH of 8.5 but the sites examined in the current study were all around pH 7. A new species of this genus has recently been proposed to accommodate such neutrophilic members (Burns et al. 2009b).

MSP8-clade of related phylogroups

19 clone sequences, forming six separate 97%-OTUs branched together in phylogenetic tree reconstructions, and deeply away from the other taxa and phylogroups (Fig. 3). The level of sequence identity of members of this clade to recognized taxa was around 74% but chimera-checking programs indicated that they were valid rRNA sequences. The clade was named after one of two related clone sequence that were recovered from an alkaline saltern pond in east Africa in 1996 (Grant et al. 1999), and that branched

specifically with the Australian sequences. The MSP8 sequence shares only 86–89% similarity to the Australian sequences, and across the entire MSP-clade there was a similar level of divergence (86–87% similarity).

Discussion

The dominant haloarchaeal group at three, geographically distant, Australian crystallizer ponds, was *Hqr. walsbyi*, and the great majority of the recovered 16S rRNA gene sequences related to this taxon diverged by less than 2% from each other, and from the type strain of this genus ($C23^T$). Sequences 99.5% identical to the type strain were present at all sites, and previous studies of crystallizer ponds in Spain (Antón et al. 1999; Bolhuis et al. 2006; Legault et al. 2006), Tunisia (Baati et al. 2008), Turkey (Mutlu et al. 2008) and Israel (Sorensen et al. 2005) have recovered sequences that show similarly close relationships to the type strain, and to each other. The type strain was isolated in 2004 from the Cheetham saltern in Moolap, Australia, only 10 km away from the Lara site examined in the present study. In our study of the Moolap site in 2004, twenty 16S rRNA gene sequences were recovered, and while most were <600 nt, they also indicated ≥99% identity to *Hqr. walsbyi* $C23^T$ (Burns et al. 2004a). Preliminary data from the genome sequencing of the $C23^T$ strain indicate that 85% of its genome sequence is closely related to that of strain HBSQ001, and that these shared sequences are, on average, 98.6% identical (manuscript in preparation). This comparison supports the close relationship of these strains inferred from 16S rRNA gene sequence data. The combined evidence points to a globally distributed and strongly coherent species that has remained dominant for at least a decade (Antón et al. 1999; Burns et al. 2004a, b; Legault et al. 2006; this study). It coexists and competes with other haloarchaea, such as *Halorubrum* that, as judged by 16S rRNA gene sequence data, display significantly greater genetic diversity.

Divergence within and between species of prokaryotes has become much easier to quantitate with the availability of many complete genome sequences. Within the Bacteria, some species show high levels of strain variation, such as those of *Pseudomonas* and *Bacillus*, while others show extremely limited divergence, particularly pathogens and obligate endosymbionts (Deloger et al. 2009). Even free-living species that usually dominate the microbial flora of specific environments show differing levels of variation. For example, the marine bacterium "Pelagibacter" (Wilhelm et al. 2007) shows considerably higher variation compared to the very limited diversity among strains of *Candidatus Accumulibacter phosphatis*, a bacterium observed in sludge bioreactors in the US and Australia (Kunin et al. 2008). Clearly, hypersaline environments also possess dominant groups that differ in their level of genetic variation, i.e., *Haloquadratum* versus *Halorubrum*. Genomic and metagenomic studies of *Haloquadratum* should help pinpoint the factors underlying these differences.

How the world-wide homogeneity of *Haloquadratum* maintained is yet to be understood but there are some grounds for proposing a combination of factors that could be important, including a global dispersal system, natural reservoirs, and strong selection pressures. Natural salt lakes often dry out completely, and the crystallizers of marine solar salterns are drained and refilled annually. Dispersal of *Haloquadratum* via the oceans is unlikely, as this organism cannot form spores, does not grow at salt concentrations less than about 14% w/v and lyses at low salt (2% w/v). However, survival inside the fluid inclusions of salt crystals has been shown for other haloarchaea (e.g., see Fendrihan et al. 2009 and the references cited), and this could allow dispersal by wind or by the migratory birds that frequent hypersaline waters. Recently, haloarchaea have been found as normal flora of the salt glands of a species of migratory bird (Brito-Echeverría et al. 2009). The ability to cope with cyclical, extreme conditions may also apply strong selective pressures that limit intraspecies divergence, and *Haloquadratum* may be better adapted to these different extremes. For example, in addition to growing well in saturating solutions of sodium chloride, *Haloquadratum* tolerates high magnesium concentrations that occur in crystallizer ponds after the precipitation of sodium chloride (Bolhuis et al. 2006). Indeed, the $C23^T$ strain achieves higher cell densities above 1 M $MgCl_2$ (Burns et al. 2007).

A surprising feature of the archaeal diversity seen in this study was the presence and relatively high frequency of sequences forming the MSP8 clade, that branched very deeply from the other haloarchaea (86–87% nt similarity). The first reported examples of such sequences (MSP8, MSP41) were recovered from a study of Lake Magadi, an East African soda lake, in 2000 (Grant et al. 1999). Similar sequences have been rarely detected, but a 2008 study of saltern ponds in Tunisia (Baati et al. 2008) recovered one clone sequence (accession FN391274) that was most closely related to phylogroups 12 and 13 (97% similarity) in the current study. The deep branching of the MSP8 clade makes it an important target for cultivation studies, to determine if the metabolic capabilities of these species differ appreciably from those of the classified haloarchaea.

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References

Antón J, Llobet-Brossa E, Rodríguez-Valera F, Amann R (1999) Fluorescence in situ hybridization analysis of the prokaryotic community inhabiting crystallizer ponds. Environ Microbiol 1:517–523

Antón J, Oren A, Benlloch S, Rodríguez-Valera F, Amann R, Rossello-Mora R (2002) *Salinibacter ruber gen.* nov., sp. nov., a novel, extremely halophilic member of the Bacteria from saltern crystallizer ponds. Int J Syst Evol Microbiol 52:485–491

Baati H, Guermazi S, Amdouni R, Gharsallah N, Sghir A, Ammar E (2008) Prokaryotic diversity of a Tunisian multipond solar saltern. Extremophiles 12:505–518

Benlloch S, López-López A, Casamayor EO, Øvreås L, Goddard V, Daae FL, Smerdon G, Massana R, Joint I, Thingstad F, Pedrós-Alió C, Rodríguez-Valera F (2002) Prokaryotic genetic diversity throughout the salinity gradient of a coastal solar saltern. Environ Microbiol 4:349–360

Bidle KA, Amadio W, Oliveira P, Paulish T, Hicks S, Earnest C (2005) A phylogenetic analysis of haloarchaea found in a solar saltern. BIOS 76:89–96

Bolhuis H, Palm P, Wende A, Falb M, Rampp M, Rodriguez-Valera F, Pfeiffer F, Oesterhelt D (2006) The genome of the square archaeon *Haloquadratum walsbyi*: life at the limits of water activity. BMC Genomics 7:169

Bolhuis H, Poele EM, Rodriguez-Valera F (2004) Isolation and cultivation of Walsby's square archaeon. Environ Microbiol 6:1287–1291

Bowman JP, McCammon SA, Rea SM, McMeekin TA (2000) The microbial composition of three limnologically disparate hypersaline Antarctic lakes. FEMS Microbiol Lett 183:81–88

Brito-Echeverría J, López-López A, Yarza P, Antón J, Rosselló-Mora R (2009) Occurrence of *Halococcus* spp. in the nostrils salt glands of the seabird Calonectris diomedea. Extremophiles 13:557–565

Burns DG, Camakaris HM, Janssen PH, Dyall-Smith ML (2004a) Combined use of cultivation-dependent and cultivation-independent methods indicates that members of most haloarchaeal groups in an Australian crystallizer pond are cultivable. Appl Environ Microbiol 70:5258–5265

Burns DG, Camakaris HM, Janssen PH, Dyall-Smith ML (2004b) Cultivation of Walsby's square haloarchaeon. FEMS Microbiol Lett 238:469–473

Burns DG, Janssen PH, Itoh T, Kamekura M, Li Z, Jensen G, Rodríguez-Valera F, Bolhuis H, Dyall-Smith ML (2007) *Haloquadratum walsbyi* gen. nov., sp. nov., the square haloarchaeon of Walsby, isolated from saltern crystallizers in Australia and Spain. Int J Syst Evol Microbiol 57:387–392

Burns DG, Janssen PH, Itoh T, Kamekura M, Echigo A, Dyall-Smith DL (2009a) *Halonotius pteroides* gen. nov., sp. nov., an extremely halophilic archaeon recovered from a saltern crystallizer in southern Australia. Int J Syst Evol Microbiol. doi: 10.1099/ijs.0.010017-0

Burns DG, Janssen PH, Itoh T, Minegishi H, Usami R, Kamekura M, Dyall-Smith ML (2009b) *Natronomonas moolapensis* sp. nov., two non-alkaliphilic isolates recovered from a solar saltern crystallizer pond. Int J Syst Evol Microbiol. doi:10.1099/ijs.0.010132-0

Cuadros-Orellana S, Martin-Cuadrado AB, Legault B, D'Auria G, Zhaxybayeva O, Papke RT, Rodriguez-Valera F (2007) Genomic plasticity in prokaryotes: the case of the square haloarchaeon. ISME J 1:235–245

Deloger M, El Karoui M, Petit MA (2009) A genomic distance based on MUM indicates discontinuity between most bacterial species and genera. J Bacteriol 191:91–99

Dyall-Smith ML (2001) The Halohandbook: protocols for halobacterial genetics. http://www.haloarchaea.com/resources/halohandbook/

Fendrihan S, Berces A, Lammer H, Musso M, Ronto G, Polacsek TK, Holzinger A, Kolb C, Stan-Lotter H (2009) Investigating the effects of simulated Martian ultraviolet radiation on *Halococcus dombrowskii* and other extremely halophilic Archaebacteria. Astrobiology 9:104–112

Fry NK, Warwick S, Saunders NA, Embley TM (1991) The use of 16S ribosomal RNA analyses to investigate the phylogeny of the family Legionellaceae. J Gen Microbiol 137:1215–1222

Grant S, Grant WD, Jones BE, Kato C, Li L (1999) Novel archaeal phylotypes from an East African alkaline saltern. Extremophiles 3:139–145

Huber T, Faulkner G, Hugenholtz P (2004) Bellerophon; a program to detect chimeric sequences in multiple sequence alignments. Bioinformatics 20:2317–2319

Ihara K, Watanabe S, Tamura T (1997) *Haloarcula argentinensis* sp. nov. and *Haloarcula mukohataei* sp. nov., two new extremely halophilic archaea collected in Argentina. Int J Syst Bacteriol 47:73–77

Janssen PH, Yates PS, Grinton BE, Taylor PM, Sait M (2002) Improved culturability of soil bacteria and isolation in pure culture of novel members of the divisions *Acidobacteria*, *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia*. Appl Environ Microbiol 68:2391–2396

Kunin V, He S, Warnecke F, Peterson SB, Garcia Martin H, Haynes M, Ivanova N, Blackall LL, Breitbart M, Rohwer F, McMahon KD, Hugenholtz P (2008) A bacterial metapopulation adapts locally to phage predation despite global dispersal. Genome Res 18:293–297

Legault B, Lopez-Lopez A, Alba-Casado J, Doolittle W, Bolhuis H, Rodriguez-Valera F, Papke T (2006) Environmental genomics of "*Haloquadratum walsbyi*" in a saltern crystallizer indicates a large pool of accessory genes in an otherwise coherent species. BMC Genomics 7:171

Ludwig W, Klugbauer S, Weizenegger M, Neumaier J, Bachleitner M, Schleifer KH (1998) Bacterial phylogeny based on comparative sequence analysis. Electrophoresis 19:554–568

Maturrano L, Santos F, Rosselló-Mora R, Antón J (2006) Microbial diversity in Maras salterns, a hypersaline environment in the Peruvian Andes. Appl Environ Microbiol 72:3887–3895

Mutlu MB, Martínez-García M, Santos F, Peña A, Guven K, Antón J (2008) Prokaryotic diversity in Tuz Lake, a hypersaline environment in inland Turkey. FEMS Microbiol Ecol 65:474–483

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 (2002) Molecular ecology of extremely halophilic Archaea and Bacteria. FEMS Microbiol Ecol 39:1–7

Oren A, Pri-El N, Shapiro O, Siboni N (2006) Buoyancy studies in natural communities of square gas-vacuolate archaea in saltern crystallizer ponds. Saline Systems 2:4

Pašić L, Ulrih NP, Črnigoj M, Grabnar M, Velikonja BH (2007) Haloarchaeal communities in the crystallizers of two adriatic solar salterns. Can J Microbiol 53:8–18

Sorensen KB, Canfield DE, Teske AP, Oren A (2005) Community composition of a hypersaline endoevaporitic microbial mat. Appl Environ Microbiol 71:7352–7365

Walsby AE (1980) A square bacterium. Nature (London) 283:69–71

Wilhelm LJ, Tripp HJ, Givan SA, Smith DP, Giovannoni SJ (2007) Natural variation in SAR11 marine bacterioplankton genomes inferred from metagenomic data. Biol Direct 2:27