

S. Leuko · F. Goh · M. A. Allen · B. P. Burns
M. R. Walter · B. A. Neilan

Analysis of intergenic spacer region length polymorphisms to investigate the halophilic archaeal diversity of stromatolites and microbial mats

Received: 12 April 2006 / Accepted: 16 August 2006 / Published online: 3 November 2006
© Springer-Verlag 2006

Abstract Hamelin Pool in Western Australia is one of the two major sites in the world with active marine stromatolite formation. Surrounded by living smooth and pustular mats, these ancient laminated structures are associated with cyanobacterial communities. Recent studies have identified a wide diversity of bacteria and archaea in this habitat. By understanding and evaluating the microbial diversity of this environment we can obtain insights into the formation of early life on Earth, as stromatolites have been dated in the geological record as far back as 3.5 billion years. Automated ribosomal intergenic spacer analysis (ARISA) patterns were shown to be a useful method to genetically discriminate halophilic archaea within this environment. Patterns of known halophilic archaea are consistent, by replicate analysis, and the halophilic strains isolated from stromatolites have novel intergenic spacer profiles. ARISA-PCR, performed directly on extracted DNA from different sample sites, provided significant insights into the extent of previous unknown diversity of halophilic archaea within this environment. Cloning and sequence analysis of the spacer regions obtained from stromatolites confirmed the novel and broad diversity of halophilic archaea in this environment.

Keywords Shark Bay · Stromatolites · Intergenic spacer region · Diversity · Hypersaline environment · ARISA

Introduction

The living stromatolites of Hamelin Pool, Shark Bay, in Western Australia are considered analogs of one of the oldest habitats of life on Earth (Monty 1977; Petrisor and Decho 2004; Reid et al. 2003). The surface of these stromatolites is covered with living mats which represent the actively growing microbial layer (Arp et al. 2001; Burns et al. 2004). Surrounding the stromatolites are living smooth and pustular mats, which may also have evolutionary significance because of their similarity to lithified stromatolites from the Proterozoic and Early Paleozoic eras (Palmisano et al. 1989). Due to the limited water exchange and the high evaporation rate in this area, the concentration of ions in the water is high, excluding most animals that would otherwise graze on the microbial mats. Despite these conditions in Hamelin Pool, including high levels of Na^+ and Cl^- (twice as high as normal seawater), recent research has revealed an extensive diversity of cyanobacteria and other bacteria (Burns et al. 2004; Papineau et al. 2005). Although the sodium chloride content of the water is only around 6% (w/v), the work by Burns et al. (2004) also revealed the presence of halophilic archaea of the family Halobacteriaceae. This diversity of as yet uncultivated halophilic archaea was surprising since these organisms grow optimally in media containing 15–35% (w/v) NaCl (Kushner and Kamekura 1988) and require at least a minimum of 9% (w/v) NaCl (Oren 2001) for active growth.

Halophilic archaea typically thrive in high salt environments and have been isolated from the Dead Sea (Oren et al. 1995), salt evaporation lagoons and ancient halite (McGenity et al. 2000; Mormile et al. 2003; Stan-Lotter et al. 2002). These environments are characterized by high ionic concentrations, and even halophiles isolated from seawater (Rodríguez-Valera et al. 1979)

Communicated by K. Horikoshi.

S. Leuko · B. P. Burns · M. R. Walter · B. A. Neilan
Australian Centre for Astrobiology,
Biotechnology Research Institute,
Macquarie University,
NSW 2109 Sydney, Australia

F. Goh · M. A. Allen · B. P. Burns · B. A. Neilan (✉)
School of Biotechnology and Biomolecular Science,
University of New South Wales,
NSW 2052 Sydney, Australia
E-mail: b.neilan@unsw.edu.au
Tel.: +612-9385-3235
Fax: +612-9385-1591

require 2 M NaCl for growth. Recent publications have raised the possibility of cultivating halophilic archaea from low salt environments. For example, Purdy et al. (2004) cultivated three novel types that were isolated from 3.2% (w/v) NaCl environments and are also capable of slow growth at sea water salinity of 2.5% (w/v) NaCl.

Screening early Earth analogs, such as the Shark Bay stromatolites, for halophilic archaea can provide novel insights into possible early life formation since halophiles are known for their longevity and their ability to survive for several million years in ancient halite (Grant et al. 1998; Radax et al. 2001; Stan-Lotter et al. 2002). Furthermore, species of the genus *Halococcus* show an increased resistance to extreme conditions, such as those of a simulated Martian atmosphere and heat shock (Leuko et al. 2002), underlying their importance in studies of early life on Earth and other possible habitable regions.

There are two established strategies to investigate the microbial diversity of a given environment, culture-dependent and culture-independent methods. Culture-dependent approaches to investigate the diversity of halophilic archaea in environmental samples are limited by two main factors: (1) the range of different nutrient requirements of different species and (2) the significant length of time to establish and grow culturable isolates. To overcome these issues, the diversity of halophilic archaea associated with Shark Bay was investigated in the present study, using automated ribosomal intergenic spacer analysis (ARISA).

Automated ribosomal intergenic spacer analysis is a culture-independent method, developed by Fisher and Triplett (1999), and has been shown to be useful for analyzing microbial communities (e.g., González et al. 2003; Ranjard et al. 2000). In comparison to the standard 16S rRNA gene cloning approach to investigate diversity, ARISA provides a rapid initial appraisal of the diversity present.

ARISA-PCR targets the intergenic spacer region between the 16S and the 23S rRNA genes (ITS region) of the ribosomal RNA operon. This region may encode various tRNAs depending on the species and has significant heterogeneity in terms of sequence length and composition (González et al. 2003; Ranjard et al. 2000). ARISA is sensitive as it can detect single nucleotide polymorphisms and is highly reproducible due to automation. As with all PCR-based methods, a concern for data interpretation is that the representation of the microbial community is accurate and reproducible (Polz and Cavanaugh 1998). This method of culture-independent analysis has proven to be a fast and reliable method for analyzing microbial communities found in solar salterns (Benlloch et al. 2001), freshwater environments (Fisher and Triplett 1999), as well as in several lakes (Yannarell and Triplett 2005). We report here the first successful application of ARISA-PCR to environmental stromatolite samples and the living microbial mats obtained from Hamelin Pool. The results describe

a previously unknown breadth of archaeal diversity indicated by intergenic spacer region polymorphisms.

Materials and methods

The following cultivated strains were obtained from Professor Masahiro Kamekura for ARISA analysis: *Haloterrigena turkmenica*, *Natrialba aegyptia*, *Halogeometricum borinquense*, *Halobaculum gomorense*, *Halococcus salifodinae*, *Halococcus morrhuae*, *Halococcus saccharolyticus*, *Haloferax volcanii*, *Natrinema pallidum* and *Halorubrum trapanicum*. *Halobacterium salinarum* NRC-1 was obtained from Prof. Stan-Lotter. All strains, except *Hbt. salinarum* NRC-1, were cultured in DSM 97 medium (DasSarma et al. 1995) with 150 g of NaCl and supplemented with 7.23 g MgCl₂·6H₂O and 2.70 g CaCl₂·2H₂O per liter, pH 7.4 (DSM 97 modified). *Halobacterium salinarum* NRC-1 was cultured in ATCC 2185 medium as described at <http://www.atcc.org/mediapdfs/2185.pdf>. Cultures were incubated at 37°C on a rocking platform for up to 2 weeks. Late exponential cultures were diluted (10⁻⁶/10⁻⁷) and gently spread onto agar plates containing the same salt concentration as the liquid media. Plates were incubated at 37°C for up to 1 month.

Extraction of DNA from environmental samples

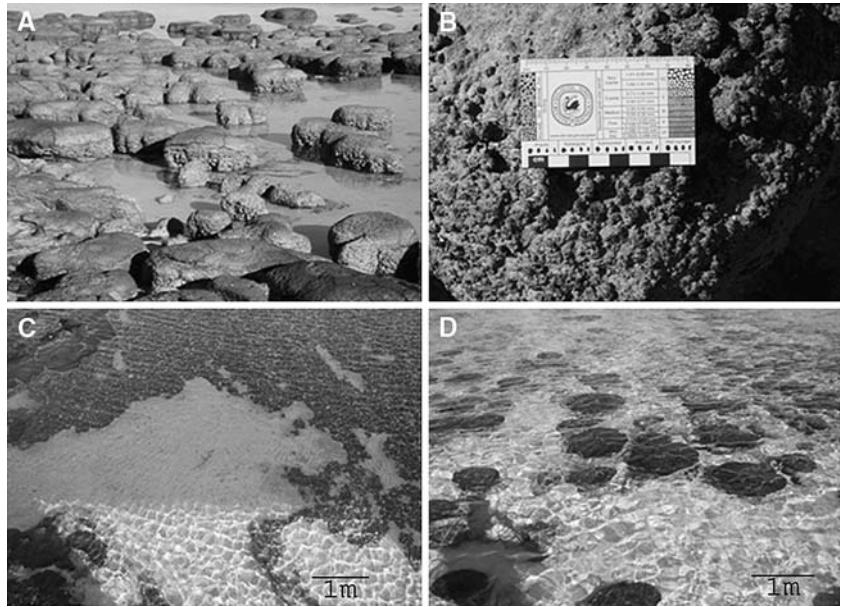
Environmental samples were collected from stromatolites (top layer), smooth and pustular mats (intertidal) at Telegraph Station, Shark Bay (26°25'00"S, 114°13'05"E) (see Fig. 1). From stromatolites, whole DNA was extracted using the XS-buffer method (Tillett and Neilan 2000). This method uses a buffer containing 1% potassium ethyl xanthogenate, 100 mM Tris-HCl pH 7.4, 20 mM EDTA pH 8, 1% sodium dodecylsulfate and 800 mM ammonium acetate. Samples were vortexed for 5 min and then incubated for 120 min at 65°C followed by a PCI (25:24:1) extraction and isopropanol purification. The purified DNA was resuspended in 100 µl dH₂O and stored at -20°C until further use.

Environmental samples from smooth and pustular mats were incubated on a shaker with 0.5 M EDTA at 37°C overnight to remove excess Ca²⁺ and Mg²⁺. Supernatant was removed and the pellet was dissolved in 567 µl TE buffer. Samples were exposed to thermal shock by freezing/thawing the solution five times. Following these steps, DNA was further extracted using the CTAB method (Wilson 1990).

Isolation of halophilic archaea from Shark Bay

Stromatolite samples were obtained and handled with sterile instruments during the course of the study as described previously (Burns et al. 2004), transported to the laboratory and stored at 4°C. A small section of the

Fig. 1 Stromatolites, smooth and pustular mats in Shark Bay. **a** Intertidal stromatolites during low tide, **b** close-up of the top of a stromatolite, **c** smooth (white patch in front) and pustular mats (dark patch at the back), **d** intertidal stromatolites during high tide



stromatolite, approximately 1 cm², was taken and ground using a mortar and pestle. The ground material was suspended in 5 ml of sterile 5% NaCl, and 100 µl aliquots were plated in duplicate onto DSM 97 (DasSarma et al. 1995) modified agar plates supplemented with 100 µg/ml ampicillin, penicillin and streptomycin to inhibit bacterial growth. To obtain pure cultures, single colonies were streaked several times onto fresh agar plates and incubated for up to 2 weeks at 37°C. Microbial mat samples were treated the same way. From the stromatolite samples, two strains of halophilic archaea were isolated and designated as 100NA1 and 100A6. Strain 100A6 was further characterized and is designated *Halococcus hamelinensis* sp. nov. (Goh et al. 2006). Strains obtained from smooth mats (A01–A07) and pustular mats (A08–A12) were also isolated as described above.

ARISA conditions

Single colonies from reference strains and the novel isolates were selected from agar plates containing 15 or

25% (w/v) NaCl, depending on the NaCl requirements of the strains, and were dissolved in 50 µl dH₂O. Cells were lysed by 3–5 cycles of freezing/thawing with a 30 s vortexing step between each cycle. One microliter of the supernatant was used as PCR template. To gain the most accurate insight into diversity present, several sets of primers were tested on type strains and on DNA from the environment, and these primer sequences are given in Table 1.

To obtain PCR products for cloning, unlabeled forward primer was used. For amplification, the following touch-down PCR conditions were used: initial denaturation step at 95°C for 3 min, followed by 10 cycles of 95°C for 20 s, 68°C for 30 s with a reduction of temperature of 1°C per step and a reduction of annealing time 1 s per step, and 72°C for 1 min, followed by 25 cycles of 95°C for 20 s, 58°C for 20 s and 72°C for 1 min with a final extension at 72°C for 10 min. PCR was performed in a 20 µl reaction volume containing 2 µl 10 × *Taq* polymerase buffer, 50 mM MgCl₂, 0.2 mM dNTPs, 10 pmol of each primer and 0.2 units of *Taq* polymerase. For DNA amplification of environmental samples, approximately 10 ng of environmental DNA

Table 1 Primers used in this study

Sequence (5'–3') ^a	Gene	Position ^b	Name	Reference
AAGTCGTAACAAGGTAACC	16S rRNA	1,424–1,446	E1492F	DeLong (1992)
TCGGYGCCCGAGCCGAGCCAT CC	23S rRNA	44–66	71R	García-Martínez and Rodríguez-Valera (2000)
TCGCAGCTTRSCACGYCCTTC		44–64	Cren 1	Benlloch et al. (2001)
GCTTATCGCAGCTTGRACAGTCC		48–71	Halo23S-70R	This study
AACGGGCAACGGTAGGTCAGT		1,130–1,151	Halo1150F	This study
CACCAGGTGGCACAGTAGCC		2–22	Halo23S-20R	This study

^aBase codes: Y: C or T; R: A or G; S: G or C

^bNucleotide positions in the *Halobacterium salinarum* NRC-1 rRNA operons

was used as template. Template DNA concentrations were measured using a Nanodrop ND-1000 spectrophotometer (Biolab). All reactions were performed in a GenAmp PCR System 2400 (Perkin Elmer, Applied Biosystems) and the products were separated with a 1% agarose gel and visualized via UV illumination after ethidium bromide staining. PCR products were purified by precipitation with polyethylene glycol (PEG, MW 8000) and ethanol as described at http://www.gator.biol.sc.edu/CGEL_ABI/PEG_Precip.html and resuspended in 20 µl dH₂O. Purified products were analyzed using a 3730 ABIPrism sequencing capillary and the results were interpreted using SoftGenetics Gene Marker 1.40. To optimize PCR conditions, the ITS of *Hbt. salinarum* NRC-1 and an environmental DNA sample (stromatolitic DNA) were both amplified using 15, 20, 25, 30 and 35 cycles, under otherwise standard conditions as described.

Cloning and sequencing of stromatolitic intergenic spacer PCR products

Subsequent to ARISA-PCR on total stromatolite DNA, the PCR products were cloned into pGEM[®]-T Easy Vector (Promega) according to the manufactures instructions. Positive clones were selected and suspended in 50 µl dH₂O. Inserts were amplified using 10 mM of the cloning vector primers MPf (5'-CCCAGTCAC-GACGTTGTAAAAACG-3') and MPr (5'-AGCGGA TAACAATTTTCACACAGG-3'). The following conditions were used for amplification: initial denaturation step at 95°C for 3 min, followed by 30 cycles of 94°C for 30 s, 55°C for 20 s and 72°C for 1 min, with a final extension at 72°C for 7 min. PCR products were purified with PEG as described above, sequenced with primers E1492F or Halo1150F using conditions as previously used by Burns et al. (2004) and analyzed using the A-biPrism 3730 capillary. All sequences were analyzed against the NCBI database.

Results

ARISA primer optimization

As the genome of *Hbt. salinarum* NRC-1 has been sequenced (Ng et al. 2000), this strain was employed as a test strain. Applying primers E1492F, 71R and Cren1 to amplify the ITS regions we obtained three distinct peaks, initially suggesting that *Hbt. salinarum* NRC-1 contains four different 16S rRNA operons. However, the published sequence indicated that *Hbt. salinarum* NRC-1 possesses only one 16S rRNA operon, suggesting an unspecific binding of these primers. Pairing of the newly designed primer 23s-70R with E1492F resulted in one distinctive peak in *Hbt. salinarum* NRC-1 that supports the type strain genomes sequence results (Ng et al. 2000). However, applying primers E1492F and 23s-70R to

environmental DNA again showed unspecific binding to bacterial DNA, observed when ITS regions obtained from stromatolitic DNA were cloned and sequenced (data not shown). The combination of Halo1150F with 23s-70R or 23s-20R, respectively, is the most accurate primer combinations for this environment. Amplifying the ITS regions with an unlabeled Halo1150F primer and subsequent cloning and sequencing showed that only halophilic Archaea were amplified (20 clones were picked and analyzed). Hence, the combination of Halo1150F and 23s-70R was chosen for the purpose of this study.

Effects of PCR cycle number on overall ARISA patterns

To optimize and validate the number of PCR cycles required for the most reliable amplification of the intergenic spacer region, cycle conditions were tested using *Hbt. salinarum* NRC-1 DNA as a template. The results obtained showed that the first pattern detected by the automated analysis occurred after 20 cycles; however, signal strength of the patterns was much stronger using 35 cycles of PCR. To minimize any PCR introduced artifacts, 35 cycles were used for environmental patterns.

ARISA analysis of halophilic representatives

Automated ribosomal intergenic spacer analysis was performed in triplicate on single halophilic strains to accurately determine the intergenic spacer region length. By employing primers Halo1150F and 23s-70R, analysis of single halophilic strains produced distinctive patterns (Table 2). The length obtained with this primer set

Table 2 Representative 16S–23S rRNA internal transcribed regions from halophilic archaea

Strain	Peaks observed (bp)			Actual ITS length ^a		
<i>Halococcus morrhuae</i>	766			395		
<i>Halococcus saccharolyticus</i>	769			398		
<i>Halococcus salifodinae</i>	755			384		
<i>Halobacterium salinarum</i> NRC-1	811			440		
<i>Haloferax volcanii</i>	740			369		
<i>Natrinema pallidum</i>	773			402		
<i>Halobaculum gomorrense</i>	740	744		371	373	
<i>Haloterrigena turkmenica</i>	770			399		
<i>Halogeometricum borinquense</i>	771			400		
<i>Natrialba aegyptia</i>	735			364		
Isolate 100 NA1 ^b	763	769		392	398	
Isolate 100 A6 ^b	763	769		392	398	
Isolate 50 A11 ^b	772			401		
Isolate A07 ^c	366	733	739	–	362	368
Isolate A12 ^d	366	733	739	–	362	368

^aCalculations were based on the positions in the *Halobacterium salinarum* NRC-1 16S rRNA and 23S rRNA operons

^bIsolate cultivated from a stromatolite

^cIsolates cultivated from smooth mat

^dIsolate cultivated from pustular mat

represents the ITS region with approximately 320 bp from the 16S rRNA operon and 50 bp from the 23S rRNA operon. The actual length was calculated by subtracting the lengths of the 3' region of the 16S rRNA operon and the 5' region of the 23S rRNA operon as previously described by Cardinale et al. (2004).

Isolates A07 and A12 possess the smallest ITS region (362 bp), and *Hbt. salinarum* NRC-1 possess the longest (440 bp) intergenic spacer regions in its ribosomal RNA operons. Most strains possess one intergenic spacer size with only *H. gomorreense* and the Isolates 100 NA1 and A6 revealing two distinctive operons. The only strains possessing three different intergenic spacer sizes were Isolates A07 and A12. One isolate from the stromatolite, 50 A11, contained one intergenic spacer region (401 bp), whereas isolates 100 NA1 and 100A6 possessed an identical ARISA profile with two distinctive bands. The recent publication by Goh et al. (2006) showed that 100 NA1 and 100A6 are in fact identical strains according to DNA–DNA hybridization.

ARISA profiles from a stromatolite

Initial PCR results revealed several different sized amplicons that provided the first indication of the archaeal diversity of a stromatolite. The actual length of the ITS region is stated in brackets.

Figure 2a shows the results of ARISA profiling of the stromatolite community showing 12 peaks within the range of 729–814 bp (358–443 bp) with the largest peak at 764 bp (393 bp). This result indicated a large diversity in the area between 729 and 814 bp (358–443 bp). No peaks between 200 and 700 bp were observed. Peaks around 100 bp were observed but considered as primer dimers or other amplification anomalies and not used for further analysis.

ARISA profile from pustular and smooth microbial mats

Results from the smooth mat (Fig. 2b) indicated a higher diversity compared to the stromatolite. Figure 2b shows that the smooth mat had 14 distinct PCR fragments with a prominent peak at 799 bp (428 bp). Furthermore, the peaks were between 568 and 805 bp (197 and 434 bp). Analysis of the pustular mat samples resulted in 18 different peaks (Fig. 2c) with the majority between 569 and 799 bp (198 and 428 bp), showing a similar pattern as observed for the smooth mat. Once again, a prominent peak was present at 730 bp (359 bp). Furthermore, the pustular mat showed a higher diversity in the area between 720 and 800 bp (349 and 429 bp).

Discussion

There are currently many genetic tools available to investigate the diversity of unknown environmental

samples. A very common and broadly used technique is the amplification, cloning and sequencing of the 16S rRNA gene (Burns et al. 2004; Radax et al. 2001) as well as DGGE profiling and terminal restriction fragment length polymorphism (T-RFLP) (Øvreås et al. 2003). ARISA was first developed by Fisher and Triplett in 1999 and was also shown to be a useful method to investigate the diversity of different environments (Acinas et al. 1999; Benlloch et al. 2001). In the present study, we targeted the intergenic spacer region between the 16S and 23S rRNA genes to investigate the diversity of halophilic archaea associated with the extant stromatolites and microbial mats of Shark Bay.

The choice of the most specific primers was very important for this work, as halophilic archaea represent a minority within this environment. To ensure that only ITS regions from halophilic Archaea were amplified, we tested four different primer combinations and screened the sequences obtained for their accuracy. Previous published primers (E1492F, Cren 1 and 71R) proved to be too unspecific for this particular environment, as they were also amplifying bacterial ITS regions. Specifically designed primers (Halo1150F, Halo23s-70R and Halo23s-20R) in the present study gave the best results as no unspecific amplification was observed.

By analyzing the ITS lengths of ten cultivated and described halophilic archaeal strains, a set of data was created which demonstrated the reproducibility and accuracy of this method as well as the validity of the primers employed. Furthermore, analyzing these cultured strains provided further indication of the expected ITS lengths of halophilic archaea. A previous study by Dennis et al. (1998) found that *Haloarcula marismortui* has two different intergenic spacers with lengths of 383 and 405 bp, which were also the range of lengths obtained in the present study. A similar length (383 bp) was also obtained for the intergenic spacer from *Haloferax mediterranei* (Briones and Amils 2000). The environmental DNA profile generated for a stromatolite contained a majority of PCR fragments with sizes between 729 and 814 bp (358 and 443 bp) (Fig. 2a). Although a database of ITS regions from known halophilic archaea was created (Table 2), and the database for intergenic spacer analysis (Ribosomal Intergenic Spacer Sequence Collection) (García-Martínez et al. 2001) was consulted, it was not possible to positively identify a known strain within the extracted DNA from stromatolites. Our data suggested that the stromatolite community is yet to be fully characterized as many novel 16S rRNA gene sequences from as yet unknown halophilic archaea were obtained. Figure 2a showed a clear peak at 763 (392) and 769 bp (398 bp), ITS lengths also obtained from the novel stromatolite isolate, 100A6 (Table 2), which has been recently characterized and named *Hcc. hamellinensis* (Goh et al. 2006). These consistent results observed for stromatolite isolates and the stromatolite sample further demonstrated the validity of this method. Furthermore, strain A07 showed a peak at 739 bp (368 bp), which could also be found in

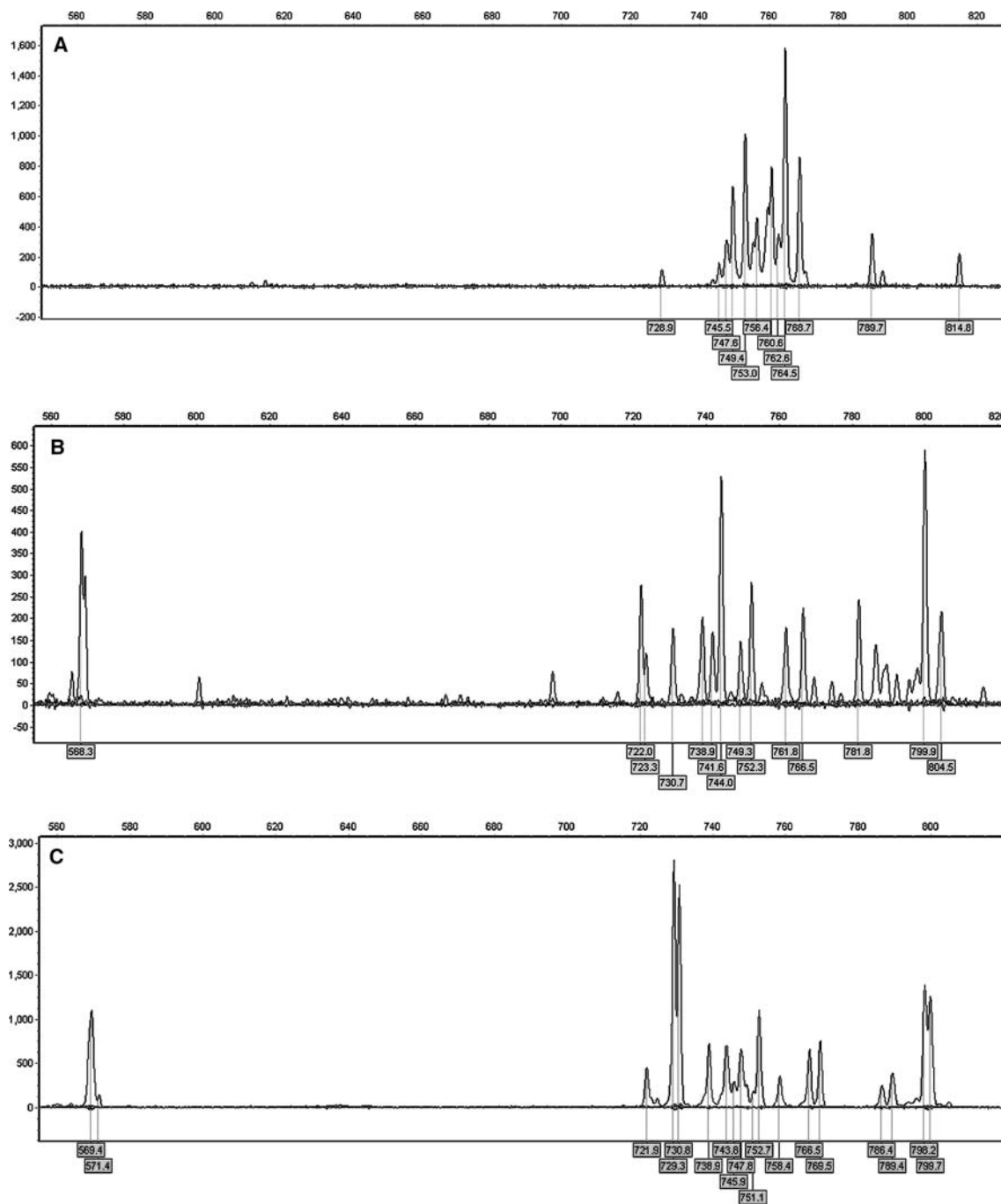


Fig. 2 Partial ARISA profile of the Archaeal communities from Shark Bay. **a** An archaeal community associated with a stromatolite. **b** An archaeal community associated with a smooth mat. **c** An archaeal community associated with a pustular mat

the smooth mat sample, the environment from which this strain was isolated (Fig. 2b). Interestingly, environmental samples from the stromatolite revealed an apparent lower diversity than in the surrounding mats. In Fig. 2, it can be clearly seen that the stromatolite contained only 13 fragments in the profile (Fig. 2a), whereas the pattern from the pustular mat showed 18 distinctive peaks. Both smooth and pustular mat had most of their amplicon fragments between 568 and 804 bp (197 and 433 bp) and both had only one or two

peaks at 568 bp (197 bp), respectively. Although the mats are more exposed to tidal changes with a transient reduction in ion concentration, this influx also provides more nutrients, whereas the top layer of stromatolites are hypersaline at all times except at peak high tide. Although the salinity in mats is not typical for members of the *Halobacteriales*, they are known to inhabit lower-salinity environments with localized NaCl concentration high enough to prevent their lyses. Similar findings of halophilic archaeal survival at low NaCl concentrations

were presented in the study of the diversity of the Zoletone spring environment (Elshahed et al. 2004).

The environmental DNA profile of the stromatolite generated 13 peaks between 729 and 814 bp (Fig. 2a) (358 and 443 bp), which initially suggested a diversity of 13 species. However, as shown in Table 2, halophilic archaea can have multiple intergenic spacer lengths. Previous work investigating the heterogeneity of 16S rRNA operons in halophilic Archaea (e.g., Amann et al. 2000; Boucher et al. 2004) confirmed that they possess multiple copies of the rRNA operon. Based on our current knowledge of the haloarchaeal ITS region, the actual diversity may therefore be between 13 and 4 species present within this environment. To reduce this uncertainty, amplified 16S rRNA fragments and the ITS region, isolated from the environment, were cloned and sequenced. Using the optimized primer set (Halo1150F, Halo23s-70R and Halo23s-20R), only halophilic archaea were identified. Closely related species to the genus *Halobacterium* were also previously reported by Burns et al. (2004) in these stromatolites. Interestingly, Isolates A07 and A12 possess distinctive peaks at 366 bp (without subtracting the primer) which suggest that they have no ITS region. Further investigations in the possibility of halophilic archaea possessing no ITS region need to be done to confirm this observation.

Optimization of DNA extraction and PCR conditions for the environmental samples was critical to overcome biases introduced from differences in the relative quantities and qualities of template (Polz and Cavanaugh 1998; von Wintzingerode et al. 1997). DNA extraction and subsequent PCR amplification from environmental samples can lead to an underestimation of the diversity present (Martin-Laurent et al. 2001). It should be acknowledged that ARISA can also underestimate microbial diversity, and intergenic spacer regions of identical length but different sequences can potentially be represented as a single peak in these profiles (Fisher and Triplett 1999).

In conclusion, we describe here the initial application of ARISA for the analysis of archaeal diversity in Shark Bay, Western Australia. This and other similar studies may provide the basis for the development and extension of bioinformatic resources and thus further improve the usefulness of ARISA in microbial diversity assessment.

Acknowledgments This research was funded by the Australian Research Council and an International Postgraduate Research Scholarship from Macquarie University, Australia. We thank Professor Francisco Rodríguez-Valera for kindly providing us with the updated URL of the RISSC database.

References

- Acinas SG, Antón J, Rodríguez-Valera F (1999) Diversity of free-living and attached bacteria in offshore western Mediterranean waters as depicted by analysis of genes encoding 16S rRNA. *Appl Environ Microbiol* 65:514–522
- Amann G, Stetter KO, Llobet-Brossa E, Amann R, Antón J (2000) Direct proof for the presence and expression of two 5% different 16S rRNA genes in individual cells of *Haloarcula marismortui*. *Extremophiles* 4:373–376
- Arp G, Reimer A, Reitner J (2001) Photosynthesis-induced biofilm calcification and calcium concentrations in phanerozoic oceans. *Science* 292:1701–1704
- Benlloch S, Acinas SG, Antón J, López-López A, Luz SP, Rodríguez-Valera F (2001) Archaeal biodiversity in crystallizer ponds from a solar saltern: culture versus PCR. *Microb Ecol* 41:12–19
- Boucher Y, Douady CJ, Sharma AK, Kamekura M, Doolittle WF (2004) Intragenomic heterogeneity and intergenomic recombination among haloarchaeal rRNA genes. *J Bacteriol* 186:3980–3990
- Briões C, Amils R (2000) Nucleotide sequence of the 23s rRNA from *Haloferax mediterranei* and phylogenetic analysis of halophilic archaea based on LSU rRNA. *Syst Appl Microbiol* 23:124–131
- Burns BP, Goh F, Allen MA, Neilan BA (2004) Microbial diversity of extant stromatolites in the hypersaline marine environment of Shark Bay, Australia. *Environ Microbiol* 6:1096–1101
- Cardinale M, Brusetti L, Quatrini P, Borin S, Puglia AM, Rizzi A, Zanardini E, Sorlini C, Corselli C, Daffonchio D (2004) Comparison of different primer sets for use in automated ribosomal intergenic spacer analysis of complex bacterial communities. *Appl Environ Microbiol* 70:6147–6156
- DasSarma S, Fleischmann EM, Rodríguez-Valera F (1995) Halophiles. In: Robb FT (ed) *Archaea: a laboratory manual*. CSHL Press, Cold Spring Harbor, pp 225–230
- DeLong EF (1992) Archaea in coastal marine environments. *Proc Natl Acad Sci USA* 89:5685–5689
- Dennis PP, Ziesche S, Mylvaganam S (1998) Transcription analysis of two disparate rRNA operons in the halophilic Archaeon *Haloarcula marismortui*. *J Bacteriol* 180:4804–4813
- Elshahed MS, Najar FZ, Roe BA, 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
- Fisher MM, Triplett EW (1999) Automated approach for ribosomal intergenic spacer analysis of microbial diversity and its application to freshwater bacterial communities. *Appl Environ Microbiol* 65:4630–4636
- García-Martínez J, Rodríguez-Valera F (2000) Microdiversity of uncultured marine prokaryotes: the SAR11 cluster and the marine Archaea of group I. *Mol Ecol* 9:935–948
- García-Martínez J, Bescós I, Rodríguez-Sala J, Rodríguez-Valera (2001) RISSC: a novel database for ribosomal 16S-23S RNA genes spacer regions. *Nucleic Acid Res* 29:178–180
- Goh F, Leuko S, Allen MA, Bowman JP, Kamekura M, Neilan BA, Burns BP (2006) *Halococcus hamelinensis* sp. nov., a novel halophilic archaeon isolated from stromatolites in Shark Bay, Australia. *Int J Syst* 56:1323–1329
- González N, Romero J, Espejo RT (2003) Comprehensive detection of bacterial populations by PCR amplification of the 16S–23S rRNA spacer region. *J Microbiol Meth* 55:91–97
- Grant WD, Gemmell RT, McGenity TJ (1998) Halobacteria: the evidence for longevity. *Extremophiles* 2:279–287
- Kushner DJ, Kamekura M (1988) Physiology of halophilic eubacteria. In: Rodríguez-Valera F (ed) *Halophilic bacteria*, vol I. CRC Press, Boca Raton, pp 109–140
- Leuko S, Weidler G, Radax C, Legat A, Koemle NI, Kargl G, Stan-Lotter H (2002) Examining the physico-chemical resistance of halobacteria with the LIFE-DEAD kit, following exposure to simulated Martian atmospheric conditions and heat. *ESA Special Publication (ESA-SP-518)*
- Martin-Laurent F, Philippot L, Hallet S, Chaussod R, Germon JC, Soulas G, Catroux G (2001) DNA extraction from soils: old bias for new microbial diversity analysis methods. *Appl Environ Microbiol* 67:2354–2359
- McGenity TJ, Gemmell RT, Grant WD, Stan-Lotter H (2000) Origins of halophilic microorganisms in ancient salt deposits. *Environ Microbiol* 2:243–250

- Monty C (1977) Evolving concepts on the nature and the ecological significance of stromatolites. In: Flügel E (ed) Fossil algae, recent results and developments. Springer, Berlin Heidelberg New York, pp 15–35
- Mormile MR, Biesen MA, Gutierrez MC, Ventosa A, Pavlovich JB, Onstott TC, Fredrickson JK (2003) Isolation of *Halobacterium salinarum* retrieved directly from halite brine inclusion. *Environ Microbiol* 5(11):1094–1102
- Ng WV, Kennedy SP, Mahairas GG, Berquist B, Pan M, Shukla HD, Lasky SR, Baliga NS, Thorsson V, Sbrogna J, Swartzell S, Weir D, Hall J, Dahl TA, Welti R, Goo YA, Leithauser B, Keller K, Cruz R, Danson MJ, Hough DW, Maddocks DG, Jablonksi PE, Krebs MP, Angevine CM, Dale H, Isenbarger TA, Peck RF, Pohlschroder M, Spudich JL, Jung KH, Alam M, Freitas T, Hou S, Daniels CJ, Dennis PP, Omer AD, Eberhardt H, Lowe TM, Liang P, Riley M, Hood L, DasSarma S (2000) Genome sequence of *Halobacterium* species NRC-1. *PNAS* 97:12176–12181
- Oren A (2001) The order Halobacteriales. In: Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebrand E (eds) The prokaryotes: an evolving electronic resource for the microbiological community, 3rd ed., release 3.2, 25 July 2001. Springer, Berlin Heidelberg New York
- Oren A, Gurevich P, Gemmill RT, Teske A (1995) *Halobaculum gomorrense* gen. nov., sp. nov., a novel extremely halophilic archaeon from the Dead Sea. *Int J Syst Bacteriol* 45:747–754
- Øvreås L, Daae FL, Torsvik V, Rodríguez-Valera F (2003) Characterization of microbial diversity in hypersaline environments by melting profiles and reassociation kinetics in combination with terminal restriction fragment length polymorphism (T-RFLP). *Microb Ecol* 46:291–301
- Palmisano AC, Summons RE, Cronin SE, Des Marias DJ (1989) Lipophilic pigments from cyanobacterial (blue-green algal) and diatom mats in Hamelin pool, Shark Bay, Western Australia. *J Phycol* 25:655–661
- Papineau D, Walker JJ, Mojzsis SJ, Pace NR (2005) Composition and structure of microbial communities from stromatolites of Hamelin Pool in Shark Bay, Western Australia. *Appl Environ Microbiol* 71:4822–4832
- Petrisor AI, Decho AW (2004) Using geographical information techniques to quantify the spatial structure of endolithic boring processes within sediment grains of marine stromatolites. *J Micro Meth* 56:173–180
- Polz MF, Cavanaugh CM (1998) Bias in template to product ratios in multitemplate PCR. *Appl Environ Microbiol* 64:3724–3730
- Purdy KJ, Cresswell-Maynard TD, Nedwell DB, McGenity TJ, Grant WD, Timmis KN, Embley TM (2004) Isolation of haloarchaea that grow at low salinities. *Environ Microbiol* 6:591–595
- Radax C, Gruber C, Stan-Lotter H (2001) Novel haloarchaeal 16S rRNA gene sequences from alpine Permo–Triassic rock salt. *Extremophiles* 5:221–228
- Ranjard L, Brothier E, Nazaret S (2000) Sequencing bands of ribosomal intergenic spacer analysis fingerprints for characterization and microscale distribution of soil bacterium populations responding to mercury spiking. *Appl Environ Microbiol* 66:5334–5339
- Reid RP, James NP, Macintyre IG, Dupraz CP, Burne RV (2003) Shark Bay stromatolites: microfabrics and reinterpretation of origins. *Facies* 49:299–324
- Rodríguez-Valera F, Ruiz-Berraquero F, Ramos-Cormenzana A (1979) Isolation of extreme halophiles from Seawater. *Appl Environ Microbiol* 38:164–165
- Stan-Lotter H, Pfaffenhuemer M, Legat A, Busse HJ, Gruber C (2002) *Halococcus dombrowskii* sp. nov., an archaeal isolate from a Permian alpine salt deposit. *Int J Syst Evol Microbiol* 52:1807–1814
- Tillett D, Neilan BA (2000) Xanthogenate nucleic acid isolation from cultured and environmental cyanobacteria. *J Phycol* 36:251–258
- Wilson K (1990) Preparation of genomic DNA from bacteria. In: Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (eds) Current protocols in molecular biology. Wiley, New York, pp 2.4.1–2.4.2
- von Wintzingerode F, Goebel UB, Stackebrandt E (1997) Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. *FEMS Microbiol Rev* 21:213–229
- Yannarell AC, Triplett EW (2005) Geographic and environmental sources of variation in lake bacterial community composition. *Appl Environ Microbiol* 71:227–239