Environmental Microbiology (2004) **6**(10), 1096–1101 doi:10.1111/j.1462-2920.2004.00651.x

Microbial diversity of extant stromatolites in the hypersaline marine environment of Shark Bay, Australia

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Summary

Stromatolites have been present on Earth, at various levels of distribution and diversity, for more than 3 billion years. Today, the best examples of stromatolites forming in hypersaline marine environments are in Hamelin Pool at Shark Bay, Western Australia. Despite their evolutionary significance, little is known about their associated microbial communities. Using a polyphasic approach of culture-dependent and culture-independent methods, we report the discovery of a wide range of microorganisms associated with these biosedimentary structures. There are no comparable reports combining these methodologies in the survey of cyanobacteria, bacteria, and archaea in marine stromatolites. The community was characterized by organisms of the cyanobacterial genera *Synechococcus*, *Xenococcus*, *Microcoleus*, *Leptolyngbya*, *Plectonema*, *Symploca*, *Cyanothece*, *Pleurocapsa* and *Nostoc*. We also report the discovery of potentially free-living *Prochloron*. The other eubacterial isolates and clones clustered into seven phylogenetic groups: OP9, OP10, Marine A group, Proteobacteria, Low G+C Gram-positive, Planctomycetes and Acidobacteria. We also demonstrate the presence of sequences corresponding to members of halophilic archaea of the divisions Euryarchaeota and Crenarchaeota and methanogenic archaea of the order Methanosarcinales. This is the first report of such archaeal diversity from this environment. This study provides a better understanding of the microbial community associated with these living rocks.

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Introduction

The living stromatolites of Hamelin Pool in Western Australia are internationally renowned as the most extensive examples on earth of extant marine stromatolites and are an outstanding example of a significant stage in the earth's evolutionary history. One of the earliest pieces of evidence of planetary biota is contained in the microfossils of stromatolites (Walter *et al*., 1980; Byerly *et al*., 1986). Although the biogenic origin of the oldest fossilized stromatolites is under debate (Brasier *et al*., 2002; Schopf *et al*., 2002), the textures and morphological features of these modern stromatolites have been considered to resemble closely ancient stromatolite assemblages (Logan, 1961; Riding, 2000), and thus may represent the oldest examples of life on earth. Owing to the restricted flow of sea water into Hamelin Pool and high net evaporation rates, surface waters have salinity twice that of normal sea water (Arp *et al*., 2001) and, by analogy, many ancient stromatolites have been considered to form in hypersaline or intertidal conditions (Monty, 1977). Many important steps in evolution may have occurred within stromatolites owing to the close proximity of diverse microorganisms and microniches (Nisbet and Fowler, 1999). In addition, the process of microbial carbonate precipitation is intrinsic to stromatolite formation, and the presence of carbonates is important in biogenicity determinations in fossilized samples (Riding, 2000). Thus, modern stromatolites are a significant resource for studying the origin, evolution and distribution of life, particularly the physiological processes that may leave preserved biosignatures in fossils on Earth and, potentially, distant biospheres.

The results reported here relate to the distinctive coarse agglutinated columns in the intertidal zone at Hamelin Pool. Surfaces of these stromatolites are covered with living mats, and this study focused on characterizing the microbial community of this actively growing layer. This is because, at the microscale most relevant to bacteria, a vital factor that affects stromatolite formation is the presence of other organisms. Apart from physical factors such as wave action, it has been suggested that stromatolite morphology will depend on the community present (Riding, 2000) and will therefore be determined by it. The environment selects for biological diversity, which in turn is reflected in these biogenic structures. Preliminary work in our laboratory reported distinct cyanobacteria in micro-

bial mats from various locations, including Shark Bay (Neilan *et al*., 2002). To describe the nature and diversity of Shark Bay stromatolitic populations better, we analysed both culturable organisms and 16S rDNA cyanobacterial, other eubacterial and archaeal clone libraries amplified directly from environmental genomic DNA extracted from the columnar stromatolite.

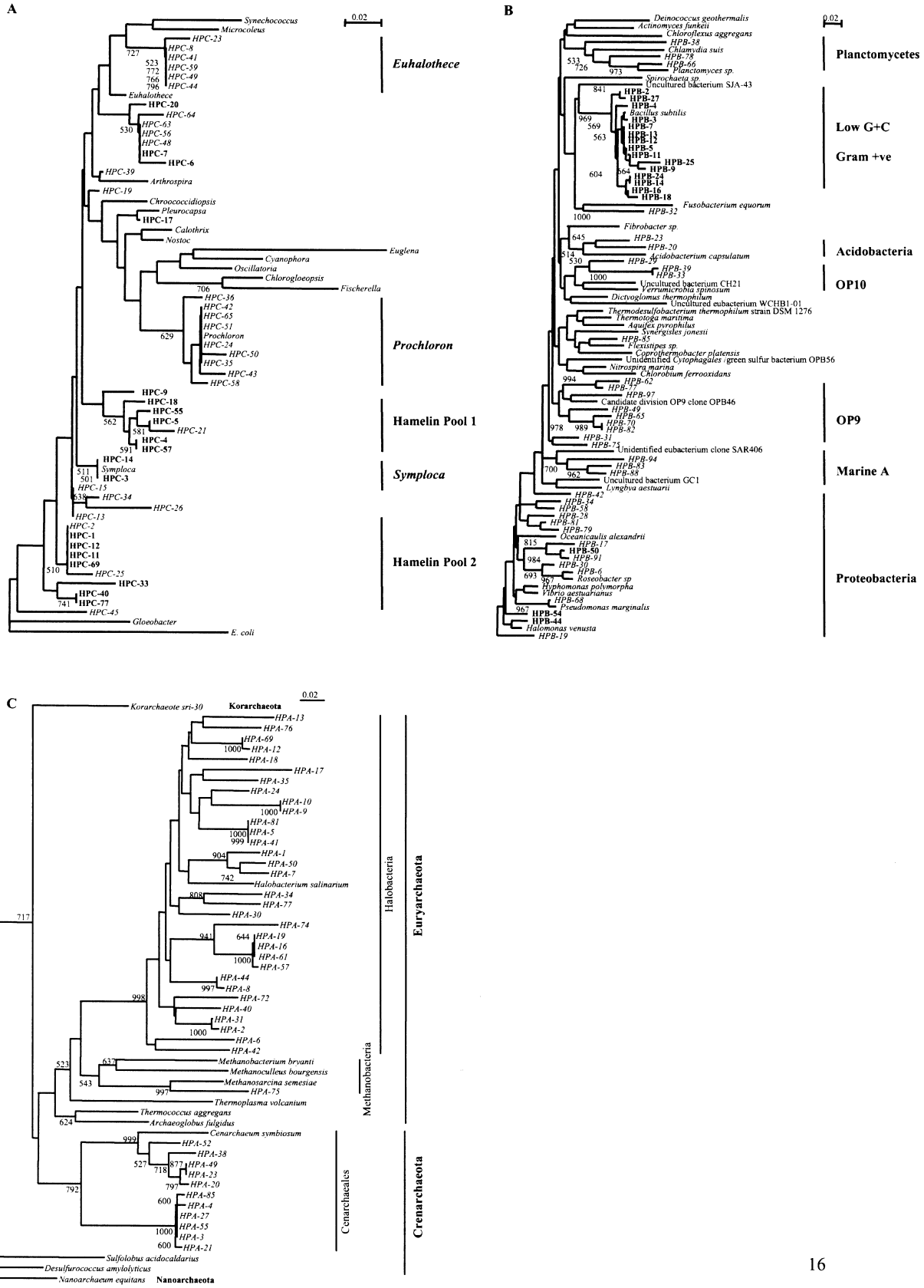
Results and discussion

Samples of intertidal columnar stromatolites were collected from Telegraph Point (26°25′00″ S, 114°13′05″ E) on the south-eastern shore of Shark Bay. Salinity of the sea water surrounding the stromatolites was 70%. The columns ranged in height from ≈20 to 50 cm and contained visible trapped sediment with grain sizes of silt, sand or larger. Samples were taken at low tide when the stromatolites were exposed, and a geological pick was used physically to remove small sections with a vertical interval of 2 cm from the top of the hard stromatolite surface. The geomorphology of these stromatolites has been described previously (Chivas *et al*., 1990) and, at a macroscale, the surface was a mix of sediment and mat material. Samples were placed in plastic specimen bags and stored at 4°C during transportation and were obtained and handled with sterile instruments throughout the course of the study. This 2 cm section was physically pulverized using a mortar and pestle to obtain a homogenized mixture. After suspension in 5% sterile saline, 100 μl aliquots of the homogenate were used directly as the inoculum in BG-11 media (Rippka *et al*., 1981), Luria–Bertani (LB) media (Sambrook *et al*., 1989) and DSM97 media (DasSarma *et al*., 1995) for the isolation of cyanobacteria, bacteria and archaea respectively. The salt concentrations for 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) to levels to mimic the elevated environmental concentrations found in Shark Bay (Arp *et al*., 2001). Bacterial and archaeal cultures were incubated at 37°C, while cyanobacterial cultures were incubated at room temperature and a constant light intensity. As isolates appeared, they were recultivated until pure.

Cultivation of cyanobacteria from the sample yielded 33 morphologically distinct cyanobacterial isolates, and the majority that we observed were filamentous, a characteristic known to aid sediment trapping in stromatolites (Reid *et al*., 2000). Analysis of cyanobacterial-specific 16S rDNA clone libraries yielded phylotypes with closest relation to a further eight genera not identified by culture. From our analysis of sequences of representative isolates and clones (Fig. 1A), we observed previously uncharacterized diversity from this environment. Earlier studies have only identified one or two cyanobacterial genera in stromatolites at Shark Bay (Golubic, 1976; López-Cortés, 1999; Neilan *et al*., 2002) and the Bahamas (Reid *et al*., 2000), the other major location for marine stromatolites. The Bahaman stromatolites are characterized by *Schizothrix* and *Solentia* (Reid *et al*., 2000), organisms not detected in our study, suggesting that these formations in different locations may be associated with disparate organisms adapted to each niche. One type of diversity here corresponds to sequences that seem to represent early branches in the cyanobacteria tree, e.g. HPC-45, as this occupies an ancestral position in the inferred phylogenies. We also discovered novel sequences that cluster separately from known strains, such as the Hamelin Pool 1 cluster comprising the clade HPC-4 plus HPC-57, the lineages HPC-55 and HPC-18 and the clade HPC-5 plus HPC-21. We include here also the potentially novel Hamelin Pool 2 cluster, comprising the sequences HPC-2, HPC-1, HPC-12, HPC-11 and HPC-69. All these exhibited at least 5% dissimilarity to the database sequences.

Isolates with closest BLAST similarity to *Pleurocapsa* (HPC-17), *Nostoc* (HPC-6), *Leptolyngbya* (the clade HPC-40 plus HPC-77) and *Symploca* (HPC-14 and HPC-3) were observed here to form a tough, sticky benthic matrix of cells in culture, a property that may be important in providing stromatolite structure. Molecular analysis revealed a number of sequences clustering with *Euhalothece* (Fig. 1A), and this group of cyanobacteria has also been identified in hypersaline microbial mats in other geographical locations (Garcia-Pichel *et al*., 1998; Nübel *et al*., 2000). Of further interest is the finding here that 34% of the clones clustered with the genus *Prochloron* (Fig. 1A), with percentage similarities between 97% and 99%. *Prochloron* is symbiotic with didemnid ascidians and, to date, there is no report of its existence as a free-living organism (Kühl and Larkum, 2002). This is the first time that *Prochloron* has been shown in this environment, and we have found no evidence either through our own observations or in the literature of ascidians in Hamelin Pool. Whether potentially free-living *Prochloron* are associated with stromatolites can only be suggested at this stage; however, future work *in situ* such as using *Prochloron*-specific probes and probes targeting ascidians may help to clarify this issue.

Although cyanobacteria primarily contribute to stromatolite morphogenesis, the metabolic activity of heterotrophic bacteria is also significant in microbial mats (Riding, 2000; Des Marais, 2003). Cultivation of non-cyanobacterial microorganisms on LB media with elevated salts and subsequent restriction fragment length polymorphism (RFLP) analysis revealed 18 unique phylotypes that clustered principally with *Bacillus* spp. with the low G+C Gram-positive bacteria (Fig. 1B). Results from Gram staining revealed that 94% of isolates were Gram-


A
0.02
Synechococcus
Microcoleus
HPC-23
HPC-8
HPC-41
HPC-59
HPC-49
HPC-44
Euhalothece
HPC-20
HPC-64
HPC-63
HPC-56
HPC-48
HPC-7
HPC-6
HPC-39
Arthrospira
HPC-19
Chroococcidiopsis
Pleurocapsa
HPC-17
Calothrix
Nostoc
Euglena
Cyanophora
Oscillatoria
Chlorogloeopsis
Fischerella
HPC-36
HPC-42
HPC-65
HPC-51
Prochloron
HPC-24
HPC-50
HPC-35
HPC-43
HPC-58
HPC-9
HPC-18
HPC-55
HPC-5
HPC-21
HPC-4
HPC-57
HPC-14
Symploca
HPC-3
HPC-15
HPC-34
HPC-26
HPC-13
HPC-2
HPC-1
HPC-12
HPC-11
HPC-69
HPC-25
HPC-33
HPC-40
HPC-77
HPC-45
Gloeobacter
E. coli
Euhalothece
Prochloron
Hamelin Pool 1
Symploca
Hamelin Pool 2
B
0.02
Deinococcus geothermalis
Actinomyces funkeii
Chloroflexus aggregans
HPB-38
Chlamydia suis
HPB-78
HPB-66
Planctomyces sp.
Spirochaeta sp.
Uncultured bacterium SJA-43
HPB-2
HPB-27
HPB-4
Bacillus subtilis
HPB-3
HPB-7
HPB-13
HPB-12
HPB-5
HPB-11
HPB-25
HPB-9
HPB-24
HPB-14
HPB-16
HPB-18
Fusobacterium equorum
HPB-32
Fibrobacter sp.
HPB-23
HPB-20
Acidobacterium capsulatum
HPB-29
HPB-39
HPB-33
Uncultured bacterium CH21
Verrumicrobia spinosum
Dictyoglomus thermophilum
Uncultured eubacterium WCHB1-01
Thermodesulfobacterium thermophilum strain DSM 1276
Thermotoga maritima
Aquifex pyrophilus
Synergisles jonesii
HPB-85
Flexistipes sp.
Coprothermobacter platensis
Unidentified Cytophagales /green sulfur bacterium OPB56
Nitrospira marina
Chlorobium ferrooxidans
HPB-62
HPB-17
HPB-97
Candidate division OP9 clone OPB46
HPB-49
HPB-65
HPB-70
HPB-82
HPB-31
HPB-75
Unidentified eubacterium clone SAR406
HPB-94
HPB-83
HPB-88
Uncultured bacterium GC1
Lyngbya aestuarii
HPB-42
HPB-34
HPB-58
HPB-28
HPB-81
HPB-79
Oceanicaulis alexandrii
HPB-17
HPB-50
HPB-91
HPB-30
HPB-6
Roseobacter sp
Hyphomonas polymorpha
Vibrio aestuarianus
HPB-68
Pseudomonas marginalis
HPB-54
HPB-44
Halomonas venusta
HPB-19
Planctomycetes
Low G+C
Gram +ve
Acidobacteria
OP10
OP9
Marine A
Proteobacteria
C
0.02
Korarchaeote sri-30
Korarchaeota
HPA-13
HPA-76
HPA-69
HPA-12
HPA-18
HPA-17
HPA-35
HPA-24
HPA-10
HPA-9
HPA-81
HPA-5
HPA-41
HPA-1
HPA-50
HPA-7
Halobacterium salinarium
HPA-34
HPA-77
HPA-30
HPA-74
HPA-19
HPA-16
HPA-61
HPA-57
HPA-44
HPA-8
HPA-72
HPA-40
HPA-31
HPA-2
HPA-6
HPA-42
Methanobacterium bryanti
Methanoculleus bourgensis
Methanosarcina semesiae
HPA-75
Thermoplasma volcanium
Thermococcus aggregans
Archaeoglobus fulgidus
Cenarchaeum symbiosum
HPA-52
HPA-38
HPA-49
HPA-23
HPA-20
HPA-85
HPA-4
HPA-27
HPA-55
HPA-3
HPA-21
Sulfolobus acidocaldarius
Desulfurococcus amylolyticus
Nanoarchaeum equitans
Nanoarchaeota
Halobacteria
Methanobacteria
Cenarchaeales
Euryarchaeota
Crenarchaeota

positive bacteria. Selected groups such as several *Bacillus* species were recovered readily by culture; however, their prevalence may be related to their observed rapid growth in the selected media, the presence of endospores and their known antibiotic production, which could preclude the isolation of slow-growing and antibiotic-sensitive organisms. However, clone library analysis revealed a greater degree of diversity (34 additional phylotypes) and emphasizes the complementary nature of culture-independent examinations. Interestingly, we identified several sequences with closest BLAST match to unidentified bacteria, such as the clade HPB-39 plus HPB-33 and the lineage HPB-29. Clones with phylogenetic affiliation with Planctomycetes were also identified here (HPB-78, HPB-38 and HPB-66), and this group has been shown to be important in nutrient cycling in other environments (Fuerst, 1995). Molecular analysis also revealed several phylotypes with closest relation to *Rhodovibrio* (HPB-58), *Rhodobacter* (HPB-91 and HPB-17), *Hyphomonas* (HPB-30) (phototrophic purple non-sulphur bacteria) and *Roseobacter* (HPB-6) (aerobic purple bacteria). This is consistent with published accounts revealing the diversity of Proteobacteria in stromatolites (Bauld *et al*., 1979; Visscher *et al*., 1998; Reid *et al*., 2000).

The third group targeted in this study was the archaea and, under the experimental growth conditions here, none could be isolated. However, cloning and subsequent analysis of archaeal-specific 16S rDNA polymerase chain reaction (PCR) products revealed 44 different phylotypes (Fig. 1C). Although one archaeal strain has been identified previously in Shark Bay (Zhilina, 1983), the present study is the first to reveal such a diversity of archaea associated with marine stromatolites. BLAST analysis revealed that 63% of the phylotypes were most closely related to uncultured archaea. The majority of the archaeal clones identified (74%) were closely related to the Halobacteria, an archaeal class known to be abundant in hypersaline settings (Oren, 2002). The presence of halophilic archaea and the anoxygenic phototrophs discussed above suggests that forms of phototrophic metabolism other than cyanobacterial oxygenic photosynthesis may contribute to the morphogenesis of the Shark Bay stromatolites. Two sequences in particular (HPA-6 and HPA-42) branched relatively deeply within this cluster (Fig. 1C), while other clades were neither deep in the tree nor specifically related to other recognized clades (e.g. HPA-34 plus HPA-77). Another group of clones clustered with the Crenarchaeota (Fig. 1C) and, as no member of the non-thermophilic Crenarchaeota has been isolated to date, the physiological characteristics of these organisms and their roles in stromatolite formation are unknown. The presence of a clone (HPA-75) related to the methanogenic archaea is another intriguing discovery for this ecosystem, particularly as this is a high sulphate environment (Arp *et al*., 2001) that may preclude most forms of methanogenesis (Amaral and Knowles, 1994). Based on these inferred physiologies, we have since developed media and growth conditions to facilitate isolation of archaea from this environment.

Conclusions

The combined use of culture-dependent and culture-independent methods has demonstrated for the first time a range of metabolically diverse prokaryotes in the Shark Bay stromatolites. Using this approach, 26% of phylotypes described could be cultured compared with 74% identified by molecular identification. All the microorganisms isolated in the present study were cultivated on high salt media, and ongoing investigations are focused on determining the genetic and physiological basis for salt tolerance in the Shark Bay stromatolites. Many of the cloned populations are unique phylotypes with no close relatives in the database, and the organisms represented by these

Fig. 1. Phylogenetic relationships of the prokaryotic community from Hamelin Pool intertidal stromatolites, inferred from 16S rDNA sequence analysis.
A. Phylogenetic tree for cyanobacteria.
B. Phylogenetic tree for bacteria.
C. Phylogenetic tree for archaea.
Sequences determined in this study were given an alphanumeric designation beginning with HPA (archaea), HPB (bacteria) or HPC (cyanobacteria); cultivated microorganisms are marked in bold, 16S rDNA clones are marked in italics. Bootstrap values (1000 resampling events) are shown for key branches; only values >500 were considered significant. The scale bar represents the number of substitutions per 100 bases. Total genomic DNA was extracted from isolates and recovered directly from the stromatolite sample, using methods established in our laboratory (Neilan *et al*., 2002). Modifications here included a longer lysozyme step (1 h) and one initial freeze–thaw cycle to improve cell lysis. PCR amplification of 16S rDNA from cyanobacteria, bacteria and archaea was carried out using specific primers and conditions described elsewhere (DeLong, 1992; Neilan *et al*., 1997). Clone libraries were constructed by cloning the gel-purified PCR products from cyanobacteria, bacteria and archaea into the pGEM-T vector (Promega). One hundred clones from each of the clone libraries were selected, and preliminary screening was carried out by RFLP analysis. For RFLP analysis, clones were amplified with specific primers via direct colony PCR. The resulting 16S rDNA PCR product was digested with the restriction enzymes *Alu*I and *Scr*FI, and the different phylotypes were grouped based on their banding patterns before sequencing. Automated sequencing was carried out as described previously (Neilan *et al*., 2002). BLAST searches (http://www.ncbi.nlm.nih.gov/blast) were used to identify similar sequences from GenBank (http://www.ncbi.nlm.nih.gov/GenBank). Phylogenetic analysis was carried out using genetic distance methods for phylogenetic inference as before (Neilan *et al*., 2002), using 400–500 homologous bases for the isolates and clones. SSU 16S rRNA sequences are available under GenBank accession numbers AY429113–AY429141, AY430099–AY430161, AY433816–AY433833 and AY435178–AY435211.

clones may also possess novel physiologies vital to the persistent morphogenesis of Shark Bay stromatolites. The close association of microorganisms in this setting may also facilitate horizontal gene transfer of evolutionally significant traits, including salt tolerance or antibiotic resistance. Our predictions of community metabolism from molecular systematic determinations will be complemented by future studies focusing on the design of more appropriate media to isolate uncultured organisms, the targeting of specific functional genes and conducting rational *in situ* experiments to detect specific enzyme activities. The data here provide us with a better understanding of the microbial diversity of these unique ecosystems and are critical for the conservation of Shark Bay stromatolites as well as the assessment of planetary exobiology.

Acknowledgements

We are especially grateful for the continuing support from the Australian Centre for Astrobiology, particularly excellent discussions with Malcolm Walter. We thank Amber Goodchild for provision of archaeal reference strains and primers, and Lucy Houghton for assistance with sample collection. Funding for this research was provided by the Australian Research Council, the Australian Academy of Science (Kanagawa Museum of Natural History Award) and the NASA Planetary Biology Internship Program.

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