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Full Length Research Paper

Assessment of alkaliphilic haloarchaeal diversity in Sua pan evaporator ponds in Botswana

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Cultivation-dependent and molecular-based culture-independent methods were used to assess alkaliphilic haloarchaeal diversity at Sua pan evaporator ponds in Botswana. Isolates belonging to the genera *Natrialba*, *Natronococcus* and *Natronorubrum* were recovered from brine samples by enrichment and identified through a series of biochemical tests as well as sequencing of 16S rRNA fragments. In addition, an environmental 16S rRNA library was constructed from brine samples of two evaporator ponds. The library comprised members of the genera *Halorubrum* (65%), *Natrialba* (14%), *Natronorubrum* (7%) and new phylotypes (14%). The new phylotypes consisted of two clones that exhibited low 16S rRNA similarity (95 – 97%) with known species and could potentially represent new species in the genus *Halorubrum,* one clone with 91% similarity to *Natronolimnobius* which could represent a new genus, as well as an unidentifiable phylotype which exhibited 79% similarity to *Methanotorris formicicus*. Two major differences were observed between cultivation- and molecular-based methods; firstly, *Halorubrum* species were largely represented in the environmental clone library but no isolates were obtained, and secondly, *Natronococcus* species were isolated but not detected in the clone library. An overlap between the archaeal isolates and the ribosomal library clones was apparent although the novel phylotypes detected in this study were not recovered through cultivation.

Key words: Haloalkaliphile, solar salterns, *Natronococcus*, *Natrialba*, *Natronorubrum*, biodiversity.

INTRODUCTION

The microbial diversity of hypersaline environments including the Dead Sea, solar salterns and soda lakes has been studied previously using both culture-independent molecular ecology methods as well as culture-based methods (Grant et al., 1999; Benlloch et al., 2001; Ochsenreiter et al., 2002; Burns et al., 2004; Rees et al., 2004; Maturrano et al., 2006). Quite often, molecular and culture-based methods yield different results and in some cases lower diversity is recovered by molecular techniques than culturable diversity, while in other instances the cultivated isolates are not related to the sequences retrieved directly from the environmental samples (Benlloch et al., 2001; Ochsenreiter et al., 2002; Burns et al., 2004; Maturrano et al., 2006). Archaeal diversity at a genus level is generally very low in these environments. However, there is considerable microdiversity, which is exhibited by the high number of strains of different species within a single genus (Benlloch et al., 2001; Maturrano et al., 2006).

Alkaliphilic haloarchaea are a specialized group of obligate extreme halophiles that, in addition to halophily also require high pH (between 8.5 and 11) and low Mg^{2+} for growth (Kanai et al., 1995; Kamekura et al., 1997; Xu et al., 2001). They are commonly isolated from soda lakes but they have also been previously isolated and detected from solar salterns and other hypersaline environments across acidic, neutral and alkaline pHs (Ochsentreiter et al., 2002; Burns et al., 2004). These haloarchaea are currently distributed in the genera *Natronobacterium*, *Natronococcus*, *Natronomonas*, *Natronolimnobius*, *Halalkalicoccus* which are exclusively alkaliphilic, and *Haloarcula*, *Halobaculum*, *Haloferax*, *Halobiforma, Natronorubrum*, *Natrialba*, as well as *Halorubrum* which comprise both alkaliphilic and neutrophilic species (Grant et al., 2004; Enache et al., 2007).

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Halorubrum and *Haloarcula* spp often form the majority of isolates obtained from neutral brines (Grant et al., 2004).

Makgadikgadi salt pans in Botswana cover an area of about 12,000 km^2 and consist of two major pans namely; Ntwetwe which is approximately 106 × 96 km and Sua pan which is 112 × 72 km (Hughes and Hughes, 1992). Botswana Ash (Pty) Ltd. manages an extensive network of solar salterns on the northern part of Sua pan from which the company produces NaCl, Na_2CO_3, Na_2SO_4 and $NaHCO_3$ salts (Eckardt et al., 2008). The brine at Sua pan is typically alkaline with a pH of 8.5 – 10, and contains a higher proportion of Na^+ and HCO_3^- and less K^+, Mg^{2+} and SO_4^{2+} than other salt pans in Southern Africa (McCullosh et al., 2008). These characteristics are similar to those of east African soda lakes such as Lake Magadi, Bogoria and Natron on the Kenyan-Tanzanian Rift Valley (Grant et al., 1999).

While studies have been carried out on the hydrogeochemistry of Sua pan (Seaman et al., 1991; White and Eckardt, 2006; Eckardt et al., 2008), little is known about the microbial ecology of the solar salterns derived from these pans. Therefore, the purpose of the current study was to assess the diversity of alkaliphilic haloarchaea in the Sua pan evaporator ponds using both culture-dependent and culture-independent methods.

MATERIALS AND METHODS

Sample collection

Fifty millilitres of brine were collected in triplicate from three different ponds (CS, E0, E4) at Sua pan solar salterns in Botswana in January 2004. The samples were kept on ice packs during transportation. The NaCl concentration of the ponds on the day of sampling ranged between (14 – 18% w/v) based on regular measurements performed on site by Botswana Ash (Pty) Ltd using titration with $AgNO_3$ (Denny, 1927). A more detailed chemical analysis of the brine samples was carried out by the Institute of Groundwater Studies at the University of the Free State.

Isolation of haloalkaliphilc archaea

Twenty five millilitres of brine samples from ponds CS, E0 and E4 were transferred to three 250 mL Erlenmeyer flasks and mixed with 25 mL of medium containing 2% (w/v) glucose and 1% (w/v) yeast extract resulting in an initial NaCl concentration of ± 8% (w/v). The flasks were incubated for 5 weeks at 37°C with shaking at 150 rpm. Crystalline NaCl was baked at 100°C for 5 h and added to the cultures on a weekly basis at a concentration increase of 5% (w/v) for three weeks, resulting in a final NaCl content of ± 23% (w/v). One hundred microlitre samples were collected weekly and spread on alkaliphile medium composed of 200 g NaCl, 2 g KH_2PO_4, 5 g casamino acids, 18 g Na_2CO_3, 20 g bacteriological agar and 1 mL trace metals per litre (Dyall-Smith, 2000). Plates were incubated at 42°C for a minimum of 7 days or until growth was observed. The incubation temperature was changed due to the slow growth of the archaeal isolates at 37°C. Single colonies were re-streaked onto fresh plates and examined microscopically to confirm purity. A rapid screen of the isolates was carried out through colony PCR using the primer set A751F (5′-CCGACGGTGAGRGRYGAA-3′) and UA1406R (5′-ACGGGCGGTGWGTRCAA-3′) (Baker et al., 2003). Colonies were picked using a toothpick and suspended in 20 µl of distilled water and heated for 5 minutes at 80°C. The cell debris was removed by centrifugation. Five microliter of the sample was then used for PCR reactions. PCR reactions were performed under the following conditions: initial denaturation at 94°C for 2 min; 25 cycles of 94°C (30 s), 55°C (45 s), 72°C (1 min) and a final extension at 72°C for 8 min. PCR amplicons were sequenced with both primers using an Applied Biosystems model 3130 X L genetic analyzer, incorporating the ABI Big Dye Terminator Cycle Sequencing kit version 3.1., and the amplicons with 100% identity were regarded as representing the same isolate. Isolates were then grouped into groups and representative isolates were then characterized further using phenotypic and biochemical properties.

Morphological characterization

Cultures were streaked out on the alkaliphile medium and incubated at 42°C for 12 days. The colonies were characterized according to Oren et al. (1997). Cell suspensions were prepared on glass slides in a drop of 0.9% (w/v) NaCl solution and air-dried. The cells were fixed with 2% acetic acid followed by Gram staining (Dussault, 1955). Cell morphology was observed at 1000 × magnification under oil emersion.

Biochemical characterization

Biochemical tests such as nitrate reduction, carbohydrate fermentation, starch and casein and gelatin hydrolysis and catalase activity were carried out in accordance with proposed minimal standards (Oren et al., 1997). All tests were carried out using the alkaliphile medium as the basic medium for growth. The medium was then supplemented with different substrates. Nitrate reduction was tested in liquid medium supplemented with 0.1% (w/v) KNO_3 while the production of acids from 1% (w/v) glucose, sucrose and lactose was examined in medium containing 0.001% phenol red (Tindall et al., 1984). To determine starch hydrolysis, archaeal isolates were streaked onto the alkaliphile medium supplemented with 1% (w/v) soluble starch and stained with iodine solution after growth was observed. Liquefaction of gelatin was tested using the alkaliphile medium supplemented with 12% (w/v) gelatin while casein hydrolysis was determined using milk agar (1% skim milk). Cultivations were carried out at 42°C for 14 days. Catalase activity was determined by adding drops of 3% H_2O_2 onto wet smear prepared from seven day old colonies. The smears were prepared on glass slides. All experiments were carried out in triplicate and the results were presented as the mean of three.

Antibiotic susceptibility tests

Antibiotic sensitivity tests were performed by spreading suspensions of actively growing cultures on alkaliphile agar medium and applying discs impregnated with the following antibiotics: Novobiocin (5 µg), Ampicillin (10 µg), Chloramphenicol (10 µg), Gentamicin (10 U), Penicillin G (10 U), Tetracycline (30 µg) and Eythromycin (15 µg). The results were recorded as sensitive or resistant after 14 days of incubation at 42°C. Isolates were defined as either sensitive or resistant in accordance with Performance Standards for Antimicrobial Susceptibility Testing; 16th Informational supplement (Clinical and Laboratory Standards Institute, 2006).

Physiological characterization

Optimum salt concentration, pH and temperature for growth were determined in liquid alkaliphile medium with shaking at 140 rpm and

Table 1. The elemental composition of evaporator ponds CS, E4 and E0.

Chemical	Evaporator pond		
	CS	E4	E0
Concn. (g/L)			
Na^+	59	65	67
K^+	5.25	4.5	4.1
Ca^{2+}	2.65	2.3	2
Mg^{2+}	1	0.92	1
Cl^-	82	83	86
SO_4^{2-}	4.8	3.7	3.7

growth was monitored by measuring optical density at 600 nm. Specific growth rates were determined. Pre-cultures were prepared in alkaliphile medium containing 20% NaCl (w/v) and used to inoculate 100 mL of the same medium at 1% inoculum size. The effect of NaCl on growth was determined by monitoring growth within a range of 10 – 30% NaCl at 37°C and pH 9. The effect of temperature was determined by monitoring growth of the archaeal isolates in the alkaliphile medium at 20% NaCl within the temperature range of 30 – 50°C. Optimum growth pH was determined by excluding Na_2CO_3 from the alkaliphile medium and rather adjusting the pH of the medium with 0.1 M phosphate-HCl buffer for pH 7 – 9 and 0.1 M phosphate-NaOH buffer for pH 10. Triplicate experiments were performed for all the tests.

16S rRNA sequence analysis

Genomic DNA was isolated using a rapid DNA isolation method (Dyall-Smith, 2000). A new set of archaeal specific primers, 6F (5′-ATTCCGGTTGATCCTGC-3′) and 1509R (5′-GYTACCTTGTTACGACTT-3′) were used to amplify the 16S rRNA using the following PCR programme: initial denaturation at 94°C for 2 min; 30 cycles of denaturation at 94°C (45s), annealing at 55°C (1 min), extension at 72°C (1 min 30 s); final extension at 72°C for 10 min. These primers were used as they amplified a larger fragment of the 16S rRNA gene and would allow better identification. The amplified products were excised from a 1% agarose gel and purified using QIAquick® Gel extraction kit from QIAGEN. The fragments were ligated to pGEM-T Easy and cloned in *E. coli* JM109, followed by routine plasmid isolation (Sambrook et al., 1989). The selected positive clones were sequenced by Inqaba Biotechnical Industries Pty. Ltd., South Africa, using an Applied Biosystems model 3130XL genetic analyzer, incorporating the ABI Big Dye Terminator Cycle Sequencing kit version 3.1. Both strands of the 16S rRNA were sequenced using the T7 and SP6 primers. The isolates were identified by carrying out BLASTn searches with 16S rRNA fragments of approximately 1400 bp.

Environmental 16S rRNA library construction

Fifty millilitres of brine samples from pond E0 and E4 were centrifuged for 15 min at 12 000 × *g* and DNA was extracted from the cell pellets using the method described in Halohandbook (Dyall-Smith, 2000). The sample from pond CS was excluded from this study due to poor DNA recovery. Community 16S rRNA genes were amplified from the DNA samples using the primer pair A751F (5′-CCGACGGTGAGRGRYGAA-3′) and UA1406R (5′-ACGGGCGGTGWGTRCAA-3′) (Baker et al., 2003). PCR reactions were performed under the following conditions: initial denaturation at 94°C for 2 min; 25 cycles of 94°C (30 s), 55°C (45 s), 72°C (1 min) and a final extension at 72°C for 8 min. The resulting amplicons were cloned *E. coli* JM109 cells as described above. Seventy two transformants were picked from each library and plasmid DNA was isolated. Purified plasmid DNA was digested with *Rsa*I, and a double digest of *Rsa*I and *Eco*RI. Fourteen clone groups were predicted based on the restriction patterns. The 16S rRNA gene was re-amplified from the plasmids and gel purified. The amplicons were then digested with *Rsa*I, *Hae*III and *Taq*I in separate reactions, followed by electrophoresis on 2% (w/v) agarose gel, to confirm the groups. The corresponding clones for each group were sequenced and identified by carrying out BLASTn searches of 655 bp 16S rRNA fragments.

Phylogenetic analysis of archaeal isolates and environmental clones

The 655 and 1400 bp 16S rRNA fragments obtained from environmental clones and archaeal isolates were compared by BLASTn search against related sequences available on the National Centre for Biotechnology Information (NCBI) database. The sequences were aligned with the ClustalW programme and edited using BioEdit Sequence Alignment Editor version 7.0.9.0 (Hall, 1999). Fragments of 630 bp were then used to construct a neighbour-joining tree using CLC Combined Workbench© (CLC bio, Denmark) for the environmental 16S rRNA clones while 1400 bp fragments were used to construct a tree of the archaeal isolates.

Nucleotide sequence accession numbers

The partial 16S rRNA gene sequences of the archaeal isolates and environmental clones from the current study have been deposited in GenBank under the accession numbers EU672837 – EU672841 and EU720380 – EU720393.

RESULTS

Properties of brine samples

The brine samples obtained from evaporator ponds CS, E4 and E0 were mainly of NaCl composition with very low levels of divalent cations such as Mg^{2+} and Ca^{2+} (Table 1). The cation pattern was $Na^+ > K^+ > Ca^{2+} > Mg^{2+}$. The brine was alkaline (pH 8 - 8.5) and the temperature of the ponds at the time of sampling was 25°C.

Identification and characterization of archaeal isolates

The diversity of cultivable alkaliphilic halorachaea was evaluated by culture enrichment followed by isolation on alkaliphile medium. Five representative isolates that formed different phenotypic characteristics were characterized further. The isolates displayed typical haloalkaliphilic growth characteristics with optimal growth at pH 9 – 10 (Table 2). Isolates Sua-CS1, Sua-E41, Sua-E42 and Sua-E01 grew optimally at 20% NaCl while Sua-E43 displayed optimum growth at 15%. Growth was observed at temperatures between 20 and 50°C, with

Table 2. Phenotypic characteristics and biochemical properties of the five archaeal isolates obtained from evaporator ponds through culture enrichment methods.

Property					
Strain number	**Sua-CS1**	**Sua-E41**	**Sua-E42**	**Sua-E43**	**Sua-E01**
Closest relative	***Natrialba chahannaoensis***	***Natronococcus occultus***	***Natrialba chahannaoensis***	***Natronococcus amylolyticus***	***Natronorubrum* sp.**
Cell shape	Rod	Cocci	Coccobacillus	Cocci	Rod
Gram stain	negative	positive	negative	positive	negative
Colony pigmentation, texture and diameter	Orange-Red; smooth; 1 - 1.5 mm	Orange-Red; rough; 2 mm	Red; smooth, raised; 1 - 1.5 mm	Orange-Red; smooth; 1 mm	Red; smooth 1 mm
Number of isolates	7	4	10	6	3
Optimum NaCl (% w/v)	20	20	20	15	20
Salt range (% w/v)	10 - 30	10 - 30	12 - 30	12 - 30	12 - 30
Optimum temp. (°C)	45	45	45	45	40
Optimum pH	9	10	9	9	10
pH range	8.0 - 11	8.0 - 10	8.0 - 11	8.0 - 11	8.0 - 11
Nitrate reduction	+	+	+	+	+
Catalase activity	+	+	+	+	+
Starch hydrolysis	+	-	-	+	-
Gelatin liquefaction	+	-	+	+	+
Casein hydrolysis	+	-	+	-	+

optimum growth at 45°C except for Sua-E01 which grew optimally at 40°C. Long lag-phases were observed at temperatures below 37°C. All the isolates were sensitive to novobiocin with inhibition zones of 10 – 16 mm diameter. Isolate Sua-E43 was also sensitive to erythromycin. All the isolates were resistant to penicillin, ampicillin, gentamicin, chloramphenicol as well as tetracycline. Nitrate reduction and catalase production was positive in all isolates. The isolates were identified as strains of *Natronococcus*, *Natrialba* as well as *Natronorubrum* species. Isolate Sua-CS1 and Sua-E42 displayed 99% similarity to each other and to *Natrialba chahannaoensis*. However, Sua-CS1 hydrolyzed starch while Sua-E42 did not hydrolyze starch (Table 2). Isolate Sua-E41 showed 98% similarity to *Natronococcus occultus* (1388/1409), while Sua-E43 displayed 99% similarity to an unidentified *Natronococcus* species (1395/1408) and 98% similarity to *Natronococcus amylolyticus* (1393/1408). The two *Natrononococcus* isolates only displayed 95% similarity to each other. Isolate Sua-E01 displayed 95% similarity to *Natronorubrum bangense* (1352/1411) and *Natronorubrum tibetense* (1354/1414).

Composition of the environmental clone library

A total of 144 clones were screened by restriction digestion using the endonucleases *Eco*RI, *Rsa*I, *Hae*III and *Taq*I. The clones were subsequently divided into 14 groups based on RFLP patterns and representatives of the groups were sequenced. *Halorubrum* species were the most abundant phylotypes and constituted about 72% of the total clones (Table 3). The representative clones affiliated to this genus displayed 96 – 98% similarity to each other. However, clones Sua-GRP9 and Sua-GRP11 were 99% similar. Clones Sua-GRP4 and Sua-GRP14 were affiliated to *Natrialba* species and they displayed 97% similarity to each other while Sua-GRP1 was affiliated to *Natronorubrum* species showing 98% similarity to *N. tibetense*. This clone also displayed 97 and 98% similarity to Sua-GRP4 and Sua-GRP14, respectively. Clones Sua-GRP5 and Sua-GRP12 only displayed 91 and 95% similarity to their closest relatives *Natronolimnobius innermongolicus* and *Halorubrum tebenquichense*, respectively while Sua-GRP6 showed low similarity (79%) to previously described taxa.

Phylogenetic placement of the archaeal isolates and 16S environmental clones

The phylogenetic affiliations of clones and isolates were studied using the 655 bp 16S rRNA gene fragment. The analysis revealed that the haloarchaeal population could be grouped into two clades. Clade one comprises species of *Natronorubrum*, *Natrialba*, and *Natronococcus*, while members of the genus *Halorubrum* formed the second clade (Figure 1). Clade one is further divided into three major clusters. *Natrialba* species form one big cluster which further separates into two subgroups. The two *Natrialba* isolates Sua-CS1 and Sua-E42 obtained in the current study cluster together with *Nab. chahannaoensis* with which 99% similarity is shared. In contrast, clone Sua-GRP4 associated with the second

Table 3. BLAST analysis of 16S rRNA sequences of representative groups in the environmental clone library.

Clone designation	Frequency (%)	Closest Validly Described Species	Accession number	% Similarity
Sua-GRP1	4.7	*Natronorubrum tibetense*	AB005656	99
Sua-GRP2	1.6	*Halorubrum aidingense* 31-Hong	DQ355813	98
Sua-GRP3	7.8	*Halorubrum xinjiangense*	AY510707	98
Sua-GRP4	4.7	*Natrialba hulunbeirensis*	AF262026	99
Sua-GRP5	14	*Natronolimnobius innermongolicus* N-1311	AB125108	91
Sua-GRP6	3.1	*Methanotorris formicicus*	AB100884	79
Sua-GRP7	1.6	*Halorubrum aidingense* 31 Hong	DQ355813	99
Sua-GRP8	3.0	*Halorubrum aidingense* JNPH-3	EU562182	97
Sua-GRP9	50	*Halorubrum tibetense* 8W8	AY149598	99
Sua-GRP10	1.6	*Halorubrum tibetense* 8W8	AY149598	97
Sua-GRP11	1.6	*Halorubrum tibetense* 8W8	AY149598	99
Sua-GRP12	3.1	*Halorubrum tebenquichense*	EF468473	95
Sua-GRP13	1.6	*Halorubrum tibetense* 8W8	AY149598	98
Sua-GRP14	1.6	*Natrialba chahannaoensis*	AJ004806	98

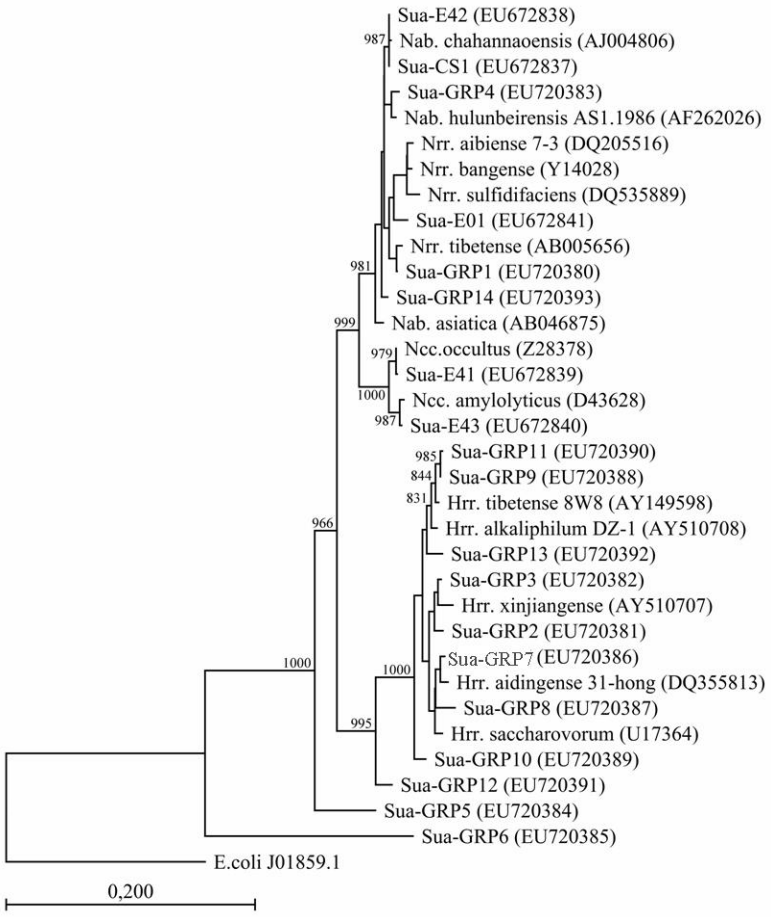

Figure 1. Neighbour-joining phylogenetic tree inferred from partial 16S rRNA gene sequences from the isolates and clones of alkaliphilic haloarchaea obtained in the current study, as well as those of closely related sequences in the database. The 16S rRNA gene sequence of *E. coli* was used as the outgroup. Numbers at the nodes indicate the level of bootstrap support on 1000 resamplings and only values greater than 700 are displayed. The numbers in parentheses are accession numbers.

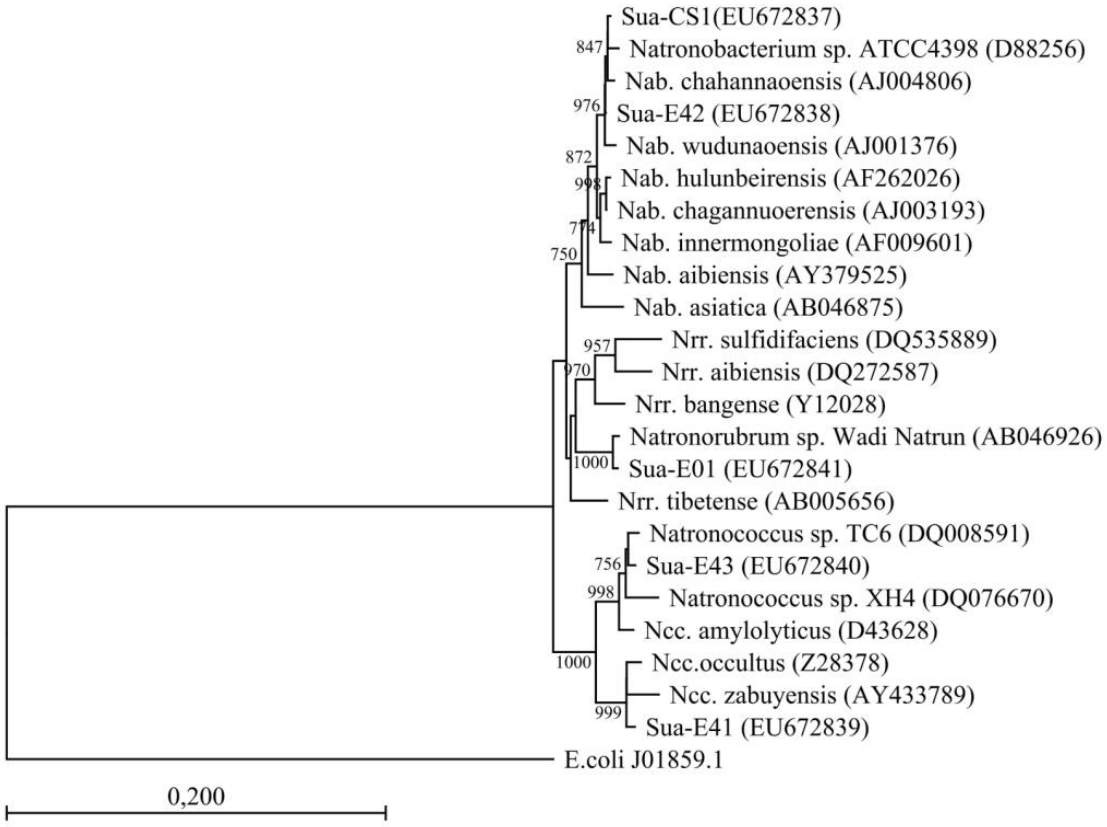

Figure 2. Phylogenetic tree reconstruction using 1400 bp 16S rRNA gene sequences of archaeal isolates obtained in the current study and related genera.

cluster of *Natrialba* species and displays 99% similarity to *Nab. hulunbeirensis*. Surprisingly, Sua-GRP14 which exhibited 98% similarity to *N. chahannaoensis* was located away from both groups, forming its own branch closer to *Natrialba asiatica*. The two major subgroups were maintained when the tree was inferred from 1400 bp gene fragments from the archaeal isolates (Figure 2).

In the second cluster, which comprises *Natronorubrum* species, clone Sua-GRP1 and isolate Sua-E01 exhibited 98% similarity to each other. However, they associated with two different subgroups of *Natronorubrum*. The isolate Sua-E01 does not show close association with any of the cultivated *Natronorubrum* species while clone Sua-GRP1 clusters with *N. tibetense*. *Natronococcus* species formed the third cluster also comprising two subgroups. The isolate Sua-E41 clustered together with the *Ncc. occultus* subgroup whereas isolate Sua-E43 formed close association with the *Ncc. amylotyticus* subgroup (Figure 1). The two subgroups were also maintained when the phylogenetic tree was inferred from the 1400 bp fragments (Figure 2).

In the second clade which only comprises *Halorubrum* species, the sequences that displayed 98 – 99% similarity form three clusters. Clones Sua-GRP11, Sua-GRP9 and Sua-GRP13 were closely associated with *H. tibetense* and *Halorubrum alkaliphilum*, Sua-GRP3 and Sua-GRP2 clustered with *Halorubrum xinjiangense*, while Sua-GRP7 and Sua-GRP8 clustered with *Halorubrum aidingense* and *Halorubrum saccharovorum*. In contrast, clones Sua-GRP5, Sua-GRP6, Sua-GRP10 and Sua-GRP12 which display ≤ 97% similarity to their closest relatives, do not cluster with any of the known taxa.

DISCUSSION

Culture-dependent and culture-independent methods were used in the current study to assess the diversity of alkaliphilic haloarchaea in solar salterns. The two methods yielded slightly different results, an observation commonly shared by many similar studies (Benlloch et al., 2001; Ochsenreiter et al., 2002; Burns et al., 2004; Maturrano et al., 2006). However, both the ribosomal DNA library clones and the archaeal isolates obtained by enrichment showed that the archaeal diversity was generally low and mainly comprised members of the family Halobacteriaceae. According to the 16S rRNA clone library, the archaeal assemblage mainly comprised species of previously characterized genera such as *Halorubrum*, *Natrialba*, *Natronococcus* and *Natronoru-*

brum. However, the environmental 16S rRNA library exhibited more diversity at the genus and species level than the culturable diversity. For instance, three genera as well as two unaffiliated phylotypes were detected by molecular methods while only three genera were recovered by cultivation. All the isolates obtained in the current study displayed antimicrobial susceptibility patterns common to halophiles. Most extreme halophiles have been shown to be resistant towards β-lactams due to the lack of peptidoglycans in the archaeal cell walls. Resistance towards ampicillin, choramphenicol, cycloserine, kanamycin, polymyxin B, erythromycin, neomycin, penicillin G, tetracycline and streptomycin and sensitivity towards aniscomycin, bacitracin and novobiocin is a common feature in the Halobacteriaceae (Birbir and Sesal, 2003).

Members of two genera (*Natrialba* and *Natronorubrum*) were successfully recovered by both the culture-dependent and culture-independent methods. Organisms with 99% similarity to *N. chahannaoensis* were the only group accurately detected by both methods. The isolates obtained in the current study (Sua-CS1 and Sua-E42) displayed optimum growth at 20% NaCl while the type strain *N. chahannaoensis* C112^T was reported to grow optimally at 15% NaCl (Xu et al*.,* 2001). In addition, isolate Sua-E42 displayed starch hydrolysis activity which is not present in isolate Sua-CS1 and *N. chahannaoensis* C112^T. The phylogenetic topology of the *Natrialba* clusters shows a definite separation between the alkaliphilic species such as *N. chahannaoensis* and *N. hulunbeirensis* from the neutrophilic *N. asiatica*. This separation was also shown to be supported by different molecular markers including 16S rRNA and DNA-dependent RNA polymerase subunit β (Wright, 2006; Enache et al., 2007), and therefore supports previous suggestion by Xu et al. (2001) that the alkaliphilic species should perhaps be placed in a different genus as they exhibit distinct polar lipid composition and phenotypic characteristics from the type species *N. asiatica*.

In the genus *Natronorubrum*, the isolate Sua-E01 and the clone Sua-GRP1 displayed 98% similarity to each other but showed higher similarity to different species. The isolate Sua-E01 only displayed 95% similarity to previously cultivated species including the type species *Natronorubum bangense* while the clone showed 99% similarity to *N. tibetense*. Commonly, a 16S rRNA gene sequence similarity and 70% DNA-DNA hybridization has been used to delineate species, while a 93 – 95% 16S rRNA similarity has often been considered to indicate different genera (Devereux et al., 1990; Gevers et al., 2005; Konstantinos and Tiedje, 2007). Therefore, Sua-E01 could represent a new species of *Natronorubrum*, however, this would need to be corroborated through sequencing of other markers and chemotaxonomic data.

Members of the genus *Natronococcus* were recovered by cultivation but were not detected in the environmental 16S rRNA clones. Similar observations were made in previous studies, and this failure to detect *Natronococcus* species in environmental samples was attributed to possible bias which might be introduced in the PCR approach due to incomplete cell lysis especially in genera such as *Natronococcus* (Ochsenreiter et al., 2002). The natronococci are known to possess a complex cell wall which differs from other archaeal cell wall structures and are difficult to break open (Niemetz et al*.,* 1997; Ochsenreiter et al., 2002). Since the DNA isolation method used in the current study did not incorporate any mechanical cell lysis it is possible that incomplete cell lysis could contribute to failure of amplification of *Natronococcus* 16S genes from environmental samples. The isolate Sua-E41 obtained in the current study displays 98% similarity to *N. occultus* but forms rough orange-red colonies and not pale brown colonies as reported for *Ncc. occultus* (Tindall et al*.,* 1984). Since, isolates that have ≥ 97% rRNA gene similarity do not always meet the 70% DNA-DNA-hybridization criterion for inclusion into the same species (Gevers et al., 2005), we cannot rule out the possibility of isolate Sua-E41 being a different species from *N. occultus*. In contrast, isolate Sua-E43 displayed similar characteristics and 98% 16S sequence similarity to *Natronococcus amylolyticus* and may thus be regarded as a strain of this species.

Halorubrum species were found to be the dominant alkaliphilic archaea present in the solar salterns as indicated by the presence of different strains recovered in the environmental ribosomal library. Previous studies have also reported *Halorubrum* to be the most abundant genus in various salterns and salt lakes (Benlloch et al., 2001; Burns et al., 2004; Xu et al*.,* 2007). Members of this genus constituted 64% of the ribosomal library in the current study. The representative clones of *Halorubrum* species in the library displayed 95 – 98% sequence similarity to previously reported sequences. The 16S clone library sequences clustered in a homogeneous *Halorubrum* phylogenetic group with the exception of clones Sua-GRP10 and Sua-GRP12. In addition, *Halorubrum* species maintained three clusters which were previously reported by Cui et al. (2006). Cluster I and II is represented by *Halorubrum xinjiangense* and *Halorubrum saccharovorum* and mainly comprises neutrophilic *Halorbrum* species while cluster III comprises alkaliphilic species such as *Halorubrum alkaliphilum* and *H. tibetense*. The *Halorubrum* clones detected in Sua pan evaporator ponds are distributed throughout these three clusters suggesting that the *Halorubrum* community in these ponds includes both neutrophilic and alkaliphilic species. The deviation of Sua-GRP10 and Sua-GRP12 from these clusters could perhaps suggest the possibility of new clusters in the future as new species are discovered. In the current study, we were not able to recover any *Halorubrum* isolates by cultivation methods which may indicate that the representatives of this genus were not favoured by the culture conditions used for enrichment, especially since various *Halorubrum* species

have been previously isolated and cultured from different hypersaline environments (Benlloch et al., 2001; Burns et al., 2004; Feng et al., 2004; Cui et al., 2006; Xu et al., 2007). However, these studies used rich media supplemented with casamino acids and yeast extract and in some cases glucose or peptone was also added, while in the current study the medium only contained casamino acids.

Most of the 16S rRNA clones and the archaeal isolates retrieved in the current study were affiliated to known species. However, two clones (Sua-GRP5 and Sua-GRP6) were found to display low sequence similarity to previously identified species. Clone Sua-GRP5 displayed 91% similarity to its closest relative *N. innermongolicus*, and may represent a new genus in the family Halobacteriaceae. In contrast, Sua-GRP6 only displayed 79% similarity to its closest relative *Methanotorris formicicus*, a hyperthermophilic methanogen of the family Methanocaldococcacea, order Methanococcales. This low similarity with known taxa suggests that clone Sua-GRP6 may represent a distinct phylotype only distantly related to methanogenic archaea. However, proper phylogenetic placement can only be clarified by comparison of different molecular markers as well as cultivation and characterization of members of this newly detected group. The presence of methanogenic archaea in a variety of hypersaline environments has been previously documented (Kevbrin et al., 1997; Grant et al., 1999; Cytryn et al., 2000). These organisms often form part of the microbial mat in various hypersaline environments and are able to utilize noncompetitive substrates such as methylamines derived from compounds that accumulate in halotolerant organisms where they serve in osmoregulation in hypersaline environments (Cytryn et al., 2000; Elshahed et al., 2004).

Conclusion

While there may be disparities between cultivation-dependent and molecular methods, it can be concluded that the combination of these methods has allowed for identification of the major alkaliphilic haloarchaeal species in Sua pan solar salterns. In addition, results obtained in the current study suggest that although the diversity of haloarchaea in solar salterns has been shown to be generally low, there is still potential to find novel taxa. However, none of these were recovered in culture, thus formulating new media and cultivation conditions that closely mimic the prevailing conditions in the extreme environments might be necessary to promote growth of a wider variety of organisms.

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REFERENCES

Baker GC, Smith JJ, Cowan DA (2003). Review and re-analysis of domain-specific 16S primers. J. Microbiol. Methods 55: 541-555.

Benlloch S, Acina SG, Antón J, López-López, Luz SP, Rodríguez-Valera F (2001). Archaeal biodiversity in crystallizer ponds from a solar saltern: Culture versus PCR. Microb. Ecol. 41: 12-19.

Birbir M, Sesal C (2003). Extremely halophilic bacterial communities in Şereflikoçchisar salt lake in Turkey. Turk. J. Biol. 27: 7-22.

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

Clinical and Laboratory Standards Institute (2006). Document M2-A9 and M7-A7, Performance Standards for Antimicrobial Susceptibility Testing; Sixteenth Information Supplement.

Cui H-L, Tohty D, Zhou P-J, Liu S-J (2006). *Halorubrum lipolyticum* sp. nov. and *Halorubrum aidingense* sp. nov., isolated from two salt lakes in Xin-Jiang, China. Int. J. Syst. Evol. Microbiol., 56: 1631-1634.

Cytryn E, Minz D, OremLand RS, Cohen Y (2000). Distribution and diversity of archaea corresponding to the limnological cycle of a hypersaline stratified lake (Solar Lake, Sinai Egypt). Appl. Environ. Microbiol. 66: 3269-3276.

Denny FE (1927). Field Method for Determining the Saltiness of Brackish Water. Ecology 8: 106-112.

Devereux R, He S-H, Doyle CL, Orkland S, Stahl DA, LeGall J, Whitman WB (1990). Diversity and origin of *Desulfovibrio* species: Phylogenetic definition of a family. J. Bacteriol. 172: 3609-3619.

Dussault HP (1955). An improved technique for staining red halophilic bacteria. J. Bacteriol. 70: 484-485.

Dyall-Smith M (2000). The Halohandbook: Protocols for halobacterial genetics. Version 3.0. University of Melbourne, Australia.

Eckardt FD, Brayant RG, McCulloch G, Spiro B, Wood WW (2008). The hydrochemistry of a sme-arid pan basin case study: Sua Pan, Makgadikgadi, Botswana. Appl. Geochem. doi:10.1016/j.apgeochem.2007.12.033.

Elshahed MS, Najar FZ, Roe BE, Oren A, Dewers TA, Krumholz LR (2004). Survey of archaeal diversity reveals an abundance of halophilic archaea in a low-salt, sulfide- and sulfur-rich spring. Appl. Environ. Microbiol. 70: 2230-2239.

Enache M, Itoh T, Fukusguna T, Usami R, Dumitru L, Kamekura M (2007). Phylogenetic relationships within the family Halobacteriaceae inferred from *rpoB'* gene and protein sequences. Int. J. Syst. Evol. Microbiol. 57: 2289-2295.

Feng J, Zhou P-J, Liu S-J (2004). *Halorubrum xinjiangense* sp. nov., a novel halophile isolated from saline lakes in China. Int. J. Syst. Evol. Microbiol. 54: 1789-1791.

Gevers D, Cohan FM, Lawrence JG, Spratt BG, Coenye T, Feil EJ, Stackebrandt E, van de Peer Y, Vandamme P, Thompson FL, Swings J (2005). Opinion: Re-evaluating prokaryotic species. Nat. Rev. Microbiol. 3: 733-739.

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

Grant WD (2004). Life at low water activity. Phil. Trans. R. Soc. Lond. 359: 1249 -1267.

Hall TA (1999). BioEdit: A user-friendly biological sequence alignment editor and analysis programme for Windows 95/98/NT. Nucl. Acid. Symp. Ser. 41: 95-98.

Hughes RH, Hughes JS (1992). A directory of African Wetlands. IUCN, Gland, Switzerland and Cambridge. ISBN: 2880329493.

Kamekura M, Dyall-Smith ML, Upasani V, Ventosa A, Kates M (1997) Diversity of alkaliphilic *Halobacteria*: Proposals for transfer of *Natronobacterium vacuolatum*, *Natronobacterium magadii*, and *Natronobacterium pharaonis* to *Halorubrum*, *Natrialba*, and *Natronomonas* gen. nov., respectively, as *Halorubrum vacuolatum* comb.

nov., *Natrialba magadii* comb. nov., and *Natronomonas pharaonis* comb. nov., respectively. Int. J. Syst. Bacteriol. 47: 853-857.
Kanai H, Kobayashi T, Aono R, Kudo T (1995). *Natronococcus amylolyticus* sp. nov., a haloaklaiphilic archaeon. Int. J. Syst. Bacteriol. 45: 762-766.
Kevbrin VV, Lysenko AM, Zhilina TN (1997). Physiology of the alkaliphilic methanogen Z-7936, a new strain of *Methanosulsus zhilinaeae* isolated from Lake Magadi. Microbiology 66: 261-266.
Konstantinos TK, Tiedje JM (2007). Prokaryotic taxonomy and phylogeny in the genomic era: advancements and challenges ahead. Curr. Opin. Microbiol. 10: 504-509.
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.
McCullosh GP, Irvine K, Eckardt FD, Bryant R (2008). Hydrochemical fluctuations and crustacean community composition in an ephemeral saline (Sua pan, Makgadikgadi Botswana). Hydrobiologia 596: 31-46.
Niemetz R, Kärcher U, Kandler O, Tindall BJ, König H (1997). The cell wall of the extremely halophilic archaeon *Natronococcus occultus*. Eur. J. Biochem. 249: 905-911.
Ochsenreiter T, Pfeifer F, Schleper C (2002). Diversity of Archaea in hypersaline environments characterized by molecular-phylogentic and cultivation studies. Extremophiles 6: 267-274.
Oren A, Ventosa A, Grant WD (1997). Proposed minimal standards for description of new taxa in the order Halobacteriales. Int. J. Syst. Bacteriol. 47: 233-238.
Rees HC, Grant WD, Jones BE, Heaphy S (2004) Diversity of Kenyan soda lake alkaliphiles assessed by molecular methods. Extremophiles 8: 63-71.
Sambrook J, Fritsch E-F, Maniatis T (1989). Molecular cloning: A laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Seaman MT, Ashton PJ, Williams WD (1991). Inland salt waters of southern Africa. Hydrobiologia 75: 75-91.
Tindall BJ, Ross HNM, Grant WD (1984). *Natronobacterium* gen. nov. and *Natronococcus* gen. nov., two new genera of haloalkaliphilic archaeabacteria. Syst. Appl. Microbiol. 5: 41-57.
White KH, Eckardt FD (2006). Geochemical mapping of carbonate sediments in the Makgadikgadi Basin, Botswana, using Moderate Resolution Remote Sensing Data. Earth Surf. Proc. Land 31: 665-681.
Wright A-DG (2006). Phylogenetic relationships within the order *Halobacteriales* inferred from 16S rRNA gene sequences. In J. Syst Evol. Microbiol. 56: 1223-1227.
Xu Y, Wang Z, Xue Y, Zhou P, Ma Y, Ventosa A, Grant WD (2001). *Natrialba hulunbeirensis* sp. nov. and *Natrialba chahannaoensis* sp. nov., novel haloalkaliphilic archaea from soda lakes in Inner Mongolia autonomous region, China. Int. J. Syst. Evol. Microbiol. 51: 1693-1698.
Xu X, Wu M, Wu Y, Zhang H, (2007). Culturable halophilic archaeal diversity of Ayakekumu salt lake located in Xinjiang, China. Acta Ecologica Sinica 27: 3119-3123.