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Comparison of Bacterial Diversity within the Coral Reef Sponge, *Axinella corrugata*, and the Encrusting Coral *Erythropodium caribaeorum*

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Abstract. We compared the Caribbean reef sponge, *Axinella corrugata,* with the Caribbean reef coral, *Erythropodium caribaeorum* for differences in their resident microbial communities. This cursory survey of bacterial diversity applied 16S rRNA gene sequences. Over 100 culture-independent sequences were generated from five different *Axinella* 16S rRNA libraries, and compared with 69 cultured isolates. The culture-independent 16S rDNA clones displayed a higher diversity of Proteobacteria, including "uncultured" or "unknown" representatives from the Deltaproteobacteria. *Arcobacterium*, and Cyanobacteria were also found. We have also confirmed that *Axinella* sponges appeared to host specific microbial symbionts, similar to the previously identified clones termed "OSO" environmental samples. In contrast, seawater samples near *Axinella* were dominated by *Pseudoalteromonas.* Adjacent sediment samples yielded clones of Planctomycetacea, Proteobacteria, sulfate-reducing *Desulfovibrio* spp, and other Deltaproteobacteria. Anaerobe-like 16S rRNA sequences were detected after the oxygen supply to one *Axinella* sample was deliberately curtailed to assess temporal changes in the microbial community. *E. caribaeorum* yielded more Betaproteobacteria relative to *Axinella* 16S libraries, and also included the Gammaproteobacteria genus *Spongiobacter*. However, *Axinella*-derived microbes appeared phylogenetically deeper with greater sequence divergences than the coral. Overall this study indicated that marine microbial community diversity can be linked to specific source hosts and habitats.

Keywords: Sponge, coral, heterotrophic bacteria, 16S rRNA, symbiont

Introduction

Studies of microbial ecology and diversity in the oceans have accelerated over the past decade, partially due to advanced 16S rRNA and metagenomic sequencing methods (Venter et al. 2004; Sogin et al. 2006), plus the recognition of some pivotal microbial functions that include ecosystem services, biogeochemical cycles and symbioses with eukaryotic hosts (Torsvik et al. 2002; Hill et al. 2006; Taylor et al. 2007).

Along these lines, our laboratories have studied marine sponge species for several years. This research includes *Axinella corrugata (Ax)*, a common reef sponge of the Caribbean and Western Atlantic. This bright orange sponge has become a model for natural products chemistry, cell biology, molecular and population genetics (Lopez et al. 2003; Pomponi 2006). Some sponge species have a microbial biomass reaching over 50% (Santavy and Colwell 1990; Fieseler et al. 2004). Similarly, corals have been shown to possess unique and diverse bacterial populations (Rohwer et al. 2001; Ritchie and Smith 2004), but some coral species appear susceptible to bacterial disease outbreaks (Ritchie et al. 2006; Halpern et al. 2008). In addition, the coral, *Erythropodium caribaeorum* (*Ec*) has been shown to produce a wide variety of biologically active diterpenes, such as the anti-mitotic agent eleutherobin (Cinel et al. 1999), and the briarane (erythrolides) and aquariane (aquariolides) skeletal classes (Taglialatela-Scafati et al. 2003).

Longstanding questions regarding the role of microbes in these marine invertebrate hosts, coral reef diseases and potential marine natural product biosynthesis remain. Could coral disease reservoirs exist in other invertebrate species

besides corals, and then jump to reef builders when environmental conditions change (Harvell et al. 2002)? Does the ultimate source of potent natural products stem from resident, symbiotic microorganisms? To provide some baseline data for answering some of these questions we have applied molecular microbiological methods to define the microbial populations associated with these invertebrates and their environments (Sfanos et al. 2005).

Methods

Both coral and sponge hosts are relatively shallow benthic species: *E. caribaeorum* was collected by SCUBA off Fort Lauderdale, Florida at a depth of 30 fsw (feet seawater). *A. corrugata* specimens were collected between 80 – 120 fsw depth off San Salvador and Little San Salvador, Bahamas in 2002. Specimens of *A. corrugata* were held in running seawater for several days to examine short-term temporal changes in the microbial community. One additional specimen was placed in a container with no running seawater to investigate microbial changes under anoxic conditions.

Genomic DNA was rigorously extracted from sponge tissues using a modified guanidium isothiocyanate method (Lopez et al. 2002). Sponge mesohyl (tissue) was obtained from the center of the sponge prior to homogenization Typically 0.5 – 1.5 g sponge mesohyl samples were ground to a fine powder in liquid nitrogen, and incubated for about one hour at 37^{o}C in 5-10 ml of GES (60% [w/v] guanidium isothiocyanate, 20 mM EDTA, 0.5% sarcosyl). DNA from marine sediment samples was extracted using a bead beating method (Mo Bio "UltraClean" soil DNA extraction kit, Solano Beach, CA) according to the manufacturer's instructions.

16S small subunit rRNA sequences were generated by PCR and universal 16S rRNA primers using standard methods previously described (Sfanos et al. 2005). Templates for the cloned 16S rRNA libraries were two different *A. corrugata* specimens. All culture-independent library sponge clones begin with a number, such as “354e”, whereas cultured isolates begin with a letter such as T473 etc (also see Sfanos et al. 2005). *Ax* isolates in this study included T295, T266, T274, T288, T473, T273, T280, T456, , T479 S982, and J586 (also see http://www.hboi.edu/dbmr/dbmr_hbmmd.html; Gunasekera et al. 2005). 16S clones derived from *E. caribaeorum* are labeled with “EC”.

Sequence and phylogenetic analyses

After confirmation of the closest sequence relative in GenBANK via BLASTN analyses (Cole et al. 2003), new *Ec*-derived sequences were deposited into GenBank and given accession numbers DQ889871-DQ889940, while *Ax* culture independent 16S and isolate sequences had the following GenBank numbers: FJ215389-FJ215423, FJ215474 - FJ215549, and FJ215561-FJ215629.

The program FastGroup II (Yu et al. 2006) was used to perform species richness estimates and rarefaction analyses of individual *Ec* and *Ax* libraries.

Phylogenetic analysis began by aligning sequences using CLUSTALX (Thompson et al. 1997). After manually checking alignments by comparing with known secondary structure models (Sfanos et al. 2005), poorly aligned SSU rRNA regions (e.g. high number of gaps or indels) were omitted from further analysis. Alignments were then imported into PAUP (phylogenetic analysis using parsimony) v 4.0b3a (Swofford 2000), which allowed a comparison of various phylogenetic algorithms and substitution models. Due to the high amount of sequence divergence in most rRNA datasets, minimum evolutionary tree topologies based on distance models were obtained using heuristic methods. Each reconstructed group was statistically evaluated by bootstrapping with a minimum number of 200 replicates (Felsenstein 1985; Nei and Kumar 2000). Most appropriate DNA substitution models for each algorithm were determined using MODELTEST (Posada and Crandall 1998). Reference and type sequences were also downloaded from GenBank in order to help identify specific sequence clusters.

Bacterial Class	***Ax***	***Ax* Sediment**	**Ax Seawater**	***Ec***
Alphaproteobacteria	19	4		15
Gammaproteobacteria	39	9	33	30
Betaproteobacteria				32
Epsilonproteobacteria	1			1
Deltaproteobacteria	22	14		4
Acidobacteria				2
Bacteroidetes	2	2		2
Chloroflexi				1
Nitrospira	1	1		
Planctomycetacea		2		2
Spirochaetes	2			
Verrucomicrobiae		1		1
Cyanobacteria	1			6
Gram positive bacteria	2		2	2
Unknown	21	3	4	
	110	36	39	98

Table 1: Bacterial 16S rDNA clones derived from *A. corrugata (Ax)*, *E. caribaeorum* (*Ec*) and environmental samples associated with *Ax*.

Results/Discussion

A total of 110 and 98 16S rRNA clone sequences were sequenced from five different *Axinella* libraries and *Erythropodium*, respectively. Environmental sample libraries were also generated from sediment (39 clones) and water samples (36 clones) collected adjacent to the *Axinella* samples.

Table 1 shows comparative profiles of culture-independent 16S rRNA sequences derived from the two invertebrates and the sediment and water samples collected adjacent to the San Salvador *Axinella* samples.

Among some of the interesting *Ax* sponge-derived sequences, clone 363AM had 96% similarity with an *Arcobacterium*, and several clones had the closest similarity to *Bdellovibrio* and spirochaetes. In addition to the *Arcobacterium*, several additional sequences weakly matching (<90%) to Epsilonproteobacteria were also found in *Axinella*, but are not included in Table 1 at this time, pending verification. A *Nitrospira* clone, 345AU, was also identified. The sponge was further distinguished by the higher proportion of Delta-Proteobacteria and unknown microbial taxa (such as 345 BO, and 345 BM), relative to *Erythropodium.*

The diversity of cultured isolates from *Ax* comprised members of the Alpha-, Gamma- (*Alteromonas* spp, *Pseudoalteromonas* spp. and *Vibrio* spp.) and a few Betaproteobacteria, *Brachybacterium paraconglomeratum* (Actinobacteria) was also found.

The most striking similarity between the two benthic invertebrates, despite geographic and taxonomic separation, was a high representation of the Proteobacteria, especially Gamma-proteobacteria. In contrast, Betaproteobacteria-like sequences were not recovered from the sponge, but represented 32% of all bacteria in *E. caribaeorum.*

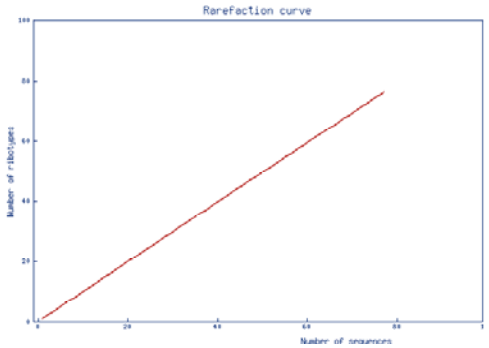

Figure 1: Rarefaction curve of one representative *Axinella* library based on 77 sequences.

About 83% of the *E. caribaeorum* sequences were representatives of the Proteobacteria, 6% of the clones were from cyanobacteria, 2% came from each of the Bacteroidetes, Actinobacteria, Acidobacteria, and Planctomycetes, while ~1% of the clones were represented by each of the *Chloroflexi*, Lentisphaerae and Verrucomicrobia taxa (Table 1).

In the coral, the largest fraction of the Proteobacteria were Betaproteobacteria (32%), followed by Gammaproteobacteria (30%), Alphaproteobacteria (15%), and Deltaproteobacteria (4%). There was only a single Epsilonproteobacteria (Table 1). The genus *Spongiobacter* dominated the Gamma-proteobacteria subdivision in this dataset. Dominating the Betaproteobacteria were representatives of the genus *Aquaspirillum*, followed by a number of clones that had "uncultured or unidentified" designations upon BLAST analysis. Moreover, six *Ec* clones showed high similarity to cyanobacteria sequences.

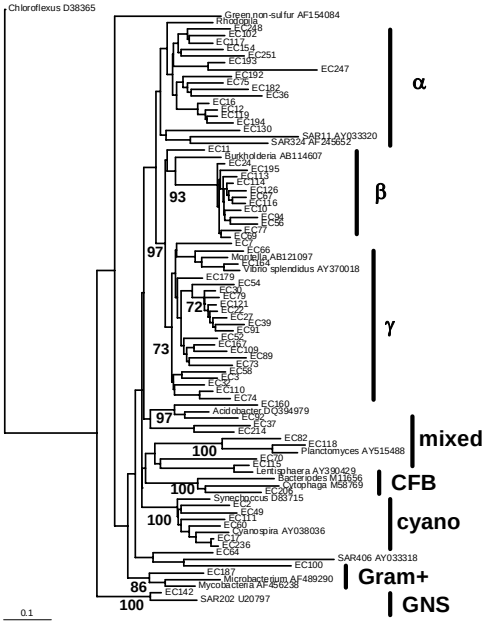

Figure 2: Neighbor-joining tree of *Erythropodium*-derived bacterial 16S rRNA. Reference sequences are listed with their GenBank numbers. Tamura-Nei correct model was implemented. Numbers below nodes are bootstrap percentages after at least 100 iterations. GNS = green non-sulfur bacterial cluster which includes *Chloroflexi.*

The rarefaction curve based on the *Axinella* 16S rRNA sequences shown in Fig. 1, confirmed that sequencing and microbial diversity analyses for this species were not exhaustive. More diversity

may be surveyed by using different 16S rRNA universal primers or DNA extraction methods.

Phylogenetic analyses

Taxonomic diversity within each invertebrate host is partially reflected in the neighbor-joining trees of *E. caribaeorum* and *A. corrugata* 16S rRNA sequences shown in Fig. 2-3, respectively. The analysis was performed primarily to provide a better understanding of taxonomic placement rather than determination of precise phylogenies of bacterial lineages. Nonetheless, the trees highlight the dominance of Proteobacteria in both species. In the *Axinella* tree, triangles denote likely species-specific symbionts (found in >1 specimen). The larger Gammaproteobacteria cluster appeared distinct from other cultured Gammaproteobacteria (e.g. *Vibrio* clade). Several bacteria cultured from

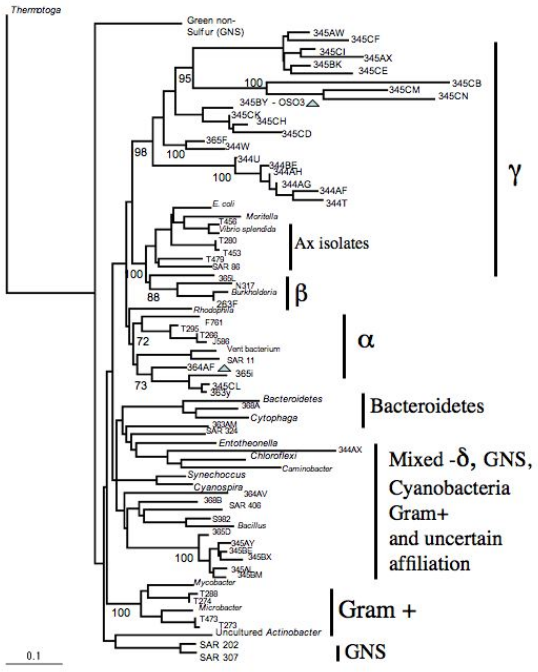

Figure 3: Neighbor-joining tree of *Axinella*-derived 16S rRNA clones. Tree reconstruction parameters and designations follow those shown for *Erythropodium* in Figure 2. A total of 77 bacterial taxa are shown.

Ax ("*Ax* isolates") appear dispersed to either the Gammaproteobacteria cluster or in the Gram+ and Alphaproteobacteria clusters at the bottom of the tree. Not all *Ax* isolates were included in the tree of Fig. 3, but the sequences have been submitted to GenBank. The previous study of cultured sponge isolates of Sfanos et al (2005) showed that most could be identified to family or genus. In contrast, several "unknown" and *Chloroflexi* bacteria were observed from these sponge culture-independent clones.

In the coral, sequences within all Proteobacteria clusters appeared less divergent, with shorter branch lengths, than *Axinella.*

These studies add to the evidence for symbiont specificity in *A. corrugata,* specifically adding to the data on "OSO" ("Orange sponge") studied by Hill et al (2006). OSO 16S rDNA sequences were found in multiple studies of geographically separated *A. corrugata* specimens. The OSO were not found among ~600 other clones generated from other sponge host species (data not shown; Lopez in preparation).

In contrast to the findings with OSO, the Gammaproteobacteria sequences derived from sediments adjacent to *Axinella* samples was uniform and dominated mostly by *Pseudoalteromonas* spp. which were not seen in the mesohyl/tissue libraries of either invertebrate.

Overall, we posit that microbial diversity patterns for both invertebrates generally reflect host species or geography, due to similar depths and water temperature of the specimens. However we cannot completely rule out temporal effects. In a limited time course experiment, some of the *Ax* clones were derived from a specimen that was oxygen deprived; this revealed an anaerobic clone (368B) with similarity to a *Clostridium*.

Although *Bacteroidetes* sequences were detected, this study did not find large occurrences of microbes previously associated with coral diseases such as *Roseobacter* and *Marinobacter* in either host reservoir. However, as the rarefaction analyses indicated, it is very possible that the sampling of total sequences remained below saturation.

It has been demonstrated that many bacterial communities associated with hard corals are largely coral species-specific, with microbial profiles reflecting phylogenetic relationships among coral species (Ritchie and Smith 2004). Previous molecular studies of hard corals have shown that the associated microbiota can be extremely diverse in species richness and abundance (Cooney et al. 2002; Rohwer et al. 2001). The same findings appear to hold for sponges, and other marine invertebrates that can serve as microbial hosts. Although a complete census of marine microbial diversity has certainly not yet been reached (http://icomm.mbl.edu/), microbial profiles are now extended with this study and with the continuing advances in high throughput sequencing technologies (Venter et al. 2004; Rusch et al, 2007).

Conclusions

The present data, in agreement with previous evidence, points to a suite of unique and interesting microbes that appear to be specifically associated with marine invertebrates.

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