

MICROBIAL DIVERSITY IN MODERN STROMATOLITES

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Abstract Poised at the biosphere–lithosphere interface, the microbial consortia associated with stromatolites have a profound impact on the evolution of Earth’s environment. In this chapter, we review the current state of knowledge of microbial diversity in extant stromatolites by examining data generated using cultivation-independent molecular techniques. Specifically, we compare natural stromatolitic mat systems of three distinctive habitats: the hypersaline pools of Shark Bay, Australia; the open ocean stromatolites of Highborne Cay, Bahamas; and the lacustrine lagoons of Ruidera Pools, Spain. We compare these natural systems to an experimental artificial microbialite, looking for fundamental differences and similarities within the microbial communities. Of the 21 bacterial phyla or sub-phyla detected in the various stromatolite ecosystems, only *Cyanobacteria* were found dominant in all habitats. Within the phylum, few cyanobacterial ecotypes were common to all ecosystems. The marine and hypersaline stromatolite ecosystems had significantly higher bacterial diversity than did the artificial microbialite or the freshwater stromatolite, and the diversity approached that observed in non-lithifying hypersaline microbial mats. Finally, we consider the ecological insights provided by the acquisition of metagenomic sequence data for understanding stromatolite diversity and function. These high-throughput metagenomic sequencing approaches have been applied to modern stromatolitic and microbialitic mat communities and have facilitated a higher resolution characterization of microbial diversity at the molecular-level, thus providing an initial glimpse into the functional complexity of these dynamic ecosystems.

Keywords Stromatolites • Microbial diversity • Cyanobacteria • Highborne Cay • Shark Bay • Artificial microbialites • Ruidera Pool • Alphaproteobacteria • Metagenomics • Functional genetics

1. Introduction

For much of the Earth’s history, stromatolites dominated the ancient landscape (Awramik, 1984). With fossils dating back over three billion years (Byerly et al., 1986; Grotzinger and Knoll, 1999), the geologic record of stromatolites clearly

shows the extensive role microbes have played in the evolution of Earth's environment (Kasting, 2001). Stromatolite morphology and structure are the direct result of microbial activities and provide key insight into the nature of these complex ecosystems (Burne and Moore, 1987).

To examine the specific role that microbes have played in the formation and accretion of stromatolites, it is necessary to delineate the community composition of the microbial mats associated with these structures. Microbial mats are multi-layered communities that drive the biogeochemical cycling of key elements within the stromatolite ecosystem. All "living" stromatolites are the products of microbial mat metabolic activity and geochemical cycling resulting in the accretion and precipitation of carbonate structures (Reid et al., 2000). However, not all microbial mats are conducive to the formation of stromatolitic structures (e.g., Dupraz and Visscher, 2005). Microbial mats capable of forming stromatolitic structures are dependent on the surrounding geochemical conditions, dominant metabolisms, and community composition (Havemann and Foster, 2008).

Prior to the molecular age, stromatolitic microbial mats were characterized primarily by morphology of the most dominant functional group (e.g., cyanobacteria), as well as the meso and macrostructure of the mineral structures (Papineau et al., 2005; Dupraz and Visscher, 2005). Now, with the advent of molecular high-throughput sequencing, ecotypes within the mat community can be delineated and compared among different stromatolite communities. The likelihood that the specific ecotypes found in modern stromatolitic mats can serve as exact proxies for Precambrian microbes is low; however, modern ecotypes may share with their ancient counterparts many basic fundamental metabolisms. Although many specific metabolic functions have been conserved over time, the exchange and rapid evolution of microbes via lateral gene transfer and interaction with viruses have made understanding the evolution of the microbial communities in stromatolites difficult. Despite such caveats, these modern analogs to Earth's microbial past offer an insightful view into the genetic and biochemical interactions between microorganisms and the ambient environment.

In this chapter, we produce a current census of stromatolite microorganisms. We compare the microbial diversities in the three prominent extant ecosystems – the hypersaline stromatolites of Shark Bay, Australia; the open ocean stromatolites of Highborne Cay, Bahamas; and the freshwater stromatolites of Ruidera Pool, Spain. We compare these natural ecosystems to an *in vitro* artificial microbialite model to ascertain if there are fundamental ecotypes associated with stromatolite formation. Finally, we discuss the emerging field of metagenomics and how this high-throughput sequencing approach has and will continue to rapidly expand our understanding of microbial diversity and functional complexity in modern stromatolites.

2. The Modern Stromatolite Habitats

Although once dominant on the early Earth, modern analogs of ancient stromatolites are far more limited in their global distribution. Although the frequency of their occurrence is low, the variety of habitats that stromatolites occupy is diverse.

In this section, we examine three aquatic habitats where microbe-dominated communities form laminated carbonate scaffolding via interconnected metabolic and geochemical processes. For our microbial census, we chose stromatolite ecosystems that have been analyzed by way of cultivation-independent cloning and sequencing of the small subunit ribosomal RNA gene (i.e., 16S rRNA). The habitats, locations, and GenBank accession numbers used in this comparative analysis are listed in Table 1. Only those sequences generated via domain-level clone libraries were used in phylogenetic and diversity analyses. As a result of variations in sequence length and position, not all sequences were appropriate for diversity analyses. Each stromatolite sample was processed by different researchers and methodologies, thus the direct comparison of these samples should be considered in this context. Likewise, we note that the statistical analyses of the sequence data can be sensitive to the number of clones analyzed for each sample (e.g., Hughes et al., 2001).

Table 1. Overview of modern stromatolite and microbial mat community 16S rRNA gene sequences.

Habitat	Location	Number of sequences	GenBank accession number	Used in analyses ^a	References
Hypersaline	Shark Bay	29	AY429113–AY429141	Yes	Burns et al. (2004)
Hypersaline	Shark Bay	63	AY430099–AY430161	Yes	Burns et al. (2004)
Hypersaline	Shark Bay	18	AY433816–AY433833	Yes	Burns et al. (2004)
Hypersaline	Shark Bay	34	AY435178–AY435211	Yes	Burns et al. (2004)
Hypersaline	Shark Bay	136	EF150675–EF150810	Yes	Goh et al. (2009)
Hypersaline	Shark Bay	478	EU851765–EU852242	Yes	Papineau et al. (2005)
Hypersaline	Shark Bay	34	AY604655–AY604688	No	Burns et al. (2005)
Marine	HBC ^b	174	EU917948–EU918121	Yes	Havemann and Foster (2008)
Marine	HBC	859	FJ911975–FJ912833	Yes	Baumgartner et al. (2009)
Marine	HBC	5	DQ822785–DQ822789	No	Unpublished
Marine	HBC	164	EU248965–EU249128	No	Foster et al. (2009)
Marine	Artificial ^c	408	EU917540–EU917947	Yes	Havemann and Foster (2008)
Freshwater	Ruidera	2	EU780449–EU780450	Yes	Santos et al. (2010)
Freshwater	Ruidera	80	EU753608–EU753687	Yes	Santos et al. (2010)
Freshwater	Ruidera	66	AY566310–AY566375	Yes	Santos et al. (2010)
Freshwater	Cuatros Ciénegas	13	AY541045–AY541057	No	Elser et al. (2005a)
Hypersaline	Guerrero Negro ^d	1655	DQ329539–DQ331020 DQ397339–DQ397511	Yes	Ley et al. (2006)

^aOnly sequences generated from domain-level clone libraries were used for the phylogenetic and diversity analyses. Where necessary, short sequences were excluded from analyses. Libraries generated from cyanobacterial primers or sequences isolated from DGGE were excluded from the phylogenetic analyses

^bSequences were derived from Type 2 stromatolites from Highborne Cay, Bahamas (HBC)

^cArtificial microbialites were derived from stromatolites isolated from Highborne Cay, Bahamas

^dHypersaline microbial mats from Guerrero Negro, Mexico were used as a non-lithifying mat reference community

Nonetheless, the combined analysis of multiple ecosystems is powerful, and reveals that each ecosystem possesses different environmental challenges, influencing the adaptation and selection pressures on the stromatolite microbial communities.

Diversity indices for all four of the different communities were generated from raw sequence data downloaded from the GenBank sequence database (Table 2). Bacterial 16S rRNA gene sequences were aligned using the online software package Greengenes (DeSantis et al., 2006b), and these alignments were imported into the phylogenetic software package MEGA (Kumar et al., 2008). A distance matrix was generated from the alignments using only positions shared by all sequences in each dataset, and using the maximum composite likelihood substitution model. The distance matrix was processed using the software package DOTUR (Schloss and Handelsman, 2005), with a 97% sequence similarity used as the threshold for inclusion in an operational taxonomic unit (OTU). Sequence characterization was also performed using the aligned sequences by implementing the online Greengenes DNA maximum likelihood (DNAML) classification tool (Fig. 1). For the generation of phylogenetic trees, the aligned sequences were imported into the software package ARB (Ludwig et al., 2004) and inserted into a 16S rRNA gene tree by implementing the parsimony option while keeping the tree topology fixed using a bacterial 50% conservation filter. This approach allows the simultaneous insertion of sequences of varying length and position into a single phylogenetic tree produced from near full-length sequences. A compressed phylogenetic tree that depicts the relationship between all examined stromatolites habitats is shown in Fig. 2.

2.1. DIVERSITY IN MARINE STROMATOLITES

Geologic evidence has indicated that the salt content of the Precambrian ocean was at least twice that of the modern ocean (Knauth, 1998; Arp et al., 1999) and that the first stromatolitic microbial ecosystems most likely formed in these hypersaline or intertidal conditions (Monty, 1977; Arp et al., 2001). Characterizing the diversity in microbial mats that form laminated stromatolitic structures in modern marine habitats, specifically hypersaline environments, may facilitate our understanding of these ancient ecosystems, as salinity has been shown to be a major driving factor in environment-specific evolution (Lozupone and Knight, 2007).

2.1.1. *Hypersaline Stromatolites in Shark Bay*

The hypersaline stromatolites of Hamelin Pool, in Shark Bay, Australia represent one of the most prominent and abundant examples of living accreting stromatolites on Earth (Burns et al., 2004; Neilan et al., 2002; Papineau et al., 2005). First discovered in 1954 (Playford and Cockbain, 1976), the stromatolites of Shark Bay have had a significant impact on stromatolite research, as they were the first described microbe-induced build-ups analogous in size and shape to fossilized

Table 2. Bacterial 16S rRNA gene library diversity analyses of clone libraries.

	Shark Bay	HBC total ^a	HBC Type 1	HBC Type 2	HBC Type 3	Artificial Model ^b	Ruidera Pool	Guerrero Negro ^b
Sequences	412	811	190	271	299	201	72	464
OTUs ^c	161	269	101	128	139	88	39	204
Singlets ^c	98	151	61	79	91	58	28	117
Doublets ^c	23	45	26	24	18	18	4	39
OTUs ^c > 5% (Sum)	3 (17%)	0 (0%)	1 (10%)	1 (9%)	1 (6%)	3 (30%)	5 (42%)	0 (0%)
Shannon (confidence)	4.54 (0.12)	4.96 (0.09)	4.29 (0.15)	4.43 (0.14)	4.51 (0.13)	3.87 (0.18)	3.36 (0.22)	4.95 (0.09)
Evenness	0.89	0.89	0.93	0.91	0.91	0.86	0.92	0.93
Chao1 (confidence) ^e	359 (273/509)	515 (427/651)	169 (136/323)	251 (196/353)	355 (257/533)	175 (132/261)	115 (66/252)	374 (308/481)
% Coverage ^f	76	79	68	71	70	71	61	75
Base pairs ^g	274	344	457	411	396	474	1,056	328

^aHBC total represents the pooled sequences of the three major mat types (Type 1–3) of Highborne Cay, Bahamas

^bThe libraries of the artificial microbialites and the Guerrero Negro hypersaline mats were split due to the absence of overlapping regions. Only results examining the forward sequences are reported. The hypersaline Guerrero Negro was included as a non-lithifying reference mat community

^cThe values were calculated based on a 97% similarity threshold

^dNumber of OTUs at 97% similarity threshold or higher that represent greater than 5% of the population. The percentage of the total community these OTUs represent are in *parentheses*

^eValues in *parentheses* represent the lower and upper 95% confidence intervals associated with Chao1 non-parametric estimator

^fPercent coverage of the clone library using the formula $(1 - [N - \text{singlets}]/[M])$

^gNumber of base pairs that were used in the comparisons for each community

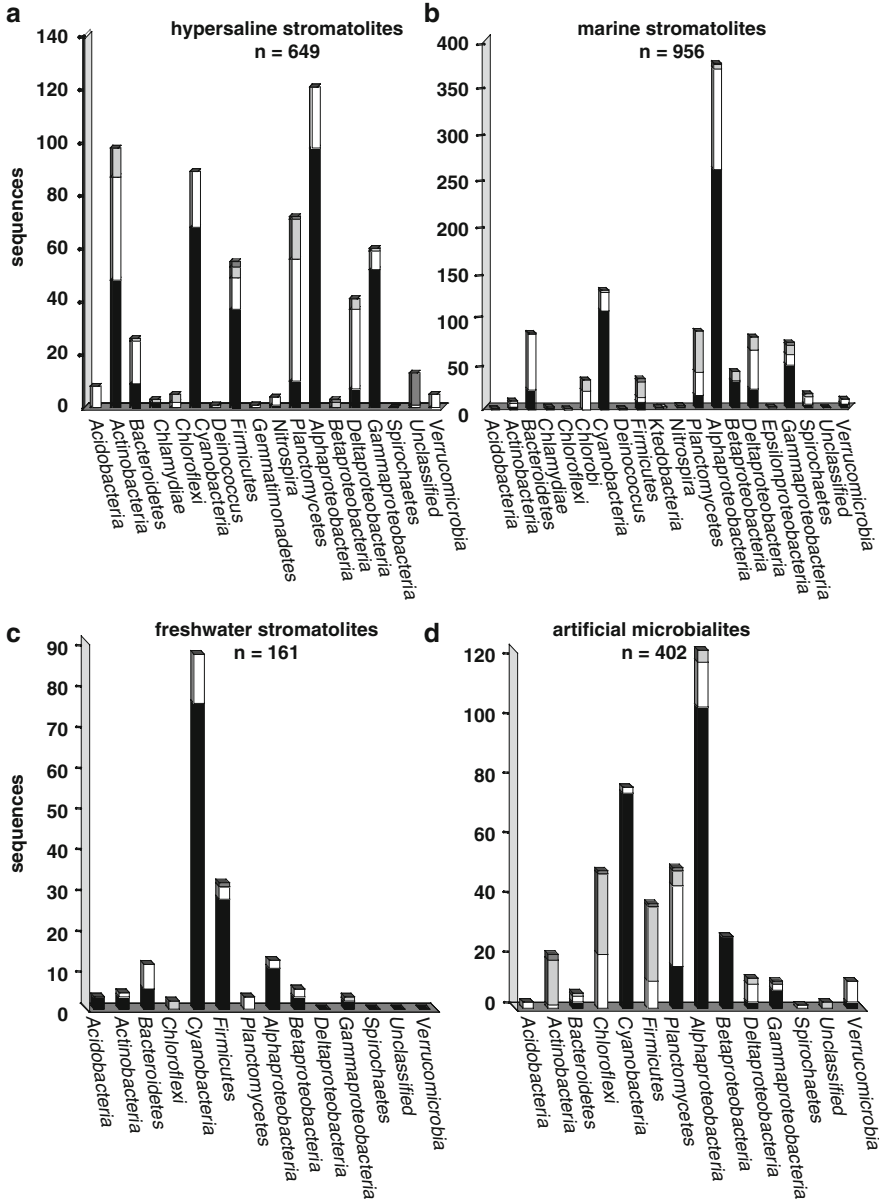


Figure 1. Overview of microbial diversity in modern stromatolite communities. *Bars* represent the total number of bacterial sequences (*n*) recovered from each phylum and separated based on their associated DNA maximum likelihood values (*black bars*, >0.90; *open bars*, 0.80–0.90; *light gray bars*, 0.70–0.80; and *dark gray*, <0.70). Graphs are also separated based on habitat and represent those sequences recovered from stromatolitic mats derived the (a) hypersaline waters of Shark Bay; (b) normal marine stromatolites of Highborne Cay; (c) freshwater lacustrine system of Ruidera Pool; and (d) laboratory cultivated microbialitic mats derived from Type 2 Highborne Cay stromatolites.

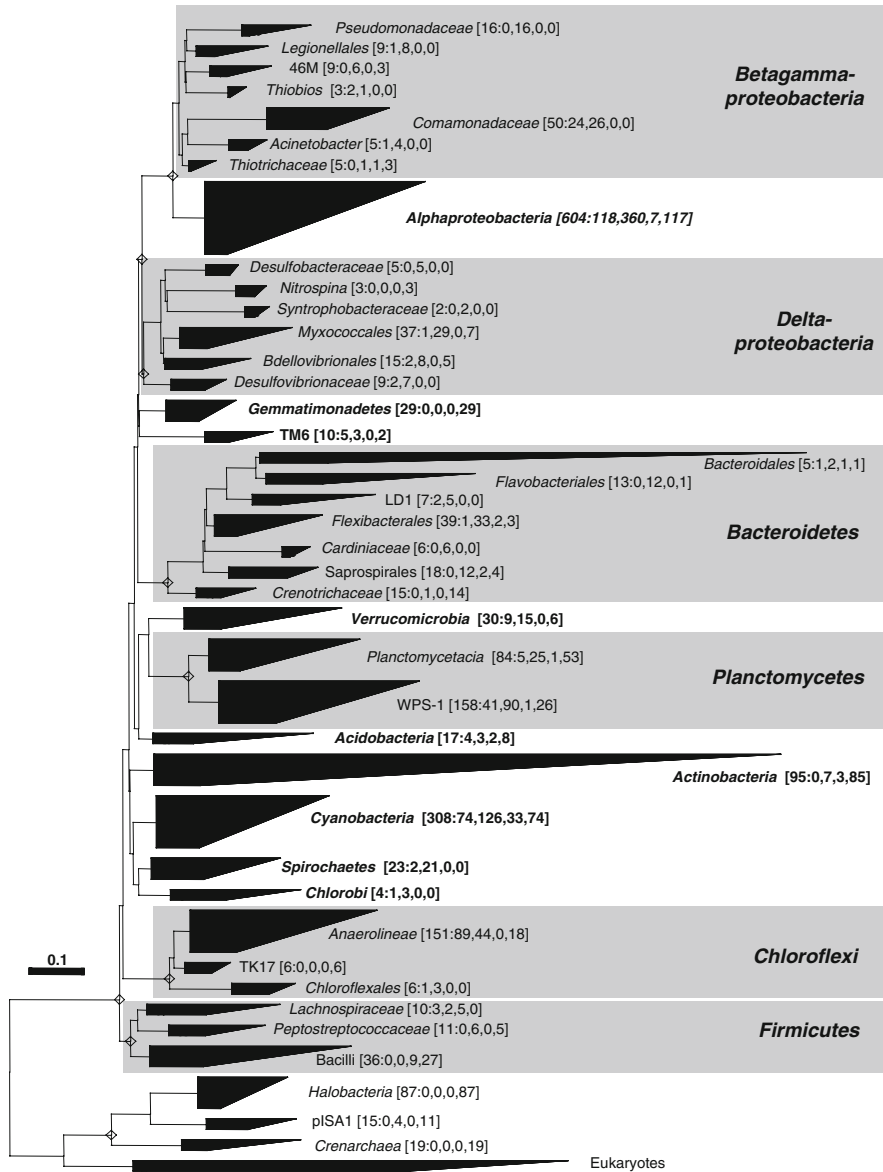


Figure 2. Phylogenetic tree of 16S ribosomal RNA (rRNA) gene sequences recovered from freshwater, marine and hypersaline stromatolites. Sequences were downloaded from GenBank, aligned using the software package Greengenes, and inserted into tree within the software package ARB using the parsimony tool and a bacterial 50% conservation filter. Major clades are compressed, and the total number of stromatolite sequences generated from clone libraries of artificial and natural stromatolite samples within each clade is indicated in *brackets*. Phyla or sub-phyla for which multiple taxa are shown (e.g., family- or order-level clades) are highlighted in *gray*. The *scale bar* represents 10% sequence divergence.

Precambrian stromatolites (Riding, 1991; Reid et al., 2003). The microfabrics of the Shark Bay stromatolites form via two major processes, the trapping and binding of organosedimentary material as well as the micritization of carbonates (Reid et al., 2003). Both processes require the involvement of microbes. Analyses of the 16S rRNA gene sequences recovered from the Shark Bay stromatolites ($n=605$) clearly show a diverse and novel community of both archaea and bacteria (Burns et al., 2004; Papineau et al., 2005). Although archaea are important in modern stromatolites, this chapter focuses only on bacterial diversity. A comparison of all Shark Bay 16S rRNA gene sequences available in GenBank detected 17 different bacterial phyla or sub-phyla (Fig. 1a). Based on these 16S rRNA gene sequences, the Shark Bay stromatolites appear to be dominated by *Alphaproteobacteria* (20%), *Actinobacteria* (16%), and *Cyanobacteria* (15%). Note, however, only those sequences generated via domain-level primers (Lane, 1991; DeLong, 1992; Table 1) were used in these clone library comparisons. Sequences derived from denaturing gradient gel electrophoresis (DGGE), functional group-specific primers (e.g., cyanobacterial), or intergenic spacer region sequencing were not included in any of the phylum-level comparisons of this analysis. The other dominant phyla or sub-phyla in the Shark Bay stromatolites included *Planctomycetes* (11%), *Gammaproteobacteria* (10%), and the *Firmicutes* (9%). The high level of phylum-level diversity expanded at narrower taxonomic levels (Table 2). The Shannon index of the Shark Bay stromatolites was high (4.54), and a Chao1 estimate of 359 phylotypes at a 97% sequence identity threshold. This level of diversity was similar to that detected in the marine stromatolite from Highborne Cay. Both the marine stromatolite and the hypersaline stromatolite diversity indices approached the diversity level detected in a highly diverse non-lithifying microbial mat from Guerrero Negro, Baja, Mexico (Table 2; Ley et al., 2006). The microbial community in the Shark Bay stromatolite samples also exhibits a high degree of sequence novelty in several key taxa (Fig. 1a). For example, more than half the recovered sequences from the *Actinobacteria*, *Deltaproteobacteria*, and *Planctomycetes* had DNAML values below 0.90 (Fig. 1a). These DNAML values were calculated by comparing the target sequences to the reference sequences in Greengenes database (i.e., ARB). The closer these values are to 1.0, the higher the matching probability with sequences in the curated database (DeSantis et al., 2006a).

2.1.2. Open Ocean Stromatolites

The well-laminated carbonate build-ups along the margins of Exuma Sound, Bahamas, represent the only known example of extant stromatolites in normal marine salinity (Dravis, 1983; Dill et al., 1986; Reid et al., 1995). One location in particular, the island of Highborne Cay, has been the subject of several recent molecular, biogeochemical, and geological investigations into the formation and development of these modern accreting stromatolites (Reid et al., 2000; Decho et al., 2005; Perkins et al., 2007; Foster et al., 2009). There are three major mat types (Type 1, 2, and 3) found in the intertidal and subtidal zones of Highborne Cay.

Each mat type is classified based on the extent of carbonate precipitation and taxonomic richness (Reid et al., 2000; Baumgartner et al., 2009; Foster et al., 2009). Comparative analysis of the available 16S rRNA genes recovered from Highborne Cay stromatolites revealed that when pooled, these three stromatolitic mat types contain the highest level of diversity found in any modern stromatolite (Table 2). Together, there were 20 different phyla detected in the Highborne Cay stromatolitic mats with the *Alphaproteobacteria* (38%) and *Cyanobacteria* (18%) dominating (Fig. 1b). The diversity of each mat type was also high even when they were examined separately (Table 2). For example, in samples of Type 2 stromatolites, which are considered to be an intermediate stage of Highborne Cay stromatolite development (Reid et al., 2000), 18 different phyla or sub-phyla were detected. These samples were similarly dominated by the *Alphaproteobacteria* (20%) and *Cyanobacteria* (18%) (Havemann and Foster, 2008). Other taxa that were detected in high abundance were *Bacteroidetes* (9%), *Planctomycetes* (9%), *Deltaproteobacteria* (8%), and *Gammaproteobacteria* (7%; Fig. 1b). DNAML analyses of the combined Highborne Cay sequences revealed the highest level of sequence novelty in the *Chloroflexi*, *Planctomycetes*, and *Deltaproteobacteria* with the majority of sequences below 0.89 (Fig. 1b). These results may suggest that several of these ecotypes may be geographically localized to the stromatolites of Highborne Cay.

2.2. DIVERSITY IN FRESHWATER STROMATOLITES

In addition to the marine habitats, stromatolitic carbonate build-ups have also been reported in freshwater ecosystems (Pedley, 2000). Many of these freshwater stromatolitic communities undergo periodic desiccation events (Pedley, 2000) and some, such as the fresh water microbialites of Cuatros Ciénegas Basin, Mexico, have biological signatures that suggest that these freshwater pools once had a marine origin (Souza et al., 2006; Desnues et al., 2008).

2.2.1. Ruidera Pools, Spain

The only two modern freshwater microbialite ecosystems that have been comprehensively examined at the molecular level are the fluvio-lacustrine system in Ruidera Pools National Park in Central Spain, and the phosphorus-poor pools of the Cuatros Ciénegas Basin in Mexico. The latter ecosystem has been examined via high-throughput metagenomic sequencing and will be discussed elsewhere in this chapter. However, the stromatolitic tufas of Ruidera Pools represent one of the most prominent sites of carbon sequestration and precipitation in Europe (Ordóñez et al., 2005; Santos et al., 2009). Currently available 16S rRNA sequences mined from GenBank reveal that 54% of the sequences recovered from the Ruidera stromatolites are *Cyanobacteria* (Fig. 1c). The other dominant phylum from the Ruidera Pools was the *Firmicutes* (19%); however, quantitative PCR results indicated

that these were not active members of the community (Santos et al., 2009). As these microbial communities are susceptible to the periodic desiccation and flooding events typical of the Ruidera Pools, the prominence of desiccation-resistant microbes such as the spore forming *Firmicutes* might not indicate a specific function essential for stromatolite formation, but merely a reflection of environmental adaptation. However, the taxonomic analyses of these freshwater microorganisms suggest that there are very few novel ecotypes in this system as most sequences had DNAML values above 0.90. In contrast to the marine and hypersaline stromatolites, the *Alphaproteobacteria* were relatively minor constituents (7%) of the total microbial community. Diversity indices reveal that the microbial consortia of the Ruidera Pool stromatolites are the lowest of all the communities compared and this may in part reflect the tremendous dominance of *Cyanobacteria* in the clone library (Table 2). Nonetheless, the clone library coverage was significantly lower (61%) for this freshwater ecosystem than the others. Additional sequencing will need to be performed to definitively demonstrate lower community diversity.

2.3. DIVERSITY IN ARTIFICIAL MICROBIALITES

To complement studies of the natural ecosystems, several researchers have developed laboratory analogs to model the initiation and development of modern stromatolites and microbialites (Fenchel and Kühl, 2000; Dupraz et al., 2006; Havemann and Foster, 2008; Foster and Mobberley, 2010). These artificial models can serve as proxies for the microbes and metabolic activity associated with microbialite formation. Comparisons between these artificial and natural communities have revealed key organisms associated with the carbon sequestration and precipitation processes (Havemann and Foster, 2008). Experimentally lithifying microbialites derived from the marine stromatolites of Highborne Cay show a decreased diversity relative to the natural stromatolites (Table 2; Fig. 1d). The decrease in diversity appears to represent a loss of diversity, as the microbial community in the experimental ecosystems was predominantly a subset of that found in the natural ecosystem (Havemann and Foster, 2008; Fig. 1b, d). The lower diversity levels can assist in developing laboratory-based models to isolate those specific ecotype and processes associated with carbonate precipitation in modern microbialites. Likewise, a lower diversity may also simplify the identification and isolation of those ecotypes essential for stromatolite formation. As in the Highborne Cay stromatolites, the artificial microbialites are dominated by *Alphaproteobacteria* (30%) and *Cyanobacteria* (18%; Fig. 1d). The representation of the other major phyla mirrors that of those in the natural system (Fig. 1d), clearly indicating that even after 1.5 years in cultivation the natural stromatolitic mat diversity was maintained under artificial conditions (Havemann and Foster, 2008). These results suggest that these artificial microbialites may serve as useful analogs that can be experimentally manipulated under simulated environmental conditions.

3. Dominant Phyla in Modern Stromatolites

Bacteria from the phylum *Cyanobacteria* are the predominant organisms present in all the stromatolites, and bacteria affiliated with the sub-phylum *Alphaproteobacteria* are the most abundant bacteria detected in the marine and hypersaline stromatolites. We discuss these organisms in greater detail later. In addition, we note the abundance of organisms affiliated with the phylum *Planctomycetes* and class *Anaerolineae* of the phylum *Chloroflexi* in the marine and hypersaline stromatolites. Although a subset of *Planctomycetes* are capable of anaerobic ammonia oxidation (ANAMMOX), many of the detected sequences in this study were not affiliated with these ANAMMOX bacteria (e.g., “Wittenberg polluted soil” or WPS bacteria of unknown physiology; Nogales et al., 2001). Likewise, bacteria from the class *Anaerolineae* are filamentous chemo-organotrophic anaerobes (Yamada et al., 2006) in a phylum known for green non-sulfur photosynthetic bacteria. We note that sequences affiliated with the *Chloroflexi* were the most abundant in the deeper, perpetually anaerobic zone of hypersaline microbial mats from Guerrero Negro, Baja, Mexico (Ley et al., 2006). Sequences affiliated with these taxa were observed to have low DNAML values (Fig. 1). This reflects a lack of isolated representatives and concurrently, a general ignorance of the role of the respective organisms in natural ecosystems. One surprising finding from the clone libraries of the marine and hypersaline systems was the absence of a substantial number of sequences affiliated with sulfate-reducing bacteria (SRB) of the sub-phylum *Deltaproteobacteria* (Fig. 2). These organisms have been shown to be abundant in non-lithifying microbial mat ecosystems (e.g., Minz et al., 1999) as well as lithifying stromatolites (e.g., Baumgartner et al., 2006), even in aerobic locations. Further work will be required to demonstrate that this is not an artifact of the sampling and analytical approach.

3.1. THE *CYANOBACTERIA*

Of all the phyla detected in modern stromatolites, none is more prominent than the *Cyanobacteria*. Often considered the driving metabolic force behind stromatolitic mat metabolism and early lithification (Pinckney and Reid, 1997; Dupraz et al., 2004; Visscher and Stolz, 2005), cyanobacteria represent a morphologically and molecularly diverse functional group (Golubic and Browne, 1996; Neilan et al., 2002; Reid et al., 2000; Stolz et al., 2001; Burns et al., 2004; Foster et al., 2009). Comparisons between the four communities revealed pronounced differences and similarities between the cyanobacterial populations (Fig. 3). One of the most surprising observations was the presence of *Leptolyngbya*-like cyanobacteria in all three natural communities (Fig. 3, Clusters 1–6). Interestingly most of the *Leptolyngbya*-like clusters contained sequences recovered from both Shark Bay and Ruidera Pool stromatolites (Fig. 3; Clusters 2–6). These ecotypes were

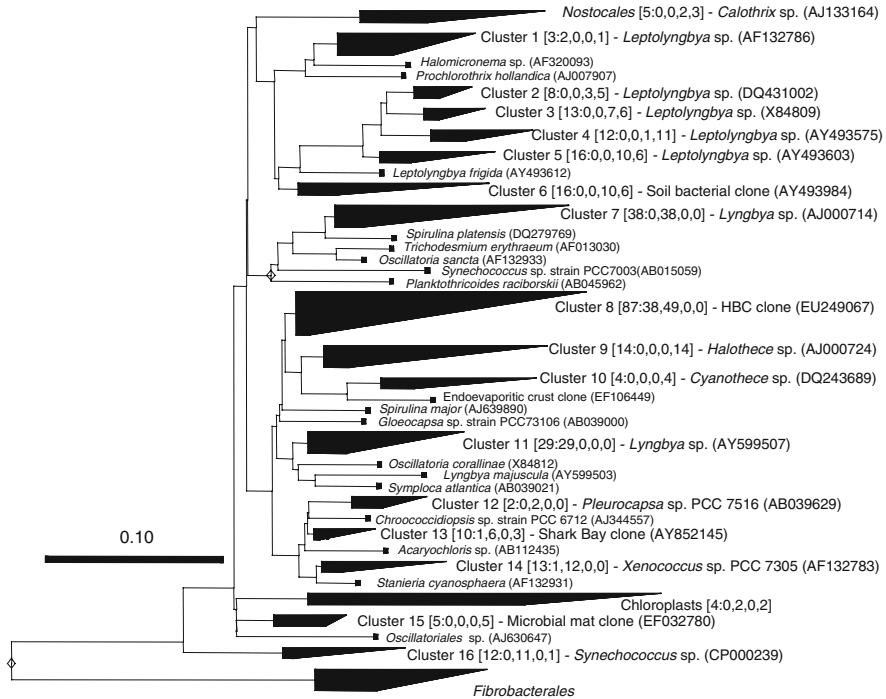


Figure 3. Phylogenetic tree of *Cyanobacteria* 16S rRNA gene sequences from clone libraries produced using general bacterial primers. Major clusters are compressed, and the total number of stromatolite sequences generated from clone libraries is indicated in brackets. The number of sequences from each of four types of stromatolites (artificial microbialite from Highborne Cay, natural stromatolite from Highborne Cay, natural stromatolite from Ruidera Pool, and natural stromatolite from Shark Bay) are indicated after a *semicolon* and separated by a *comma*. Representative sequences of each cluster are indicated. The *scale bar* represents 10% sequence divergence.

not detected in either the natural or artificial marine stromatolites, but together with several sequences from the *Nostocales*, they represented all the Ruidera Pool *Cyanobacteria*. Only *Leptolyngbya*-like organisms identified in Cluster 1 were common to Shark Bay and Highborne Cay. *Leptolyngbya* ecotypes have filamentous morphotypes and have been shown to exhibit key physiological characteristics associated with stromatolite formation (Foster et al., 2009). Despite some shared *Leptolyngbya* ecotypes, in general, there was limited overlap in cyanobacterial community composition between Shark Bay and Highborne Cay stromatolites. The dominant Shark Bay cyanobacteria included unicellular diazotrophs belonging to the *Cyanothece*-*Halothece* cluster (Fig. 3; Clusters 9 and 10). Such organisms have been found in several hypersaline non-lithifying microbial mat communities such as Guerrero Negro, Mexico (Omeregíe et al., 2004; Ley et al., 2006; Green et al., 2008). Highborne Cay stromatolitic mats contained a highly distinctive cyanobacterial community, including representatives of the unicellular

Synechococcus spp. (Cluster 13) and *Xenococcus* spp. (Cluster 14) as well as filamentous *Lyngbya* spp. (Clusters 7 and 11). The recovery of 16S rRNA gene sequences from these putatively diazotrophic cyanobacteria in Highborne Cay stromatolites correlates well with previously described nitrogenous biochemical activities and *nifH* gene profiles of these communities (Steppe et al., 2001; Paerl et al., 2001). Interestingly, heterocystous cyanobacteria were essentially absent from these stromatolite communities. A few sequences affiliated with the order *Nostocales* (e.g., *Calothrix* sp.) were detected in the Ruidera Pool and Shark Bay stromatolites, but represent only a small fraction of the total cyanobacterial community (Fig. 3). Prior studies have shown that heterocystous cyanobacteria are absent from submerged hypersaline microbial mats, even under lowered salinity and sulfate conditions (e.g., Stal, 1995; Bebout et al., 2004; Green et al., 2008). This absence appears to extend to lithifying microbial mat communities as well, with an exception described later in this chapter.

3.2. THE ALPHAPROTEOBACTERIA

The *Alphaproteobacteria* represent one of the most metabolically diverse bacterial sub-phyla and are found in most stromatolite and microbial mat communities (Ward et al., 1998; Ley et al., 2006; Havemann and Foster, 2008). The stromatolitic mats of Highborne Cay and Shark Bay are dominated by this sub-phylum with particularly high representation by photoheterotrophic purple non-sulfur bacteria of the orders *Rhodobacterales* and *Rhizobiales*. Heterotrophic *Alphaproteobacteria*, such as those from the genus *Parvularcula* and prosthecate bacteria of the genera *Hyphomonas* and *Hyphomicrobium*, are also abundant in the HBC stromatolites. By far, phototrophic and heterotrophic *Alphaproteobacteria* were the most abundant and diverse in the natural Highborne Cay stromatolites, with representative sequences from half of the defined clusters (Fig. 4; Clusters 1, 4, 5, 9–14, 20). The artificial microbialite exhibited lower diversity of *Alphaproteobacteria* than the native system, though one salient shift in the *Alphaproteobacteria* community composition was the dramatic increase in relative abundance of the organisms affiliated with Cluster 13 (Fig. 4). In the artificial microbialite, these organisms represented approximately half of the total alphaproteobacterial community, and less than 10% of the native community. No specific physiology can be ascribed to these organisms, though their sequence similarity to other *Rhodobacterales* such as *Rhodopseudomonas* spp. may indicate a photosynthetic lifestyle. Although a number of genera of freshwater anoxygenic phototrophic bacteria are known (e.g., Yurkov and Beatty, 1998), these organisms, and *Alphaproteobacteria* in general, were essentially undetected in the freshwater stromatolites from Ruidera Pool (Santos et al., 2009). As with the total bacterial community, the alphaproteobacterial community diverged significantly between the marine HBC stromatolites and the hypersaline Shark Bay stromatolites. Although both systems contained organisms from the same clusters, the proportion of these organisms diverged

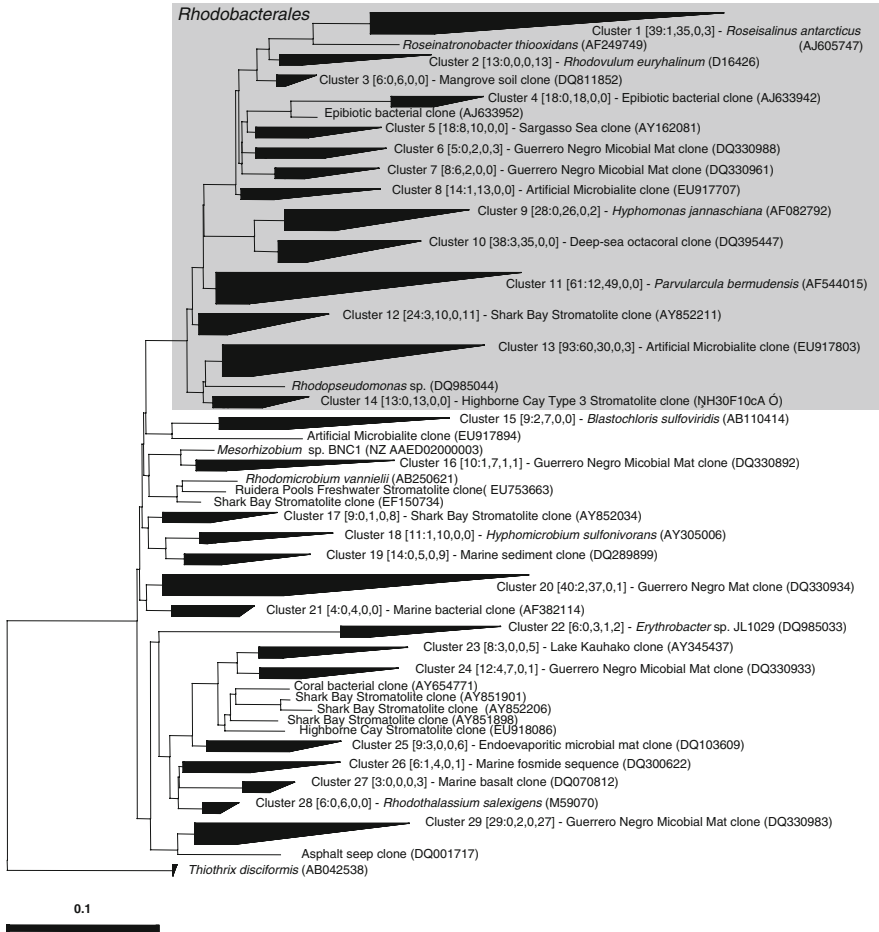


Figure 4. Phylogenetic tree of *Alphaproteobacteria* 16S RNA gene sequences from clone libraries produced using general bacterial primers. Major clusters are compressed, and the total number of stromatolite sequences generated from clone libraries is indicated in *brackets*. The number of sequences from each of four of stromatolites (artificial microbialite, natural stromatolite from Highborne Cay, natural stromatolite from Ruidera Pool, and natural stromatolite from Shark Bay) are indicated after a *semicolon* and separated by a *comma*. Representative sequences of each cluster are indicated. Sequences belonging to the order *Rhodobacterales* are highlighted in *gray*. The *scale bar* represents 10% sequence divergence.

significantly between the systems (e.g., Fig. 4; Clusters 1, 9, 13, 20, 29). Sequences affiliated with bacteria from the genus *Rhodovulum* (marine purple non-sulfur phototrophic bacteria; Fig. 4; Cluster 2; Hiraishi and Ueda, 1994) were the most abundant alphaproteobacterial sequence of the order *Rhodobacterales* detected in the hypersaline stromatolite. The other dominant *Alphaproteobacteria* detected

in the Shark Bay stromatolite were unaffiliated with any described species, and have close representatives from clone libraries of submerged and endoevaporitic hypersaline microbial mats (Fig. 4; Clusters 25 and 29; Ley et al., 2006; Sørensen et al., 2005). A number of the defined alphaproteobacterial clusters in this study had no affiliation with described or isolated bacterial species, and these groups are in general composed largely of sequences recovered from marine or hypersaline microbial mats and stromatolites (Fig. 4; Clusters 6–8, 12–14, 20, 29). The physiology of these ecotypes has yet to be explored.

4. A New Beginning: The Future of Microbial Diversity Studies in Stromatolites

With the emergence of high-throughput pyrosequencing and the capacity for genome–genome comparisons, the reign of the 16S rRNA gene as the sole target for meaningful phylogenetic comparisons is coming to an end. The use of this gene for future phylogenetic comparisons will have to be taken in context with the rest of the genome and perhaps one day, the metagenome. Although this day has yet to arrive, new data have emerged that have rapidly increased our understanding of the microbial diversity and functional complexity of modern stromatolites.

4.1. METAGENOMICS IN MICROBIALITIC MATS

Comparing the collective genomes of microbes within complex communities via metagenomics (Handelsman, 2004) has facilitated the analysis of microbialitic mats at the molecular level. The first microbialitic mat communities to be examined via metagenomic sequencing were the freshwater microbialites of Cuatro Ciénegas Basin, Mexico, and the marine stromatolites of Highborne Cay, Bahamas (Breitbart et al., 2008; Desnues et al., 2008). Although to date neither of these microbialite ecosystems has been sequenced to saturation, these initial analyses have greatly expanded our understanding of the microbial ecotype composition and metabolic potential within the microbialite communities. The high diversity of these ecosystems, as observed in a metagenome analysis of the non-lithifying microbial mats from Guerrero Negro, Baja, Mexico (Kunin et al., 2008), will present a challenge for full characterization of these ecosystems.

Both the taxonomic richness and the functional gene diversity of these microbialite communities were determined by comparing the sequences to SEED, the preeminent database for metagenomic data sets (Overbeek et al., 2005). Sequences acquired via 454 pyrosequencing were compared against the SEED non-redundant database by BLASTX and mapped on to subsystems through the MG-RAST system (Meta Genome Rapid Annotation using Subsystem Technology; Overbeek et al., 2005). This method has been used successfully to identify the taxonomic diversity and the metabolic potential of bacteria from metagenomic sequences in a wide variety of microbial mat ecosystems including

deep mine biofilms (Edwards et al., 2006), thermophilic hot springs (Bhaya et al., 2007), and non-lithifying hypersaline mat communities (Kunin et al., 2008).

In the Cuatros Ciénegas Basin, microbialites are found in a series of oligotrophic freshwater pools with the lowest levels of phosphorus ($<0.1 \mu\text{M}$) known in continental waters (Minckley, 1969; Elser et al., 2005a, b; Breitbart et al., 2008). Taxonomic profiles generated for the freshwater microbialites sampled from two pools of the Cuatros Ciénegas Basin, Pozas Azules (domal thrombolites) and Rio Mesquites (spherical oncolites), revealed that both were dominated by *Bacteria* (87% Pozas Azules; 95% Rio Mesquites; Breitbart et al., 2008; Desnues et al., 2008). Archaeal sequences were present in low abundance in both freshwater communities, with 1.8% in Pozas Azules and 0.7% in Rio Mesquites. Of the recovered archaeal sequences, roughly 86% were homologous to sequences of *Euryarchaeota*; few *Crenarchaeota* sequences were recovered. Eukaryotic diversity in these communities is also low, with only 10.6% of the recovered Pozas Azules metagenome sequences and 4.4% of the Rio Mesquites sequences sharing homology to eukaryotic organisms (Breitbart et al., 2008). The depleted phosphorus level appears to inhibit eukaryotic grazing, resulting in prokaryotic dominated communities (Elser et al., 2005a, b; Breitbart et al., 2008).

Interestingly, the taxonomic profile generated via metagenomics in the Rio Mesquites pool correlates to the 16S rRNA gene profiles generated for the freshwater stromatolites in Ruidera Pools, Spain. In both cases, the *Cyanobacteria* are dominant, comprising 74% and 54%, respectively, of 16S rRNA gene clone libraries. The Rio Mesquites microbialites contain a substantially higher proportion of *Cyanobacteria* from the order *Nostocales* relative to the Ruidera Pools stromatolite (44–21%, respectively) of the cyanobacterial community. These metagenomic analyses coupled with rRNA gene sequencing suggest that freshwater microbialites are dominated by cyanobacteria capable of nitrogen fixation, and can include heterocystous forms.

4.2. CORRELATING DIVERSITY WITH FUNCTION

Once the dominant ecotypes of the stromatolitic mats have been identified by rRNA gene sequence and through metagenomic analysis, the next challenge is to identify the function of each key organism within the community. Metagenomic sequencing from the Cuatros Ciénegas Basin microbialites has shown a significant environmental adaptation to the low phosphorus pools. SEED analyses of the recovered sequences showed a shift toward high affinity phosphate transporters, phosphatases, as well as phosphate sensing and regulation genes (Breitbart et al., 2008). Other key subsystems have been identified in these metagenomic sequencing efforts including genes associated with biofilm formation (e.g., tight adherence macromolecule transporters), EPS production (e.g., alginate and colanic acid production), and interspecies communication, such as the S-adenosylmethionine synthetases gene known to facilitate production of autoinducer-2 (Breitbart et al., 2008).

Such metagenomic data substantiate “common knowledge” about complex ecosystems, but also can provide surprising evidence for microbial processes that were not previously considered. For example, in a metagenomic analysis of a Guerrero Negro microbial mat, genes involved in photosynthesis were unsurprisingly heavily represented in the surface 2 mm (Kunin et al., 2008). However, this study showed an increase in the relative abundance of genes encoding for enzymes involved in sugar degradation with depth. Such data are at the first stage of analysis. By coupling the specific ecotypes to the expression of functional genes within the community (i.e., metatranscriptomics), isotopic signatures and geochemical cycling, we can potentially delineate the evolutionary adaptations of stromatolitic microbial mat community.

With the emergence of comparative metagenomics, a revolution of the species concept in microbes is underway (Ward et al., 2008). Organisms that share high levels of sequence similarity in 16S rRNA genes can have extensive differences in genome size and organization. One of the most compelling examples is of cyanobacteria in the thermophilic hot springs mats of Yellowstone. The complete genomes of closely related species of the genus *Synechococcus* (OS-A and OS-B', sharing >95% sequence similarity in the 16S rRNA gene) were recently analyzed and found to share little gene synteny (Bhaya et al., 2007). Such studies clearly show that despite morphological and 16S rRNA gene similarities, functional metagenomic data not only can identify these ecotypes, but also ascertain their physiological capacities and ecological significance within the community.

Such disparity within the genomes of organisms that share similar 16S rRNA gene sequences could be the result of genetic exchange with other cells and viral phages. Viruses are the most abundant biological entities in aquatic ecosystems (Suttle, 2005) and often outnumber prokaryotes 10:1 (Desnues et al., 2008). The role of phages in the marine stromatolites of Highborne Cay, Bahamas was recently examined. The vast majority of recovered phage sequences (>95%), acquired via metagenomic sequencing, were novel, suggesting biogeographical isolation of the Bahamian stromatolite community (Desnues et al., 2008). Phages, along with geochemical constraints on the microbial community, are likely driving forces for the evolution of new species within the stromatolitic microbial mat communities. Only through multi-lateral approaches of metagenomic sequencing coupled with biogeochemical and mineralogical analyses will the comprehensive microbial diversity of stromatolite ecosystems be delineated and understood.

5. References

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