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ORIGINAL ARTICLE

Determining the specific microbial populations and their spatial distribution within the stromatolite ecosystem of Shark Bay

Falicia Goh[1], Michelle A Allen[1], Stefan Leuko[2], Tomohiro Kawaguchi[3], Alan W Decho[3], Brendan P Burns[1,2] and Brett A Neilan[1,2]

[1]*School of Biotechnology and Biomolecular Sciences, University of New South Wales, Sydney, New South Wales, Australia;* [2]*Australian Centre for Astrobiology, University of New South Wales, Sydney, New South Wales, Australia and* [3]*Department of Environmental Health Sciences, Arnold School of Public Health, University of South Carolina, Columbia, SC, USA*

The stromatolites at Shark Bay, Western Australia, are analogues of some of the oldest evidence of life on Earth. The aim of this study was to identify and spatially characterize the specific microbial communities associated with Shark Bay intertidal columnar stromatolites. Conventional culturing methods and construction of 16S rDNA clone libraries from community genomic DNA with both universal and specific PCR primers were employed. The estimated coverage, richness and diversity of stromatolite microbial populations were compared with earlier studies on these ecosystems. The estimated coverage for all clone libraries indicated that population coverage was comprehensive. Phylogenetic analyses of stromatolite and surrounding seawater sequences were performed in ARB with the Greengenes database of full-length non-chimaeric 16S rRNA genes. The communities identified exhibited extensive diversity. The most abundant sequences from the stromatolites were α- and γ-proteobacteria (58%), whereas the cyanobacterial community was characterized by sequences related to the genera *Euhalothece, Gloeocapsa, Gloeothece, Chroococcidiopsis, Dermocarpella, Acaryochloris, Geitlerinema* and *Schizothrix*. All clones from the archaeal-specific clone libraries were related to the halophilic archaea; however, no archaeal sequence was identified from the surrounding seawater. Fluorescence *in situ* hybridization also revealed stromatolite surfaces to be dominated by unicellular cyanobacteria, in contrast to the sub-surface archaea and sulphate-reducing bacteria. This study is the first to compare the microbial composition of morphologically similar stromatolites over time and examine the spatial distribution of specific microorganismic groups in these intertidal structures and the surrounding seawater at Shark Bay. The results provide a platform for identifying the key microbial physiology groups and their potential roles in modern stromatolite morphogenesis and ecology.
The ISME Journal (2009) **3**, 383–396; doi:10.1038/ismej.2008.114; published online 18 December 2008
Subject Category: microbial population and community ecology
Keywords: stromatolite; cyanobacteria; FISH; microbial community; geomorphology; phylotype; halophilic archaea

Introduction

Stromatolites are abundant throughout the Earth's geological records. The oldest examples of these preserved formations are more than 3 billion years old and are found mainly in Western Australia and South Africa (Lowe, 1980; Walter *et al.*, 1980; Byerly *et al.*, 1986). Recent studies have reflected a widespread and growing acceptance of the oldest stromatolites from the Pilbara region of Western Australia as biogenic (Allwood *et al.*, 2006). However, the limited information preserved in fossil stromatolites has prompted studies on extant systems as crucial to understanding the process of their formation. Prominent examples of modern stromatolites can be found in the hypersaline region of Hamelin Pool, Western Australia (Hoffman, 1976; Playford and Cockbain, 1976), and in the open marine waters of Exuma Sound, Bahamas (Dravis, 1983; Reid *et al.*, 2000). These modern analogues provide us with a template of how Earth and its early biosphere may have co-evolved.

Sediment trapping and binding and/or mineral precipitation by microorganisms led to the formation of the Bahaman stromatolites (Reid *et al.*, 2000). In particular, filamentous cyanobacteria of the genus *Schizothrix* were shown to be responsible for the binding and trapping of sand grains, whereas coccoid cyanobacterial populations provided structural support for the growth of the stromatolites. In addition, both heterotrophic aerobic and anaerobic bacteria, which synthesize low-molecular weight organic compounds and amorphous exopolymers, were also present in these structures (Visscher *et al.*, 1998). In contrast to the Bahaman stromatolites, the Shark Bay communities exhibit a greater diversity of archaea, cyanobacteria and heterotrophic bacteria (Burns *et al.*, 2004; Papineau *et al.*, 2005; Leuko *et al.*, 2007). Shark Bay stromatolite communities are characterized by cyanobacteria belonging to the genera *Synechococcus*, *Xenococcus*, *Microcoleus*, *Leptolyngbya*, *Plectonema*, *Symploca*, *Cyanothece*, *Pleurocapsa*, *Nostoc* and *Prochloron* (Burns *et al.*, 2004; Papineau *et al.*, 2005). These initial studies identified more than 20 of the 52 known divisions of bacteria, as well as archaeal communities, including both Euryarchaeota and Crenarchaeota gene sequences. Comparison between stromatolites of differing morphology was also related to specific changes in the microbial community present. Recent studies on both Shark Bay and Bahaman stromatolites have revealed that eukaryotes are scarce in these formations (Reid *et al.*, 2000; Burns *et al.*, 2004; Papineau *et al.*, 2005), though a variety of flagellates have been documented from Shark Bay (Al-Qassab *et al.*, 2002). It has been proposed that stromatolites thrive in environments that exclude many higher organisms, particularly those that may graze on the resident microorganisms.

To date there have been no studies comparing the microbiology of morphologically similar stromatolites over temporal scales, or assessing the prokaryotic populations of the surrounding seawater. This latter feature is particularly important in attempting to assign populations specific to the geological morphogenesis and to further our understanding of the ecology and dynamics of modern stromatolite communities. Here, apart from these microbial diversity and functional assignments, scanning electron microscopy and fluorescence *in situ* hybridization were also performed on stromatolite sections to examine the spatial distribution and interaction of specific microorganism groups within these stromatolites.

Materials and methods

Sample description and sample sites

Hamelin Pool in Western Australia spans an area of ca. 1220 km^2 with an average tidal range of ca. 60 cm (Logan and Cebulski, 1970). High net evaporation rates and average water temperatures throughout the year between 17 and 27 °C create a hypersaline environment with at least twice the salinity of seawater (Arp *et al.*, 2001). Intertidal columnar stromatolites (Figure 1) were collected during the
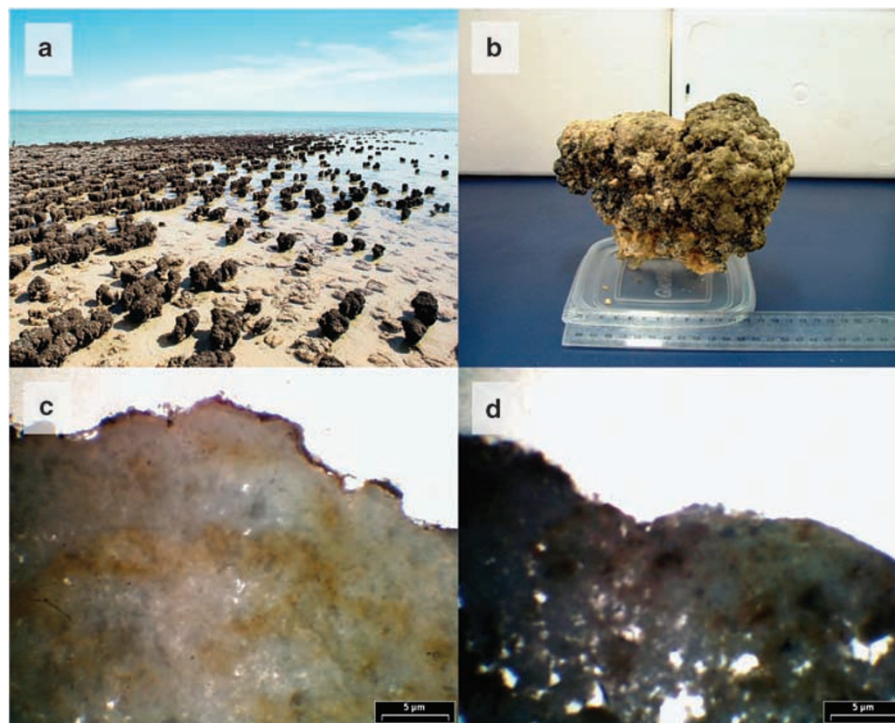

Figure 1 Images of stromatolites and the sample site at Hamelin Pool, Shark Bay. (**a**) Low tide at Telegraph Point showing stromatolites. (**b**) Sample HPS2. (**c**) A slide of sample HPS1 (Burns *et al.* (2004)) and (**d**) a slide of sample HPS2. Both (**c**) and (**d**) were embedded in epoxy resin and sectioned to a width of 30 μm; photograph taken using light microscopy (magnification = × 4).

late afternoon in November 2002 from Telegraph Point (26°25′00″S, 11H°13′05″) on the south eastern shore of Hamelin Pool, Shark Bay, Western Australia, using methods described earlier (Burns *et al.*, 2004). Samples were collected during low tide and a rock hammer was used to physically remove small sections with a vertical interval of ~ 2 cm from the top of the stromatolite. Seawater surrounding the stromatolites was collected in sterile containers immediately before the collection of stromatolite samples.

Samples were placed in sterile specimen bags and stored in the dark at 4 °C during transportation. All samples were collected and handled with sterile instruments throughout the course of the study. The stromatolite sample analysed here was designated as HPS2 and another stromatolite surface section collected from the same location was designated as HPS1 (Burns *et al.* 2004). Although both samples were obtained from different stromatolites, they were obtained from morphologically identical columnar stromatolites in the intertidal region, both exhibiting a dark green and black pustular surface. These samples (HPS1 and HSP2) also have the same morphology as the stromatolite sample (HPIRR) analysed by Papineau *et al.* (2005). In contrast, sample HPDOM (Papineau *et al.*, 2005) had a smooth domal surface. All samples were collected in the late afternoon. The interior of the sample analysed in this study consisted of fine grains as shown in the image of the cross-section from HPS2 (Figure 1d). The interior of HPS1 (Figure 1c) also showed that this sample consisted of similar fine grains. These both resembled the intertidal fine-grained stromatolites built by cyanobacteria as described in earlier studies by Awramik and Riding (1988). All samples were stored at 4 °C and DNA extraction and microorganism isolation were conducted immediately upon sample return.

Scanning electron microscopy
Fractured stromatolite samples were fixed in 3% glutaraldehyde solution, washed with 0.1 M sodium cacodylate buffer and stored at 4 °C. The samples were dehydrated in an ethanol series (50–100%), vacuum dried and sputter coated with gold using EmiTech K550× gold sputter coater. Specimens were examined in a Leica Cambridge S360 scanning electron microscope set at an accelerating voltage of 20.0 kV with a lens-to-specimen working distance of 25 mm and a beam current of 100 pA.

Culturing and isolation
Isolation of bacteria, cyanobacteria and archaea from HPS2 and seawater was carried out by modifying BG-11 media (Rippka *et al.*, 1981), Luria–Bertani media (Sambrook *et al.*, 1989) and DSM97 (DasSarma *et al.*, 1995), respectively. The salt concentrations of the different media were modified by the

addition of NaCl (852 mM), KCl (18.6 mM), CaCl$_2$ (18.4 mM), MgCl$_2$ (90 mM) and MgSO$_4$ (54 mM). This was conducted to mimic the hypersaline conditions of Hamelin Pool as described earlier (Burns *et al.*, 2004), to isolate heterotrophic bacteria, cyanobacteria and halophilic archaea from this hypersaline environment. Owing to the higher concentration of sodium chloride in DSM97, no addition of this salt was made to this medium. To further facilitate the isolation of the slow-growing halophilic archaea from the faster growing bacteria, three antibiotics at 100 mg ml^{-1} (streptomycin, penicillin and ampicillin) were added to the modified DSM97 media (Wais, 1988).

DNA isolation and PCR amplification of 16S rRNA genes
Genomic DNA extraction of isolates was performed with an extraction protocol utilizing xanthogenate (Tillett and Neilan, 2000). The total genomic DNA from ground stromatolite was extracted using the TNE buffer extraction protocol (Neilan *et al.*, 2002). Modifications to this protocol were carried out to improve cell lysis by extending the lysozyme step (> 1 h) and the inclusion of four freeze–thaw cycles. Genomic material from two litres of seawater was concentrated by centrifugation and then extracted using the same extraction protocol as for the stromatolites. PCRs were performed using 1 U *Taq* polymerase (Fischer Biotech, Perth, Western Australia, Australia), 2.5 mM MgCl$_2$, 10× *Taq* polymerase buffer (Fischer Biotech), 0.2 mM dNTPs (Fischer Biotech) and 0.2 pM of each primer. Thermal cycling was performed in a GeneAmp PCR System 2400 Thermocyler (Perkin Elmer, Norwalk, CT, USA). Primer pairs used for the amplification of the bacterial, cyanobacterial and archaeal 16S rRNA genes were 27F1(5′-AGAGTTTGATCCTGGCTCA G-3′)/1494Rc (5′-TACGGTTACCTTGTTACGAC-3′)/ 809R (5′-GCTTCGGCACGGCTCGGGGTCGATA-3′) and 21F(5′-TTCCGGTTGATCCYGCCGGA-3′)/958R (5′-YCCGGCGTTGAMTCCAATT-3′), respectively. Thermal cycling conditions for the amplification of bacterial 16S rRNA genes were initial denaturation step at 92 °C for 2 min, followed by 30 cycles of denaturation at 92 °C for 10 s, primer annealing at 52 °C for 15 s, strand extension at 72 °C for 90 s and a final extension step at 72 °C for 7 min. Thermal cycling conditions for the amplification of cyanobacteria were as per standard protocols (Jungblut *et al.*, 2005), and the amplification of archaeal 16S rRNA genes was carried out as described earlier (DeLong, 1992).

Construction of 16S rDNA clone libraries
Amplified archaeal-, universal bacterial- and cyanobacterial-specific 16S rRNA gene PCR products from total genomic DNA extracted from HPS2 and seawater were cloned into the p-Gem T Easy Vector

System (Promega, Madison, WI, USA). Ligations and transformations were performed according to the manufacturer. For each sample and set of primers used, at least 100 positive (insert containing) clones were selected. Restriction fragment length polymorphism (RFLP) analysis was performed with PCR products using restriction enzymes *Alu*I and *Scr*F1 (Fermentas, Hanover, MD, USA) (Jungblut *et al.*, 2005). Clones were then grouped according to their different restriction patterns and a clone representing each pattern was sequenced. Automated sequencing was carried out using the PRISM Big Dye cycle sequencing system and ABI 3730 Capillary Applied Biosystem (Foster City, CA, USA) using Polymer 7.

Estimation of microbial diversity

Distance matrices were generated in ARB using the neighbour-joining method with the Jukes and Cantor correction. The program DOTUR (Schloss and Handelsman, 2005) was used to cluster sequences into operational taxonomic units by pair-wise identity (95–100% ID) with a furthest-neighbour algorithm and a precision of 0.01. Rarefaction curves, richness estimations (Chao1) and diversity indices (Shannon–Wiener) were calculated using DOTUR with default settings and 1000 random sample repetitions. Coverage (*C*) values were calculated by the method of Good with the equation $C = [1 - (n/N)] \times 100$, where *n* is the number of phylotypes in a sample represented by one clone (singletons) and *N* is the total number of clones examined (Good, 1953).

Phylogenetic sequence analysis

All sequences were analysed for chimaeras using methods described by Huber *et al.* (2004) and Cole *et al.* (2003). Chimaeras were not detected in the clones obtained. Sequence identities were determined by Basic Local Alignment Search Tools (BLAST) results and phylogenetic analysis. The 16S rRNA gene sequences were aligned and analysed using the ARB software package (Ludwig *et al.*, 2004). The resulting alignments were manually checked and corrected when necessary. Phylogenetic trees were constructed using the maximum likelihood method (default parameters for ARB and fastDNAml) and the alignment of near full-length sequences (>1000 bp) of reference strains. Aligned partial 16S rRNA gene sequences (<1000 bp) were then inserted without changing the overall tree topology using the parsimony tool within ARB. This was necessary as sequences from a study on these stromatolite populations conducted by Papineau *et al.* (2005), samples designated as HPDOM and HPIRR, targeted different regions of the 16S rRNA genes as compared with this study and the study by Burns *et al.* (2004). Bootstrap values were obtained for branching patterns by using the

PHYLIP software package (Retief, 2000), and values >50% were included for the main nodes of the tree. 16S rRNA gene sequences from earlier studies on the Shark Bay stromatolites (Burns *et al.*, 2004; Papineau *et al.*, 2005) were included for the determination of differences in the populations.

Fluorescence *in situ* hybridization of archaea and sulphate-reducing bacteria

Stromatolite samples (1 cm vertical section from the surface) for fluorescent *in situ* hybridization were fixed in 4% paraformaldehyde solution, washed with 1:1 phosphate-buffered saline–ethanol buffer and stored at 4 °C until required. Samples were then embedded in epoxy resin and sectioned to a width of 30 μm. Hybridization buffer containing an appropriate concentration of formamide and 50 ng of each probe (SRB385 5′-CGGCGTCGCTGCGTCAGG-3′ or Arch915 5′-GTGCTCCCCCGCCAATTCCT-3′) was applied to the slides, followed by incubation at 46 °C for 2 h. The samples were washed for 10–15 min in wash buffer pre-heated to 48 °C and subsequently rinsed with Milli-Q water. The slides were dried in the dark. Mountant and coverslip were applied, viewed and imaged using a confocal laser scanning microscope with argon–krypton laser.

Accession numbers

Sequences of the 16S rDNA clones and isolates are available under GenBank accession numbers EF150676–EF150838.

Results and discussion

Microbial communities present in the stromatolites and the seawater surrounding these formations in Shark Bay were analysed with molecular phylogenetic methods. Isolation of cultivable microorganisms was also achieved by modifying the three culture media (Luria–Bertani, BG11 and DSM97 media) used in this study. The stromatolite sample (HPS2) analysed here had an irregular morphology and a dark green and black pustular surface. This is the same morphology as that described for both the samples studied previously, HPIRR (Papineau *et al.*, 2005) and HPS1 (Burns *et al.*, 2004). The sample interior of HSP2 consisted of fine grains as shown in the image of a cross-section (Figure 1d), similar to the cross-section of HPS1. Scanning electron micrographs indicated an association between carbonate and sand grains with filamentous bacteria (Figure 2), and boreholes, approximately 5–10 μm in diameter, were also evident. It is difficult at this stage to assign exact identities to these borers in the Shark Bay stromatolites; however, potential candidates include the range of unicellular coccoid cyanobacteria identified here (Table 1). These findings are consistent with the study carried out on Bahaman stromatolites (Reid *et al.*, 2000), where boring
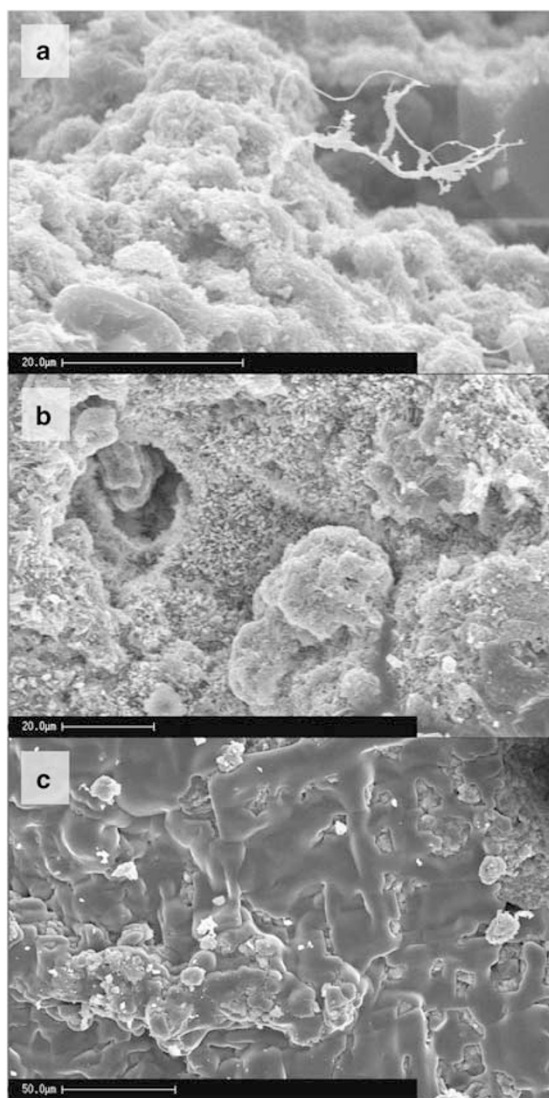
Halobacterium NCIMB 718, an uncharacterized halophilic archaeon. One of these isolates was determined to be a new species belonging to the genus *Halococcus* (Goh *et al.*, 2006). Bacteria belonging to the genera *Bacillus*, *Salinivibrio*, *Halomonas*, *Marinobacter*, *Porphyrobacter*, *Idiomarina* and two isolates with highest identity to the bacteria K2-13 and K3 were cultured from HPS2 (summarized in Table 1). Isolates with similarity to *Alteromonas*, *Vibrio*, *Paracoccus* and *Roseibium* were cultivated from the seawater. The cyanobacterial isolates had closest identity to *Euhalothece*, *Gloeocapsa*, *Cyanothece*, *Oscillatoria*, *Geitlerinema*, *Xenococcus*, *Chroococcidiopsis*, *Gloeothece*, *Lyngbya* and *Dermocarpella* (Table 1). No cyanobacteria were isolated from the surrounding seawater.

Clone library analysis of HPS2 and surrounding seawater

Amplification of 16S rDNA using universal bacterial, specific cyanobacterial and archaeal primers, and subsequent clone library construction was conducted on both total community DNA from the finely ground stromatolite samples and the surrounding seawater. Unique clones were identified by RFLP analysis. Clones of representative RFLP types were sequenced bi-directionally and compared with known sequences by BLAST analysis. From the archaeal clone libraries (93 clones), 10 RFLP groups were identified with BLAST results of sequences having closest identity to halophilic archaea. No archaeal 16S rRNA genes were amplified from the genomic DNA isolated from the seawater. Thirty-three unique RFLP groups were obtained from the stromatolite bacterial clone libraries (85 clones), whereas 19 groups were obtained from the seawater libraries (94 clones). In HPS2, the highest proportion of sequences were related to α-proteobacteria (29%) and γ-proteobacteria (29%), whereas the majority of clones in the seawater had sequence identities related to α-proteobacteria (69%) (Figure 3). A total of 96 clones were obtained for the stromatolite cyanobacterial library, with another 82 clones obtained from the seawater. Five groups were identified from the RFLP analysis of the stromatolite cyanobacterial library whereas eight groups were obtained from the seawater. The relative abundance of cyanobacterial clones is shown in Figure 3. The most abundant clones from HPS2 were affiliated with *Gloeothece* (52%) and *Gloeocapsa* (42%). The most abundant clones (56%) from the seawater cyanobacterial library were related to *Euhalothece*.

Estimated coverage and richness

The coverage and richness of the clone libraries were assessed by the method of Good (1953) for the calculation of coverage, the non-parametric estimator Chao1 for richness (Chao, 1984) and the

Figure 2 Scanning electron micrographs of Hamelin Pool stromatolite (HPS2). (**a**) Filamentous cyanobacteria binding carbonate sand grains. (**b**) Borehole, as shown by the arrow, is approximately 5–10 μm in diameter filled with needle-like microcrystalline structure, most probably aragonite (another form of calcium carbonate), and (**c**) welding of carbonate sand grains and in-filling of boreholes as a result of continued microboring.

activities of microorganisms (microboring) was characterized by boreholes surrounded by needle-like aragonite crystals. Lithified layers of micritized carbonate sand grains were also evident (Figure 2), and this is likely to be a result of continuous microboring.

Isolation of microorganisms from stromatolite sample HPS2 and surrounding seawater

A total of 25 archaeal isolates and 63 bacterial isolates (of which 42 isolates belonged to cyanobacterial genera) were cultivated. Their identities were determined based on GenBank BLAST analysis of their 16S rRNA genes. All 25 archaeal isolates showed a maximum of only 92% identity to

Table 1 Summary of bacteria isolated from HPS2 and seawater surrounding Shark Bay stromatolites

Sample	Media	Isolate ID	Accession no.	Sequence analysis		
				Nearest relative in GenBank[a]	% Identity	Accession no.
HPS2	LB[b]	HSB25	EF150759	*Bacillus* sp. SD-18	98	AF326372
		HSB26	EF150761	*Bacillus* sp. BA-54	95	AY557616
		HSB28	EF150762	*Bacillus hwajinpoensis*	99	AF541966
		HSB29	EF150758	*Bacillus marismortui* strain 123	99	AJ009793
		HSB9	EF150760	*Halomonadaceae* bacterium LA44	99	AF513453
		HSB55	EF150747	Bacterium K2-13	99	AY345436
		HSB30	EF150749	*Bacillus* sp. PL30	98	AF326370
		HSB31	EF150750	*Halobacillus* sp. D-8	95	AY351395
		HSB32	EF150752	*Halobacillus trueperi* strain GSP38	99	AY505522
		HSB13	EF150753	*Marinobacter* sp. MED104	98	AY136120
		HSB14	EF150754	*Salinivibrio costicola* strain GSP14	97	AY553070
		HSB33	EF150755	*Bacillus* sp. SG-1	98	AF326373
		HSB34	EF150757	*Bacillus litoralis*	99	AY608605
		HSB8	EF150748	*Halomonas alimentaria* strain GSP27	98	AY553077
		HSB10	EF150751	*Idiomarina* sp. NT N118	99	AB167034
		HSB35	EF150742	*Bacillus megaterium* strain SAFB-011	99	AY167865
		HSB36	EF150744	*Bacillus* sp. KMM 3737	97	AY228462
		HSB6	EF150743	*Porphyrobacter tepidarius* DSM10594	97	AF465839
		HSB37	EF150745	*Bacillus firmus*	97	AJ509007
		HSB56	EF150746	Bacterium K34	97	AY345475
Seawater		SWB5	EF150705	*Alteromonas* sp. V4.BE.32	98	AJ244758
		SWB6	EF150706	Vibrionaceae bacterium PH25	97	AF513466
		SWB3	EF150700	*Paracoccus* sp. MBIC 4036	92	AB025192
		SWB4	EF150701	*Roseibium denhamense*	98	D85832
		SWB7	EF150702	*Alteromonas* sp. NBF18	97	AF343939
		SWB8	EF150703	*Pseudoalteromonas* sp. A25	98	AF227237
		SWB9	EF150704	Vibrionaceae bacterium PH25	99	AF513466
HPS2	BG-11[a]	HSC29	EF150800	*Euhalothece* sp. strain MPI95AH13	96	AJ000710
		HSC25	EF150791	*Cyanothece* sp. ATCC 51142	92	AF132771
		HSC31	EF150793	*Gloeocapsa* sp. PCC 73106	94	AF132784
		HSC36	EF150796	LPP-group MBIC10087	97	AB058225
		HSC37	EF150794	*Geitlerinema* sp. PCC 7105	92	AF132771
		HSC22	EF150797	*Xenococcus* PCC 7305	92	AF132783
		HSC20	EF150774	*Xenococcus* sp. Cyano35	96	DQ058859
		HSC17	EF150776	*Lyngbya hieronymusii*	94	AF337650
		HSC19	EF150780	*Chroococcidiopsis* sp. PCC 6712	94	AJ344557
		HSC3	EF150785	*Dermocarpella incrassata*	98	AJ344559
		HSC34	EF150783	*Gloeothece* sp. KO11DG	92	AB067577
		HSC24	EF150784	Uncultured *Chroococcus sp.*	94	DQ058856

[a]Modified BG-11 media (Burns *et al.*, 2004) used for the isolation of cyanobacteria.
[b]Modified Luria–Bertani media (Burns *et al.*, 2004) used for the isolation of heterotrophic bacteria.

Shannon–Wiener index for diversity (Table 2). Sequences were collected into operational taxonomic units by pair-wise identity (% ID) with a furthest-neighbour algorithm and a precision of 0.01. The estimated coverage for all the libraries constructed was ⩾50% (Table 2), and collector curves for coverage for all libraries approached asymptotic (data not shown). This indicated that the coverage of the populations was comprehensive for these samples, and the libraries were representative samples of the microbial diversity. The cutoff for differentiation of species was chosen as 97% sequence identity; however, this probably underestimated the total bacterial diversity. The Chao1 richness estimate for HPS2 was highest for the bacterial population. The number of observed species from the total bacterial library was 33 with a Chao1 estimate of 44.67 (36.45–73.40), indicating that when sampled to completion, there would be between 4 and 40 more species obtained. Only 10 taxa were obtained from the archaeal library and when sampled to completion 3–20 more species would be obtained. The cyanobacterial library was sampled to completion. The number of operational taxonomic units, Chao1 richness estimates and the Shannon–Wiener diversity estimates also indicated that total bacterial diversity from HPS2 was greater than the surrounding seawater, whereas the opposite was observed for the estimates of cyanobacterial diversity.

Comparison with known Shark Bay stromatolite microbial communities

Phylogenetic analysis of HPS2 and its surrounding seawater sequences was performed in ARB with the
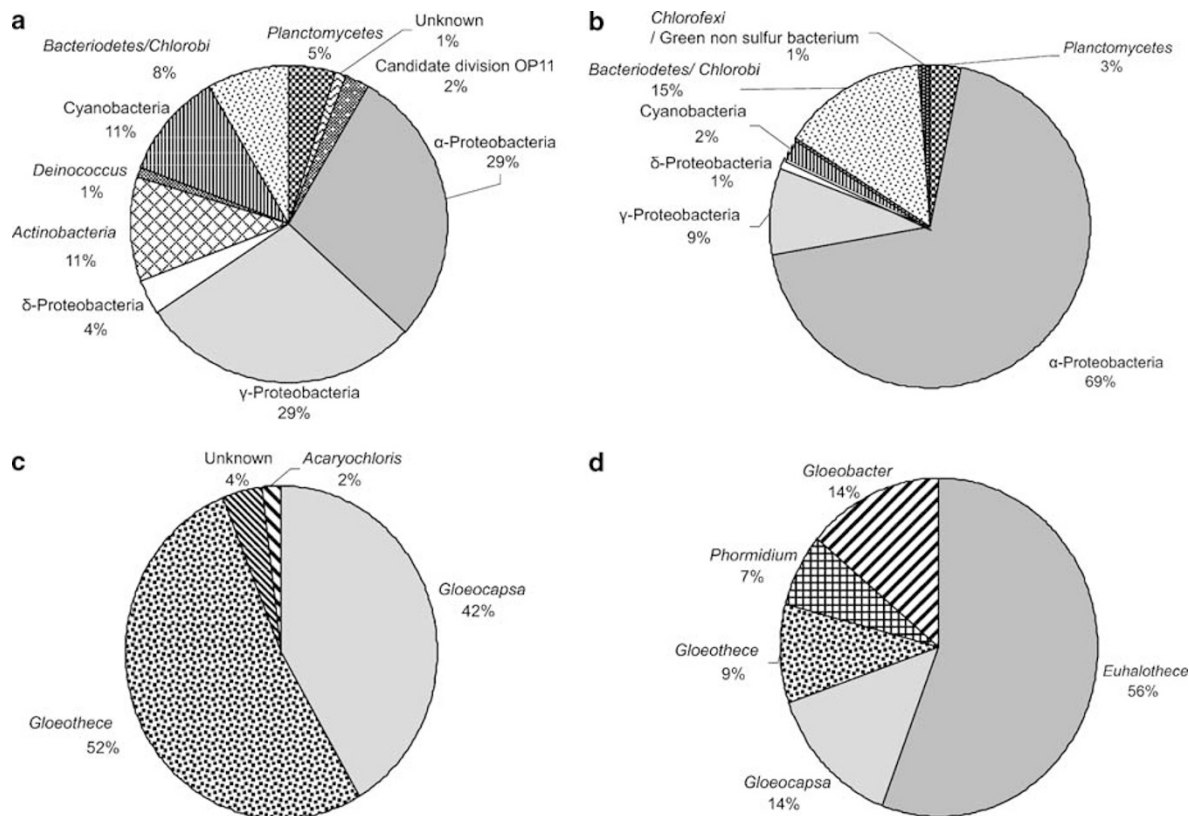
Figure 3 Comparison of the most abundant sequences in the 16S rDNA clone libraries constructed for total bacteria and cyanobacteria from the sample HPS2 and seawater. (**a**) Total bacteria in sample HSP2. (**b**) Total bacteria in surrounding seawater. (**c**) Cyanobacteria in sample HSP2. (**d**) Cyanobacteria in surrounding seawater.

Greengenes database of full-length non-chimaeric 16S rRNA gene sequences (DeSantis *et al.*, 2006). Partial sequences from earlier studies on samples HPS1, HPDOM and HPIRR (Burns *et al.*, 2004; Papineau *et al.*, 2005) were included for comparison to obtain a comprehensive understanding of the microbial diversity in this environment. The phylogenetic trees in Figures 4–6 show the relative topologies of the sequences for the archaeal domain, the bacterial domain and for the cyanobacteria, respectively. As shown in Figure 4, all archael clones from HPS2, HPS1, HPDOM and HPIRR were related to Halobacteria from the Euryarchaeota. In particular, the *Halococcus* genus was represented by both clones and isolates from HPS2 and HPS1, an indication of their intrinsic persistence in this environment. In contrast to the archaeal population of HPS1, HPDOM and HPIRR, HPS2 contained no representatives of the Crenarchaeota. The results presented here indicate that there has been a decrease in the observed taxonomic units in archaeal diversity in these stromatolites possessing similar morphologies and from the same location. The halophilic archaeal sequences obtained from different samples over time generally had close phylogenetic affinities. However, no archaea were identified in the seawater surrounding the stromatolites using either culturing techniques or total DNA analyses.

Although it is possible that archaea are present in concentrations in the seawater that are below the limits of detection of the methods employed here, it would seem that these halophilic organisms are specific to these biosedimentary structures (Goh *et al.*, 2006). The role of archaea in stromatolites is not well understood, and thus it is difficult to assess their contributions. Could archaea also contribute to stromatolite formation? Although we can only speculate at this stage, it has been shown that some Haloarchaeal species are also capable of fixing carbon dioxide (Javor, 1988). Furthermore, the association of sulphate-reducing bacteria with archaea can result in the coupling of methane oxidation with sulphate reduction and can also lead to carbonate precipitation (Michaelis *et al.*, 2002).

Bacterial 16S rDNA sequence analysis revealed 16 groups belonging to α-proteobacteria, γ-proteobacteria, δ-proteobacteria, acidobacteria, Bacteriodetes/chlorobi, two unknown clades, candidate division OP-11, firmicutes, candidate division TM6, planctomycetes, actinobacteria, chloroflexi, cyanobacteria, *Deinococcus* and thermotogales (Figure 5). Groups containing sequences from the seawater comprised the α- and γ-proteobacteria, Bacteriodetes/chlorobi, planctomycetes, chloroflexi and cyanobacteria. The cyanobacterial cluster contained clones from both HPS2 and seawater (Figure 5).

Table 2 Coverage, observed phylotype richness, richness and diversity indices for clone libraries constructed from HPS2 and seawater

	Phylotype cutoff (%)	HPS2					Surrounding seawater				
		No. of clones analysed	Coverage good (%)[a]	OTUs	Richness index Chao1 (95% CI)[b]	Diversity index Shannon–Wiener (95% CI)[c]	No. of clones analysed	Coverage good (%)[a]	OTUs	Richness index Chao1 (95% CI)[b]	Diversity index Shannon–Wiener (95% CI)[c]
Archaea	100	93 (659 bp)	84	15	18 (15–37)	2.01 (1.78–2.25)					
	99		86	13	18 (13–45)	1.57 (1.29–1.85)					
	98		89	10	13 (10–32)	1.12 (0.85–1.40)			NA		
	97		89	10	13 (10–32)	1.12 (0.85–1.40)					
	96		89	10	13 (10–32)	1.12 (0.85–1.40)					
	95		90	9	12 (9–31)	0.93 (0.66–1.19)					
Total bacteria	100	87 (628 bp)	59	35	47 (39–74)	3.21 (3.00–3.41)	92 (667 bp)	75	23	24 (23–34)	2.81 (2.64–2.99)
	99		60	34	46 (38–73)	3.19 (2.99–3.38)		77	21	23 (21–33)	2.72 (2.55–2.88)
	98		60	34	46 (38–73)	3.19 (2.99–3.38)		77	21	23 (21–33)	2.72 (2.55–2.88)
	97		62	33	45 (36–73)	3.16 (2.97–3.36)		78	20	22 (20–32)	2.62 (2.44–2.80)
	96		62	33	45 (36–73)	3.11 (2.91–3.30)		78	20	22 (20–32)	2.62 (2.44–2.80)
	95		63	32	44 (35–71)	3.11 (2.91–3.30)		79	19	21 (19–31)	2.53 (2.35–2.71)
Cyano-bacteria	100	96 (717 bp)	92	7	7 (7–13)	1.33 (1.17–1.49)	43 (781 bp)	79	9	9 (9–14)	1.84 (1.59–2.09)
	99		92	7	7 (7–13)	1.33 (1.17–1.49)		79	9	9 (9–14)	1.84 (1.59–2.09)
	98		92	7	7 (7–13)	1.33 (1.17–1.49)		79	9	9 (9–14)	1.84 (1.59–2.09)
	97		93	6	6	1.23 (1.14–1.42)		79	9	9 (9–14)	1.84 (1.59–2.09)
	96		93	6	6	1.23 (1.14–1.42)		79	9	9 (9–14)	1.84 (1.59–2.09)
	95		93	6	6	1.23 (1.14–1.42)		81	8	8 (8–13)	1.47 (1.14–1.80)

Abbreviations: CI, confidence interval; OTUs, operational taxonomic units.
[a]The coverage index was calculated by the method of Good (1953).
[b]The richness index was calculated by the method of Chao1.
[c]The diversity index by the method of Shannon–Wiener was calculated using the software program DOTUR (Schloss and Handelsman, 2005).

These clones from HPS2 showed highest sequence identity to *Cyanothece*, *Gloeocapsa* and *Phormidium*, whereas clones from the surrounding seawater had highest sequence identity to *Euhalothece*. However, using cyanobacterial-specific PCR primers, a greater diversity of cyanobacteria was observed (Figure 6). Analyses of the total bacterial community in this study indicated that the identities of cultivated organisms also differed from those of the 16S rDNA clone libraries. In particular, culturable *Bacillus* sp. appeared to dominate the Shark Bay stromatolites. One of the adaptive physiological responses to environmental stresses by *Bacillus* is spore formation (Nicholson, 2002), and the difficulty in extracting DNA from spores is a possible explanation for the lack of *Bacillus* 16S rRNA genes detected in the total genomic DNA from stromatolites in this and earlier studies. The majority of the stromatolite microbial communities were members of the proteobacteria/purple bacteria, firmicutes/Gram-positive bacteria, cyanobacteria and green sulphur bacteria, consistent with other reports on stromatolite-associated biodiversity (Bauld *et al.*, 1979; Visscher *et al.*, 1999; Reid *et al.*, 2000). Bacteria belonging to the groups planctomycetes, proteobacteria/purple bacteria, cyanobacteria and the green sulphur bacteria were

also identified in the surrounding seawater. These groups may therefore not be unique to these benthic geological communities; however, the dispersal of microorganisms from biofilms is a common phenomenon in the environment (Characklis, 1990; Brading *et al.*, 1995).

The Shark Bay stromatolite cyanobacterial 16S rDNA sequences were affiliated to the orders Pleurocapsales, Chroococcales and Oscillatoriales. The *Prochloron* group was identified only from HPS1, whereas the surrounding seawater sequences were related to Chroococcales and Oscillatoriales. Three Pleurocapsales clades were identified to be *Chroococcidiopsis* (sequences from HPS1 and HPS2), *Dermocarpella* (sequences from HPS2) and *Pleurocapsa* (sequences from HPS2). Under the order Chroococcales, sequences from HPS1, HPS2 and the surrounding seawater were related to *Gloeocapsa* and *Euhalothece* spp. The *Gloeothece* group contained sequences from HPS2, HPIRR and HPDOM, whereas the *Cyanothece* group contained sequences from HPS1 and HPS2. Within the Oscillatoriales, most sequences from HPS1 belonged to *Symploca*, *Plectonema* and a novel clade of filamentous cyanobacteria. Sequences from HPS2 clustered with *Leptolyngbya* and *Geitlerinema,* whereas the HPIRR and HPDOM sequences clustered with *Microcoleus* sp.
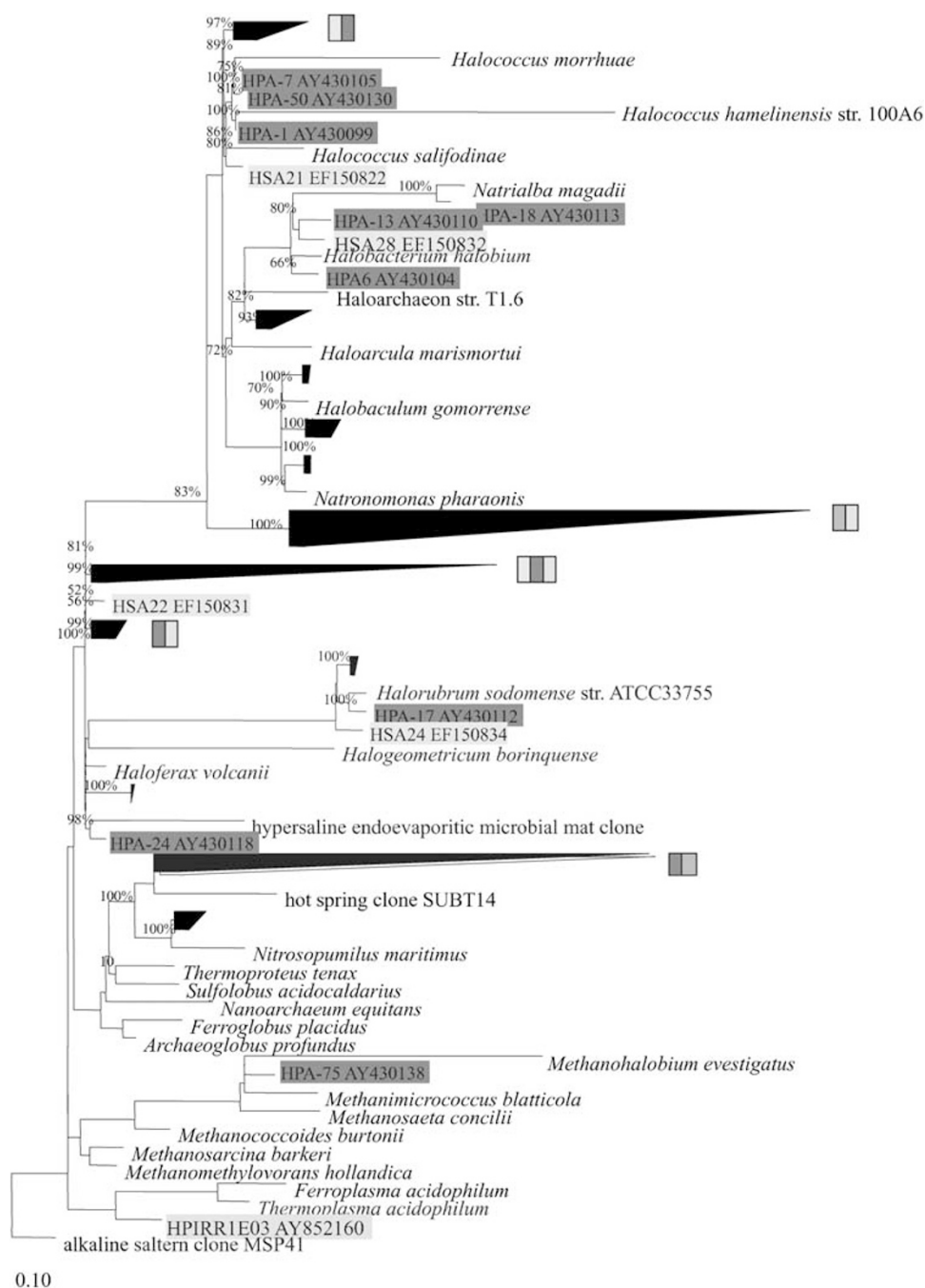
Figure 4 Phylogenetic relationships of archaea from Shark Bay intertidal stromatolites. Sequences from stromatolites HPS2, HPS1 (Burns *et al.*, 2004), HPDOM and HPIRR (Papineau *et al.*, 2005) were highlighted in yellow, red, orange and pink, respectively. A coloured box beside a filled triangle indicates that a group of sequences were clustered together. Only bootstrap values >50% were considered significant. The scale bar represents 0.1 nucleotide changes per nucleotide position. SSU 16S rDNA sequences are available under GenBank accession numbers EF150811–EF150838. The color reproduction of this figure is available on the html full text version of the manuscript.

The identification of sequences related to *Schizothrix* sp. from HPS2 agrees with reports carried out on the microbial composition of the Bahaman stromatolites (Reid *et al.*, 2000), and these and other filamentous cyanobacteria identified here may play roles in stromatolite formation in Shark Bay through the binding and trapping of sediments (Reid *et al.*, 2000). This corroborates the recent detection of unicellular coccoid cyanobacteria in modern stromatolites (Macintyre *et al.*, 2000). Although the specific coccoid cyanobacterium found to be involved in the boring and in-filling of Bahaman stromatolites (*Solentia* sp.) was not identified here, unicellular coccoid cyanobacterial isolates with 16S rDNA sequences similar to *Chroococcidiopsis* sp., *Xenococcus* sp. and *Dermocarpella* sp. from the
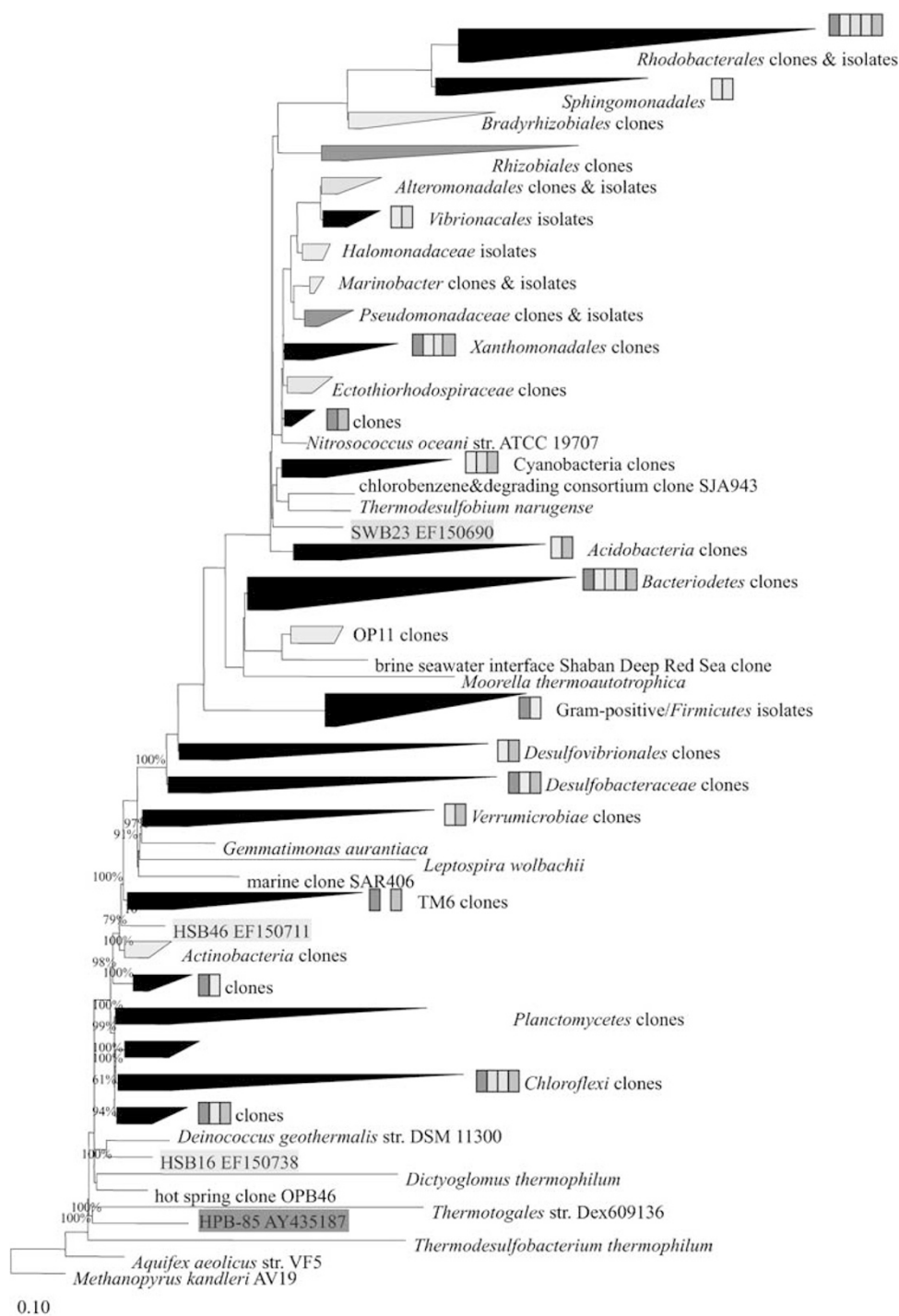
Figure 5 Phylogenetic relationships of total bacteria from Shark Bay intertidal stromatolites. Sequences from stromatolites HPS2, HPS1 (Burns et al., 2004), HPDOM and HPIRR (Papineau et al., 2005) and seawater were highlighted in yellow, red, orange, pink and blue, respectively. A coloured box beside a filled triangle indicates that sequences from more than one study were present. Only bootstrap values >50% were considered significant. The scale bar represents 0.1 nucleotide changes per nucleotide position. SSU 16S rDNA sequences are available under GenBank accession numbers EF150676–EF150762. The color reproduction of this figure is available on the html full text version of the manuscript.

same order (Pleurocapsales) were present in all the three Shark Bay samples. In particular, HPS2 indicated the presence of a higher diversity of unicellular cyanobacteria compared with HPS1, whereas the overall diversity in HPS2 was lower

compared with the other studies. In addition to the involvement of filamentous cyanobacteria in the formation of stromatolites, the identification of these unicellular cyanobacteria sp. (*Euhalothece* sp., *Gloeothece* sp., *Gloeocapsa* sp. and *Chroococci-*
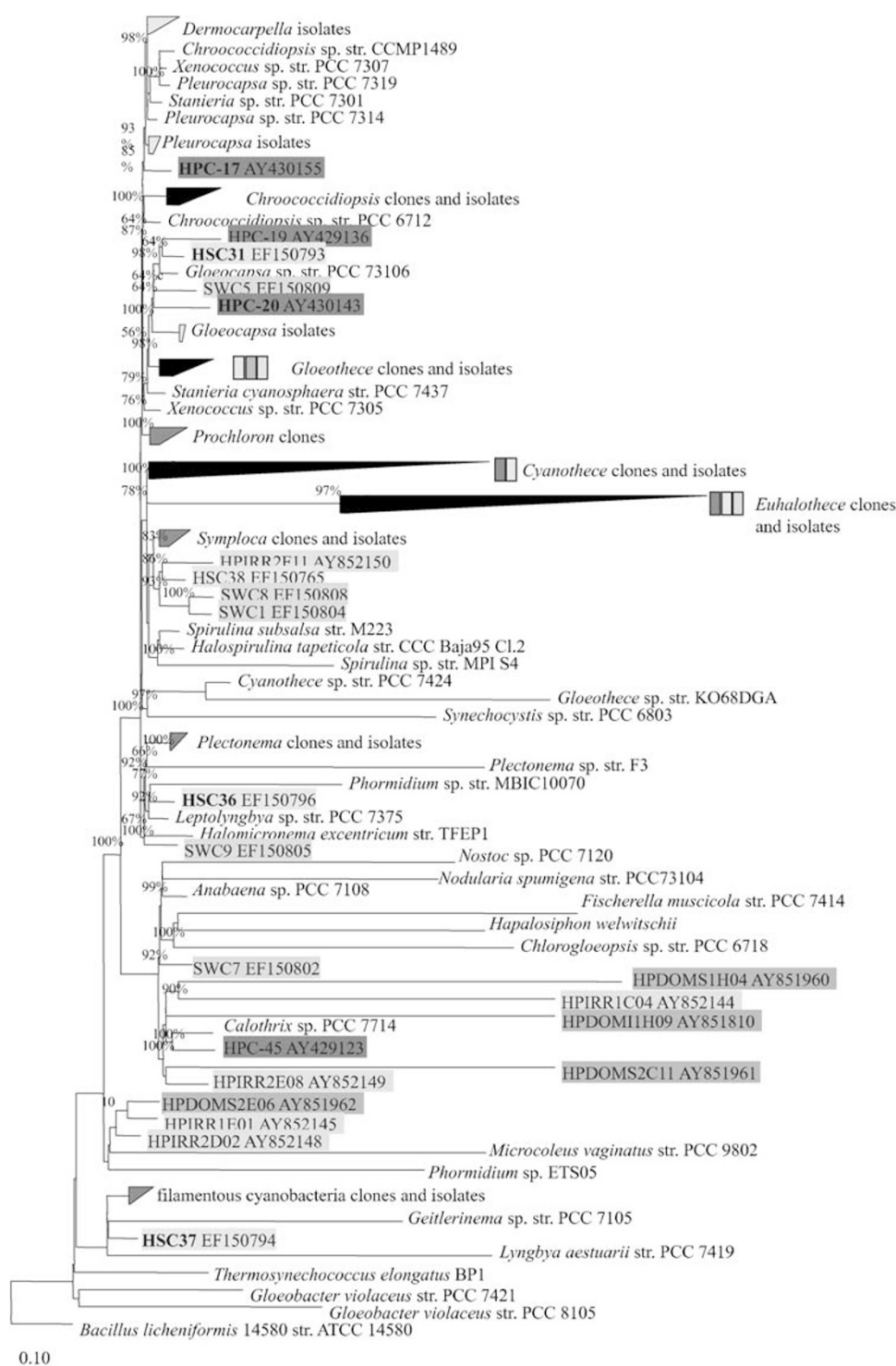
Figure 6 Phylogenetic relationships of cyanobacteria from Shark Bay intertidal stromatolites. Sequences from stromatolites HPS2, HPS1 (Burns et al., 2004), HPDOM and HPIRR (Papineau et al., 2005) and seawater were highlighted in yellow, red, orange, pink and blue, respectively. A coloured box beside a filled triangle indicates that sequences from more than one study were present. Only bootstrap values >50% were considered significant. Scale bar represents 0.1 nucleotide changes per nucleotide position. SSU 16S rDNA sequences are available under GenBank accession numbers EF150763–EF150810. The color reproduction of this figure is available on the html full text version of the manuscript.

diopsis sp.) mirrors the composition of other hypersaline ecosystems (Garcia-Pichel et al., 1998; Nubel et al., 2000).

Microscopy
In earlier studies of the Bahaman stromatolites, cyanobacteria and sulphate-reducing bacteria were

shown to be involved in their formation (Visscher, 2000). In both the present and earlier studies on microbial diversity in the Shark Bay stromatolites, microbial groups were detected based on phylogenetic affinities. To determine the spatial distribution of these microorganisms, thin sections from the top 1 cm of stromatolite samples were analysed by confocal microscopy. In addition, archaeal probes were employed to investigate the distribution of halophilic archaea in Shark Bay stromatolites. Cyanobacteria were detected by the autofluorescence of phycocyanin whereas archaea and sulphate-reducing bacteria were detected using labelled specific DNA probes. Cyanobacteria were located throughout the stromatolite section and, in particular, at the surface (Figure 7a). Archaeal cells were not located on the surface of the stromatolites, but were closely associated with the cyanobacterial cells below the surface (Figure 7b). Sulphate-reducing bacteria were localized to the surface and intermediate layers of the stromatolite section, and were also in close association with cyanobacteria. Although the cyanobacteria formed a distinct layer on the surface, there were no other apparent discrete layers of microorganisms observed within the stromatolite sections (Figure 7). The archaea and sulphate-reducing bacteria were instead irregularly localized with the cyanobacterial cells (Figure 7c).

Conclusion

The microbial communities of modern stromatolites and surrounding seawater have been characterized and compared with the microorganisms identified earlier in this ecosystem. The presence of diverse metabolic groups of microorganisms indicated that this environment has a rich pool of genetic diversity and strongly suggests a high level of biological complexity in this ecosystem. The relative abundance of these microbial communities may influence the variety of geomorphologies observed; however, biases inherent in the present microbial and molecular analyses affords only limited quantitative interpretation of the data. The use of controlled and broadly accepted procedures ensures that the results can be readily related to similar investigations.

In terms of the microorganisms identified in both the stromatolites and the surrounding seawater, earlier studies have shown that the dispersal of biofilm members in surface water is common and is particularly relevant in this environment given that these microbial communities are subjected to abrasion, sloughing and erosion due to physical forces or nutrient depletion. Differences in microbial diversity of Shark Bay stromatolites apparent from the studies conducted to date may be in part due to these various environmental challenges leading to microbial succession, as shown to occur in the Bahaman stromatolites (Reid et al., 2000). The concept of succession is common in planktonic
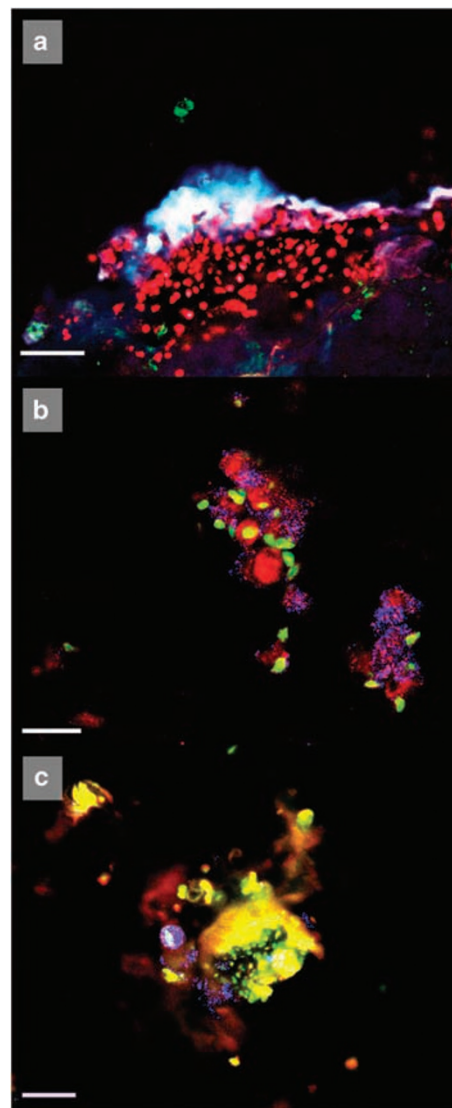

Figure 7 Krypton–Argon confocal images of FISH of cross-sections of Shark Bay stromatolite samples. (**a**) Image of a stromatolite cross-sectioned vertical surface dominated by unicellular cyanobacteria that constitute the black surface layer (Figures 1a and c). The autofluorescence of cyanobacteria is denoted in red whereas DNA in the sample was stained by SyBr Green and is denoted in green. Scale = 10 μm. (**b**) FISH image of the interaction of cyanobacteria with archaea in the middle region of the stromatolite section with an FITC archaeal probe (green) and autofluorescence of cyanobacteria (red) is denoted in red. Scale = 5 μm. (**c**) FISH image of the middle region of the stromatolite section showing sulphate-reducing bacteria (stained with FITC-SRB probe green) with cyanobacteria (denoted in yellow due to the overlap between autofluorescence of cyanobacterial pigments, phycocyanin red and phycoerythrin green). Scale = 5 μm. Both (**b**) and **c**) were taken approximately 100 μm below the surface. FISH, fluorescence in situ hybridization; FITC, fluorescein isothiocyanate.

cyanobacterial and other autotrophic communities (Stevenson, 1983), as it is in biofilm populations (Santegoeds et al., 1998; Jackson et al., 2001). If the measured fluxes in these stromatolite populations are real, certain metabolisms critical for the geological formation would also respond to the prevailing

conditions. One such factor is hypersalinity of the environment. Salinity gradients are formed due to tidal flows and the evaporation of seawater in the intertidal regions of Hamelin Pool. These gradients result in the presence of microbial species adapted to different ranges of salinity and could result in a reduction in the diversity of bacterial and archaeal communities at higher salinities (Benlloch et al., 2002; Jungblut et al., 2005). High levels and fluctuations in salinity also result in an increase in biodiversity and favour the growth of halophilic microorganisms (Wieland and Kuhl, 2006; Abed et al., 2007). It is evident from this study, and regardless of time and the type of stromatolite, that certain phenotypic groups, such as both unicellular and filamentous cyanobacteria and halophilic archaea, are consistent components of these environments and therefore likely to be intrinsic to the development of the stromatolites at Shark Bay. The information presented here is a platform to examine other functional characteristics of these systems, including salt tolerance, cell signalling and secondary metabolism (Burns et al., 2005). This study has also confirmed the importance of microbial community analyses of the Hamelin Pool stromatolites to understand the relationship between microbial populations, the conservation and ecophysiology of this particular environment, and how this may correlate with the morphology of extant and extinct stromatolites.

Acknowledgements

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