Florida reef sponges harbor coral disease-associated microbes

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Abstract Sponges can filter large volumes of seawater and accumulate highly diverse and abundant microbial communities within their tissue. Culture-independent techniques such as fluorescent in situ hybridization (FISH), 16S small subunit (SSU) rRNA gene analyses, and transmission electron microscopy (TEM) were applied to characterize the presence and distribution of microbes within sponges abundant on south Florida reefs. This study found that coral disease-associated bacteria (CDAB) are harbored within *Agelas tubulata* and *Amphimedon compressa*. FISH probes detected several potential bacterial pathogens such as *Aurantimonas coralicida, Cytophaga* sp., *Desulfovibrio* spp, *Serratia marcescans*, and *Vibrio mediterranei* within *A. compressa* and *A. tubulata* host sponges. Spatial differences in the distribution of targeted bacteria were seen within sponge hosts. Transmission electron microscopy of *A. compressa* indicated there was a higher concentration of bacteria in the choanosome compared to the ectosome. These observed spatial distributions support the presence of internal sponge niches, which could play a role in the location of the CDAB within the sponges.

Keywords Sponge · Coral disease · Bacteria · FISH · Spatial arrangement

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1 Introduction

Sponges are ancient organisms, with fossils dating back to the Late Precambrian (Li et al. 1998; Love et al. 2009). These included microbial communities likely to have had a similarly long association with their sponge hosts. Specific bacterial symbionts are purported to provide benefits to sponges such as nutrient acquisition (Vacelet et al. 1995), UV radiation protection (Sara 1971), nitrogen fixation and nitrification (Wilkinson and Fay 1979), and secondary metabolites (Schmidt et al. 2000; Unson et al. 1994). Moreover, it has been shown that bacterial symbiont community composition can differ spatially within a single individual host (Koren and Rosenberg 2006). Close examination of inner sponge anatomy reveals potentially diverse microbial niches within sponge mesohyl (extracellular matrix). For example, *Aplysina cavernicola* exhibits a high mesohyl concentration of Delta-*Proteobacteria* and *Bacteroidetes*. Both groups of bacteria are capable of

anaerobic metabolism but are not abundant in the ambient seawater. Thus, it is likely that these bacteria have acquired adaptations allowing them to thrive in diverse microhabitats such as anaerobic portions of sponge mesohyl (Friedrich et al. 1999). In addition, the sponge *Geodia barretti* exhibits higher levels of oxygen in the ectosome, no oxygen at 4–6 mm below the surface (cortex) and a peak in oxygen at 17 mm within the sponge. When the sponge stops pumping water, oxygen disappears almost completely in the cortex (Hoffman et al. 2005). This spatial difference would then have an affect on the location of anaerobic and aerobic bacteria within the sponge.

The majority (>90%) of microbial symbionts are difficult to isolate and culture (Webster and Hill 2001; Sfanos et al. 2005; Taylor et al. 2007), and it has been estimated that less than 0.1–1.0% of the microbial sponge community is culturable (Santavy et al. 1990; Webster and Hill 2001). Recent advances in molecular genetics technology have facilitated in-depth taxonomic surveys and have confirmed the utility of culture-independent techniques to study microbial communities (Rappe' and Giovannoni 2003; Pace 2009). The use of 16S rRNA gene libraries of associated microbial diversity within sponges has also become widely used, helping to gain insight into their taxonomy and ecology (Lopez et al. 1999, 2010; Webster et al. 2001; Sfanos et al. 2005; Hill et al. 2006; Meyer and Kuever 2008; Radwan et al. 2010; Mohamed et al. 2010). For example, Hentschel et al. (2002) describe only 190 sponge-derived 16S rRNA gene sequences in the GenBank database. By 2007 the number had increased to approximately 1,500 rRNA sequences (Taylor et al. 2007), providing a larger database for bacterial identification.

Sponges can filter up to 24,000 L of seawater/kg/day, leaving the expelled water nearly sterile (Reiswig 1974; Turon et al. 1997). This filtration of large volumes of water results in relatively high bacterial concentrations within the sponge compared to the surrounding seawater. An example of bacterial accumulation in sponges has been shown in *Escherichia coli* populations that are higher in the sponge *Spongia officinalis* than the surrounding coastal seawater (Kefalas et al. 2003; Stabili et al. 2008). Therefore, the sponges' high filtration rates may result in them acting as reservoirs for bacteria that are present at levels below detection in ambient seawater. This may be a reason that sponges contain unique bacterial communities not present in other marine organisms (Hentschel et al. 2002; Schmitt et al. 2007). Moreover, many symbiotic bacteria found in sponges appear distinct from those that occur in ambient seawater (Hill et al. 2006), marine sediment, and in plankton (Santavy et al. 1990). Fieseler et al. (2006) has proposed a candidate phylum 'Poribacteria' for all the bacteria that have been found to occur uniquely within sponges. Several studies have provided support for the concept that sponges in different spatial, temporal and geographic habitats may harbor similar microbial communities (Olson and McCarthy 2005; Hentschel et al. 2002; Webster et al. 2004; Hill et al. 2006; Vishnyakov and Ereskovsky 2009). However, sponges of the same species located in temperate or tropical waters have been found to contain different microbial communities (Taylor et al. 2004, 2005).

Many sponge species share benthic habitats with scleractinian corals, the primary builders of tropical coral reefs. However, coral populations have suffered due to exposure to a number of deleterious physical and biochemical changes, which include over-fishing, increased carbon dioxide inputs due to anthropogenic sources, warming sea surface temperatures, eutrophication, sedimentation and pollution (Hughes et al. 2003; Lesser 2004). These factors can contribute to physiological stress, resulting in increased opportunities for coral disease associated bacterial (CDAB) pathogens (Sutherland et al. 2004; Lesser et al. 2007; Teplitski and Ritchie 2009). However there is limited knowledge on possible CDAB reservoirs. The algae *Halimeda opuntia* bearing the CDAB, *A. coralicida*, has been documented to trigger White Plague II in the coral *Montastraea faveolata* simply by contact (Nugues et al. 2004). Marine sediments (Richardson 1997) and fire worms (Sussman et al. 2003) also may serve as possible reservoirs, since CDAB have been found in these environments.

Studies have failed to find CDAB in the water column surrounding corals (Cooney et al. 2002; Richardson 1997). Locating reservoirs of potential coral pathogens may help in the formulation of strategies to help protect corals and, ultimately, contribute to a decline in the incidence of coral disease. Two primary hypotheses were tested in this study: (1) sponges act as a reservoir for specific bacterial taxa associated with coral diseases, and specific bacteria associated with coral diseases should be present in multiple species of sponges along with multiple individuals of the same sponge species; (2) the spatial distribution of CDAB species will be similar in different sponge species, due to similar ecological functions.

2 Methods

Agelas tubulata (Demospongiae, Agelasida)*, Amphimedon compressa* (Demospongiae, Haplosclerida) and *Aplysina fistularis* (Demospongiae, Verongida) sponge samples were collected by SCUBA off the coast of Fort Lauderdale, Florida at three reef sites on July 22, 2008 and October 8, 2008. Three individuals of *A. tubulata* and *A. fistularis* were collected at Site 1 (N 26°20844 and W 80°03418) at a depth of 17 m. Two individuals of *A. compressa* were collected at Site 2 (N 26°08785 and W 80°05780) at a

depth of 6 m and a third individual from Site 3 (N 26° 011414W 80°059973) at a depth of 9 m. Sponges for FISH and transmission electron microscopy (TEM) were fixed immediately in 4% paraformaldehyde or 2% gluteraldehyde fixative respectively and stored at 4°C. Portions of each sponge sample were also frozen for DNA extraction.

2.1 Fluorescent *in situ* hybridization (FISH)

Strain-specific probes for presently known CDAB (*Serratia marc*escans, *Vibrio mediterranei*, *Aurantimonas coralicida* and *Cytophaga* sp.) were designed (Table 1). In addition, FISH probes for the major group Firmicutes and *Desulfovibrio* spp. were also used since they have been shown to be abundant in Black Band Disease (BBD) (Frias-Lopez et al. 2002; Cooney et al. 2002; Richardson 1996). Probes for Actinobacteria and Gammaproteobacteria were designed as they were found to be abundant microbes via the 16S rRNA cloning data in this study of *A. compressa* and *A. tubulata* respectively.

In order to design new strain specific probe sequences, 16S rRNA alignments were made using the CLUSTAL program within The Biology Workbench 3.2 (Subramaniam 1998). Probes were tested on the NCBI PROBE Database (www.ncbi.nlm.nih.gov/probe) and Microbial Ribosomal Databases Probe Match (Cole et al. 2007; http://rdp.cme. msu.edu/ probematch/search.jsp) and were strain specific based on these tests. Alignments are available upon request from the authors. Three probes sequences (*Desulfovibrio* spp. EUB338, and Firmicutes) were obtained from PRO-BEBASE (Loy et al. 2007; www.microbial-ecology.net/ probebase/) (Table 2). All pre-labelled probes were purchased from Invitrogen and labeled with fluoroscein dye.

Fixed sponges were transferred to 70% ETOH at −20°C. Paraffin-embedded sponge blocks were sectioned to a width of 7 μm to 8 μm with Accu-Edge low profile microtome blades (Sakura Finetek) and placed on Platinum Line StarFrost slides (Mercedes Medical). Slides were incubated for at least 12 h at 56°C and then at 58°C for 1.5–2 h. The slides were dewaxed in xylene (100%) and graded ethanol treatments (90–50%).

Formamide concentration for the hybridization buffer was determined by using assays (20–45%) for each new probe designed, and the corresponding hybridization buffer (EX: 35% formamide) was made (360 μl 5 M NaCl, 40 μl 1 M Tris-HCl, 700 μl formamide, 900 μl H20, 2 μl 10% SDS). Hybridization buffer (45 μl) was mixed with 5 μl of the desired probe and placed on each section. The slides were then placed inside a humidity chamber (a 50 ml Falcon tube containing a paper towel saturated with hybridization buffer) for 2 h at 46°C, put in the corresponding wash buffer (700 μl 5 M NaCl, 1 ml 1 M Tris-HCl, 500 μl 0.5 EDTA, 50 ml H_2O and 50 μl 10%

SDS) for 20 min at 48°C, quickly rinsed with H_2O and air dried (Sharp et al. 2007). The slides were examined in an epi-fluorescent microscope (Olympus FV1000) at magnifications of 100×, 200×, and 400× in order to assess bacterial distributions. Photos of the sections were taken with an Olympus digital camera and contrast and brightness adjusted in Abode Photoshop Elements 5.0.

FISH was performed on three individuals of *A. compressa* and *A. tubulata*, with four sections each (total of 144 sections) (Tables 1, 2). The control experiments included the universal bacteria probe (EUB338) (Fig. 3f), a reverse complemented negative nucleotide probe and no probe for autoflourescence assessment. In addition, FISH was performed targeting an abundant bacterium that was found in the cloning data for each sponge (Fig. 3e).

Two control runs testing for non-specific binding of the probes were carried out. One trial involved performing FISH sequentially on the same section with two of the same probes (one with a fluorescent dye and one without). Here, the unlabelled probe blocked the binding site from the fluorescently labeled probe. The second trial involved simultaneous use of two probes with different nucleotide sequences and fluorescent dyes. The first probe was EUB338 with a fluorescent dye (green) and the second probe was *V. shiloi* AK1 with a CY3 dye (red). Since the EUB338 probe (targeting all bacteria) was green, and the *V. shiloi* AK1 probe was red, all of the *V. shiloi* AK1 bacteria with both probes binding to it resulted in an orange fluorescence and no red fluorescence were visible. Henceforth *V. shiloi* will be referred to as *Vibrio mediterranei* as per Thompson et al. (2001).

Once all FISH slides were completely scanned, the relative abundance of CDAB was determined by comparing the amount of positive probe signals to each other (Fig. 3). Factors including the presence of some bacteria only on one section (*S. marcescans*), and presence in only one area of the section compared to the whole section were taken into consideration. An abundance category, with plus (+) signs, indicative of a higher abundance was assigned accordingly to each CDAB and a zero (0) was used for when no probe signals were present (Table 3).

Generalized Estimating Equations in SPSS 17.0 analyzed data supporting the presence of the bacteria in the sponges, with a 95% confidence level and a binomial distribution (SPSS 2008).

2.2 SSU 16S rRNA cloning and sequence analysis

Environmental 16S rRNA microbial libraries were made for both *A. compressa* and *A. tubulata* specimens. Microbial genomic DNAs were isolated using the UltraClean Microbial DNA Isolation Kit (MO BIO Laboratories, Inc.). Approximately 750–800 bp of the 5' half of the 16S small

Table 1 Newly designed FISH probes

Bacterial species/group	Strains	Location in Ecoli	Probe sequence 5'-3'	TM	Formamide (%)
Serratia marcescans	AB435613	453	ACGTATTAAGCTCACCACCT	58	25
	POXD3L10	451			
Vibrio mediterranei	AF007115	655	CTACAGTACTCTATTCCGC	60	45
Aurantimonas coralicida	AJ786361	764			
	EF512741	792	ATCCTCCGACGGCTAGCTTC	64	35
	EF512716	802			
	AY065627	791			
Cytophaga sp.	AB015260	178	ACCATGCGATAAAACATTGTT	56	45
Actinobacter	EF076123	1,311	GTTCGCCACTCGATTTTCC	58	40
Gamma proteobacteria	EU315515-37	1,262	GCCGTCAAACTAATCGGAC	58	40
No bacteria (negative)			*TGAGGATGCCCTCCGTCG*	*55*	*35*

subunit rRNA gene was amplified by PCR with universal ECO9 and LOOP27 primers (Lopez et al. 1999; Sfanos et al. 2005). The 30.25 µl PCR mix consisted of 1 µl template DNA (13 ng/µl to 14 ng/µl after purification), 3 µl of deoxyribonucleoside triphosphate, 1 µl of each primer (30 mol/µl), 1.25 units of *Taq* DNA polymerase (ProMega), 3 µl 10× buffer, and 21 µl DH$_2$O. This mixture was amplified for 35 cycles under the conditions of denaturing at 95°C for 30 s, annealing at 53°C for 30 s and extension at 72°C for 1 min. PCR products were purified using QIAquick PCR Purification Kit (Qiagen, CA) and cloned using TOPO-TA cloning kit following manufacturer's protocol. Cloning products were then sent to SymBio Corporation (Menlo Park, CA) for sequencing.

DNA sequences were initially analyzed by BLAST programs (Altschul et al. 1997), followed by the Ribosomal Database Project (RDP) Classifier function for bacterial classification (http://rdp.cme.msu.edu/index.jsp; Cole et al. 2007). The FastGroup II website was used for rarefaction analysis and to calculate the Shannon-Wiener Index (Yu et al. 2006). After checking the quality of sequences by chromatograph inspections, sequences >300 bp were submitted. GenBank accession numbers were GU984049–GU984226 for all 178 new sequences described herein.

Phylogenetic analyses began with multiple sequence alignments of all or representative subsets of the sponge 16S rRNA fragments, including typed reference 16S rRNA sequences from GenBank, using ClustalW (Larkin et al, 2007). Poorly aligned regions were removed before phylogenetic reconstruction. A distance algorithm such as Neighbor-joining was performed with PAUP 4.0b4* (Swofford 2001). Most appropriate DNA substitution models for 16S rRNA datasets 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. Typical reconstructions used random stepwise addition with at least five replications, tree-bisection-reconnection and branch swapping, and 100–500 replicates in bootstrap analyses.

2.3 Transmission electron microscopy (TEM)

Sponge samples were placed in a gluteraldehyde fixative (2% gluteraldehyde in 0.05 M Na cacodylate buffered 0.22 micron filtered seawater) for a minimum of 2 h to 3 h. They were then rinsed in three changes of 0.0 5 M Na cacodylate buffer for 10 min each. Samples were then postfixed in 1% osmium tetroxide in buffer for 45 min and rinsed in buffer for three changes at 10 min each. Samples were dehydrated in a graded series of ethanol (20%, 40%, 60%, 70%, 90% and 100%). Following dehydration, samples were then embedded in Spurr© embedding resin in flat embedding molds for 24 h at 60°C. Blocks were sectioned using a Diatome 35 degree angle diamond knife. Sections were then stained with lead citrate (Reynolds 1963) and examined in a JEOL JEM 1200 Transmission Electron

Table 2 Probes obtained from ProbeBase

Bacterial group	Probe sequence 3'-5'	Accession #	TM	Formamide (%)	Reference
Desulfovibrio spp.	GGTCGCCCCCCGACACCT	pB-01332	59	30	Dar S.A. et al. (2007)
Firmicutes	CGGAAGATTCCCTACTGC	pB-00196	50	35	Meier H. et al. (1999)
EUB338 (all bacteria)	GCTGCCTCCCGTAGGAGT	pB-00159	55	35	Amann R.I. et al. (1990)

Table 3 Targeted bacteria relative abundance

	Aurantimonas coralicida	*Seratia marcescans*	*Vibrio mediterranei*	*Firmicutes*	*Cytophaga* sp.	*Desulfovibrio* spp.
Agelas tubulata						
1-Site 1	+[a]	0	++	+	++	0
2-Site 1	0	+	++	+	0	+
3-Site 1	++	+	++	+	++	0
Amphimedon compressa						
1-Site 2	++	+	++++	+++	+++	++
2-Site 2	+++	+	++++	+++	+++	++
3-Site 3	+	0	++++	+++	+++	0

[a] Plus signs (+) are indicative of the relative abundances of the corresponding probe fluorescence. An increase in the amount of (+) represents a higher abundance. The zeros (0) are indicative of no probe fluorescence.($N_{number\ of\ sections}$ = 144)

Microscope (TEM). Micrographs were taken with a Gatan digital camera mounted on the TEM.

3 Results

3.1 SSU 16S rRNA cloning and sequence analysis

In the 16S rRNA gene library analyses, 98 *Agelas tubulata* and 83 *Amphimedon compressa* sequences were generated. For *Agelas tubulata*, applying these sequences to RDP Classifier (Cole et al. 2007) indicated that unclassified Gammaproteobacteria represented the largest relative proportion of bacteria (48%). In *Amphimedon compressa*, unclassified bacteria appeared as the largest percentage (49%) of bacteria, followed by unclassified Gamaproteobacteria, unclassified Acidomicrobidae and *Acidomicrobidae*. Unclassified Oceanospirillales and unclassified Bacteriodetes were only found in *A. tubulata*, while

Nitrospira spp., unclassified Acidomicrobidae, unclassified Deltaproteobacteria, unclassified Rhodabacteria and *Acidomicrobidae* were only found in *A. compressa*. It was interesting that *A. tubulata* had low numbers of Alphaproteobacteria (1%). Major bacterial groups identified for each sponge led to the design of an Actinobacteria probe for *A. compressa* and a Gammaproteobacteria probe for *A. tubulata* based on their clone sequences (see Table 1 and next section). These 16S rRNA clone libraries were not expected to be exhaustive or inclusive, which is reflected by rarefaction curves (not shown) (Fig. 1)

Phylogenetic analyses of representative sets of 16 rRNA sequences from both sponges were also performed to confirm some of the above taxonomic categories. Determining the precise branching topology of respective clades *per se* was not a primary goal of this exercise, especially since many of the pairwise distances between some sequences were very large (>40%). This observation also led to the application of the neighbor-joining (NJ) method.
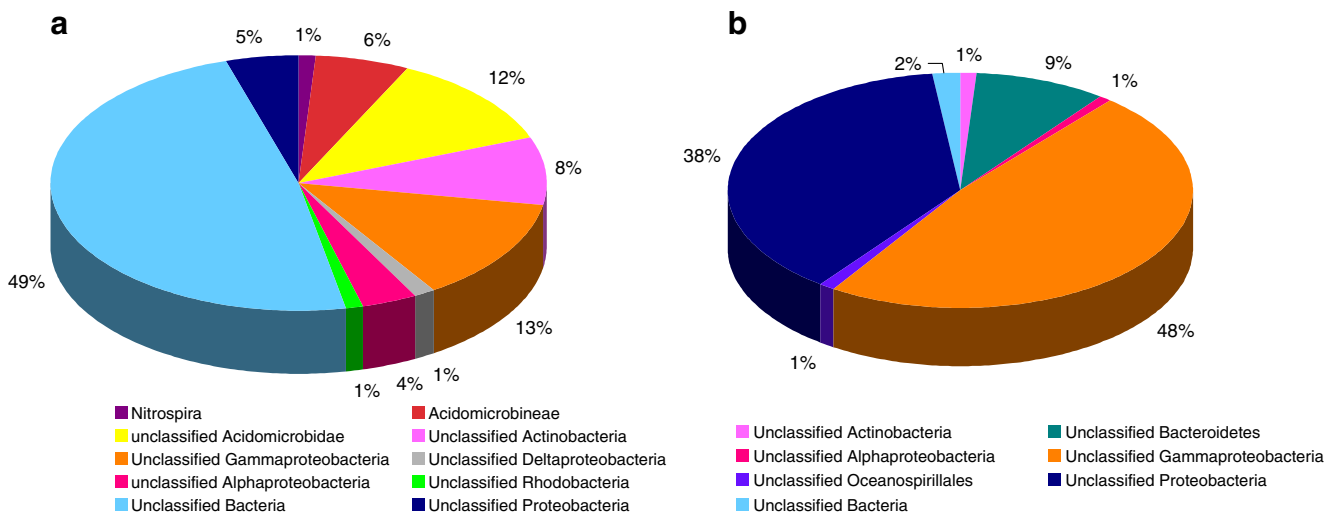

a Legend:
- Nitrospira
- Acidomicrobineae
- unclassified Acidomicrobidae
- Unclassified Actinobacteria
- Unclassified Gammaproteobacteria
- Unclassified Deltaproteobacteria
- unclassified Alphaproteobacteria
- Unclassified Rhodabacteria
- Unclassified Bacteria
- Unclassified Proteobacteria

b Legend:
- Unclassified Actinobacteria
- Unclassified Bacteroidetes
- Unclassified Alphaproteobacteria
- Unclassified Gammaproteobacteria
- Unclassified Oceanospirillales
- Unclassified Proteobacteria
- Unclassified Bacteria

Fig. 1 Composition of major bacterial taxonomic groups within cloned two16S rRNA sequence libraries of **a** *Amphimedon compressa* and **b** *Agelas tubulata*. Each library was queried with the RDP Classifier for taxonomic identification

The NJ tree in Fig. 2 confirms the large Gammaproteobacteria group composed mostly of *A. tubulata* clones in contrast to the large primarily "unclassified Bacteria" in *A. compressa*. Thus some of the more unique and basal taxa occur within *A. compressa*, such as clone E06 which showed similarity to *Nitrospira*, suggesting *A. compressa* had a slightly more diverse community of microbes compared to *A. tubulata*. This finding supports the Shannon-Weiner Diversity Index for the microbial communities in *A. compressa* and *A. tubulata*, which were $H'_A=3.042$ nats and $H'_B=2.896$ nats respectively. The tree also appears to show a clear partitioning of sequences that correlates with the source sponge host, as mostly *A. compressa* clones comprise the Gram-positive clade. A smaller Bacteroidetes clade holds only *A. tubulata* sequences

3.2 Fluorescent *in situ* hybridization (FISH)

Three of the five control FISH experiments consisted of the following—i) no probe, ii) negative probe, and iii) sequential non-fluorescent probe followed by a fluorescently labeled probe. All of these exhibited only autofluorescence and no positive FISH signals were detected. Control FISH experiments performed with two probes at the same time having different binding sites (EUB338-green label and *Vibrio mediterranei*-red label) resulted in green and orange (combined green and red) fluorescence. The absence of red fluorescence confirmed the specificity of the *Vibrio mediterranei* probe (not shown). The fifth positive control, EUB338 probe, produced abundant green fluorescence.
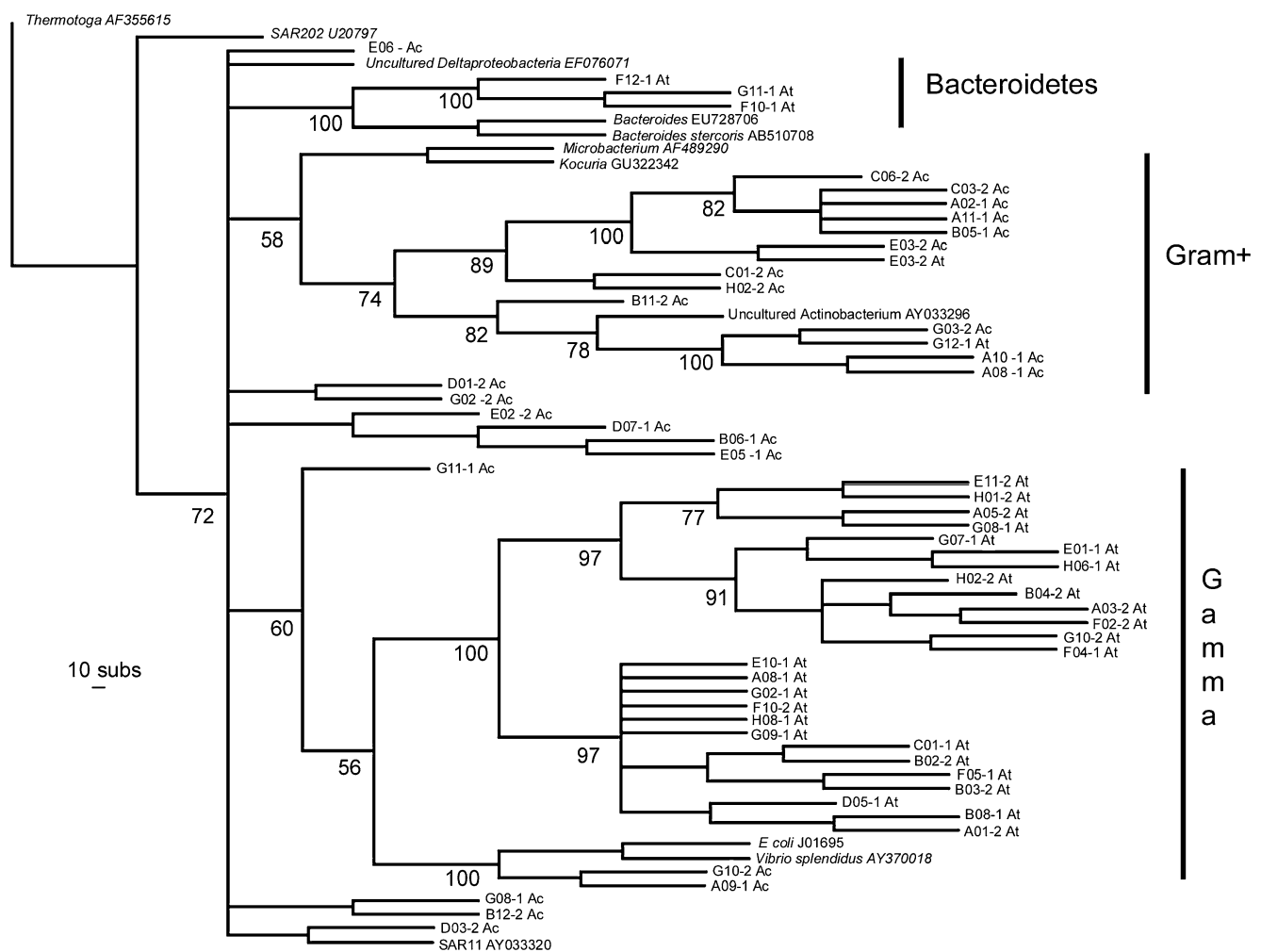

Fig. 2 Minimum evolution phylogeny of representative 16S rRNA sequences derived from host sponges *Amphimedon compressa* and *Agelas tubulata*. The Neighbor-Joining algorithm was applied on 67 sequences from both *Amphimedon compressa* (Ac) and *Agelas tubulata* ("At"), which are indicated by clone ID from each respective library. Previously typed or reference 16S rRNA sequences retrieved from GenBank are included with their respective accession numbers and assisted in designating the three major clades, including Gammaproteobacteria (Gamma). A Tamura-Nei model for base substitution was applied after running the sequence alignment through MODELTEST. *Thermotoga* sp. TMO (AF355615) rooted the tree. The scale bar indicates branch lengths in substitutions (subs). Numbers below a node indicate bootstrap percentage support after 500 iterations for the indicated clade

Not all fluorescent signals were uniform in size. For example if Figs. 3 and 4 are compared, different numbers of target bacteria are detected depending on the FISH probe applied. Several FISH target signals in Fig. 3c–f could represent individual bacteria. However, due to resolution limits, individual bacteria could not be resolved and the detection of multiple bacteria in aggregates (>1 um to 2 um in diameter) could not be precluded. However, large numbers of aggregates in the species utilized in the TEM component of this study were not observed. Alternatively, the larger fluorescent signals observed in Fig. 3c or Fig. 4c may result from differences in fluorescence exposure.

Although the targeted CDAB taxa were found in both species of sponges, they were not all present in every specimen (Fig. 3). For instance, *A. compressa* individuals collected from different localities (Table 3), exhibited differences in bacterial presence and abundance. The Generalized Estimating Equation is commonly used for correlated binary data (Hanley et al. 2003), and was used to determine that there was no statistically significant difference in the presence or absence of the six identified bacteria in *A. tubulata* and *A. compressa* (*Aurantimonas coralicida* $p=0.373$: *Cytophaga* sp. $p=0.928$: Desulfovibrio $p=0.299$: Firmicutes $p=0.480$: *Serratia marcescans* $p=0.841$: *Vibrio mediterranei* $p=0.121$). The relative abundance of all targeted bacteria was consistently lower in *A. tubulata* compared to *A. compressa*, even when the controls are compared (Table 3).

3.3 Spatial arrangements

Vibrio mediterranei bacteria were found in relatively high abundance in both sponges (Table 3) and were commonly localized at distinct areas of the sponge mesohyl (Fig. 4b). Even though some bacteria were found relatively evenly distributed throughout the sponge mesohyl (Firmicutes, *Cytophaga* sp. and Actinobacteria) (Fig. 4a), Gammaproteobacteria, *Vibrio mediterranei*, *S. marcescans* and *Desulfovibrio* spp. were found localized in high concentrations. However, some bacteria, such as *S. marcescans*, were found evenly distributed in one histological section and locally concentrated on another (Fig. 4c). It should also be noted that due to size of the fluorescence, the detection of bacterial aggregates seems to be common within the sponges. *Desulfovibrio* spp. and *S. marcescans* were the only CDAB detected within comparatively large aggregates by FISH (Fig. 4c and d).

3.4 Transmission electron microscopy (TEM)

The marked autofluorescence of *Aplysina fistularis* precluded its intended use in FISH analysis, but this sponge was useful for comparative TEM study, which partially corroborated FISH data. Both *A. fistularis* and *A. compressa* exhibited similar high concentrations of bacteria in the choanosomal mesohyl relative to the ectosome (Fig. 5). Furthermore, *A. fistularis* mesohyl contained a type of bacteriocyte, which is a typical sponge cell containing bacteria isolated from the surrounding cells and mesohyl and found in many other sponge species (Fig. 5c). These cells, also known as "pocket cells" (Boury-Esnault and Rützler 1997), have a spherical or oval shape with a diameter of 10–15 μm. The bacteriocyte has a single large cavity and is surrounded by a thin (0.12 μm to 0.4 μm) cytoplasmic layer. The cavity is often open to the intercellular mesohyl and bacteria inside bacteriocyte appear similar to those on the outside in the sponge mesohyl. The same cell type is described in other species of the demosponge genus *Aplysina* (Verongida) (Vacelet 1970, 1975) and *Petrosia ficiformis* (Haplosclerida) (Vacelet and Donadey 1977; Maldonado 2007). An occasional virus was observed in the sponge tissue, but viruses were not abundant in the tissue of either species of sponge examined. Other than large viruses and non-bacterial microbial populations were not apparent in the ultrastructural analysis.

4 Discussion

4.1 Sponges and coral disease-associated bacteria

Coral disease associated bacteria (CDAB) are rarely detected in seawater (Cooney et al. 2002; Frias-Lopez et al. 2002; Richardson 1997), likely due to their low concentrations. CDAB also appear to be missing in recent large-scale metagenomic surveys of bacterioplankton (Rusch et al. 2007). It is possible that CDAB occur below the limits of detection of current methods and sampling strategies (Wintzingerode et al. 1997; Cottrell and Kirchman 2000). In contrast, this study has found CDAB present in two species of marine sponges utilizing FISH. The filtering activity of sponges appears to have facilitated accumulation of live bacteria resulting in an increased detection level, and suggests a role for sponges as potential bioaccumulators of CDAB in seawater. Unequivocal evidence that CDAB populations are actively dividing and growing within these sponge hosts could not be shown, and proof of CDAB vertical transmission also requires further research. Nonetheless, this study demonstrates that sponges may serve as a proxy for profiling ambient water column bacterial composition. Combining FISH with 16S rRNA PCR methods described in this study is a step in this direction, even when the 16S rRNA sequencing is not exhaustive. Overall, these approaches could prove useful especially within sensitive benthic habitats, or when adjacent to critically endangered species, such as in the coral genus *Acropora* that can have
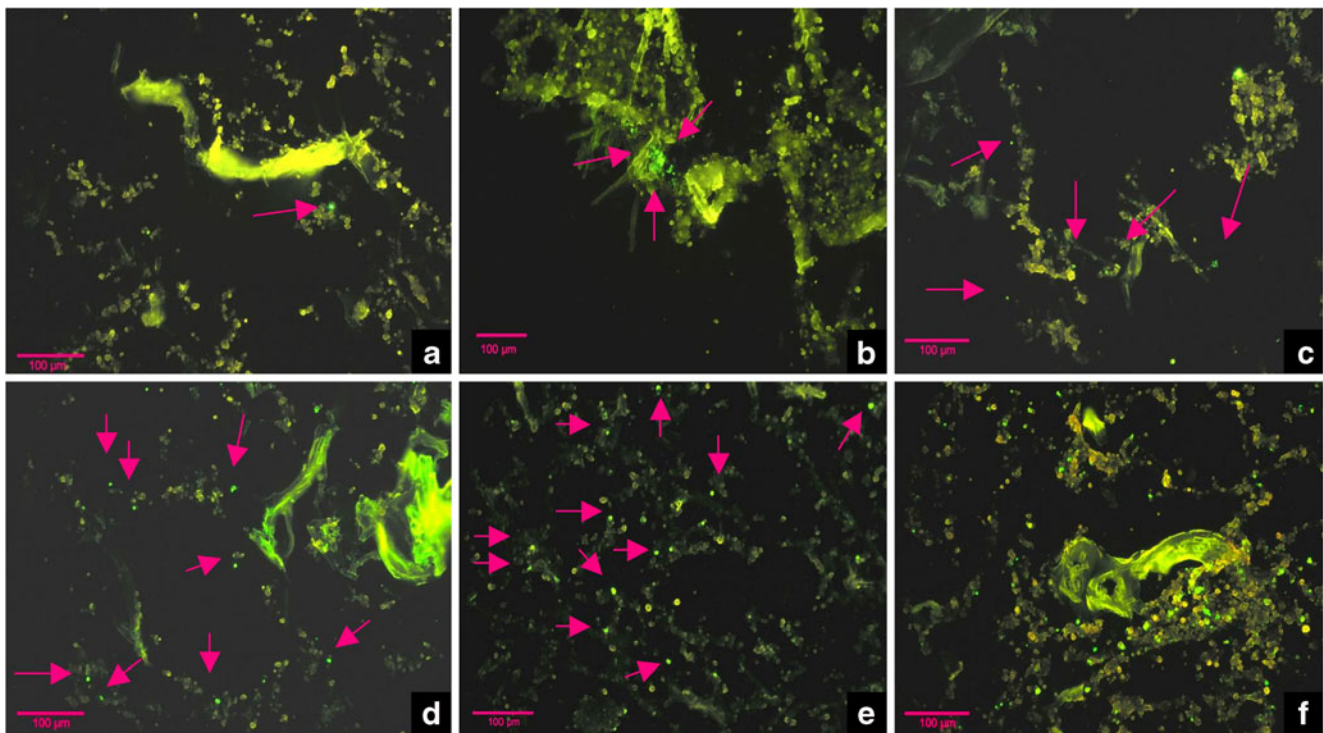
Fig. 3 FISH depicting bacterial relative abundance categories **a** +, *Amphimedon compressa* with *Serratia marce*scans probe (only one aggregate found on a section); **b** ++, *Agelas tubulata* with *Vibrio mediterranei* probe; **c** +++, *A. compressa* with *Cytophaga* sp. Probe; **d** ++++, *A. compressa* with *Vibrio mediterranei* probe; **e** +++++, *A. compressa* with Actinobacteria probe; **f** ++++++, *A. compressa* with EUB 338 positive control probe. Scale bar=100 μm

restrictions on invasive sampling (Acropora Coral Conservation and Restoration Workshop, November 2009).

Sewage and terrestrial runoff are contributing factors in some coral disease outbreaks including black band disease and white plague type II (Voss and Richardson 2006; Kaczmarsky et al. 2005; Denner et al. 2003). Therefore, geographic location may be a factor in the detection of CDAB within sponges, and potentially explain why CDAB were not found in every individual of *A. compressa* (Table 3). Although causative agents are often difficult to determine in coral disease studies, as already discussed here, there is growing evidence that specific bacteria have an important role in coral disease. Additional causative agents, such as viruses and protozoans are under study (Wilson et al. 2005; Patten et al. 2008; Thurber et al. 2008). In this study, sponge ultrastructural examination did not reveal viruses or other non-bacterial microbes sequestered in the sponges studied to any significant extent. This suggests that the filtration apparatus within the sponges may not be able to capture and concentrate the relatively small size classes (less than 1 μm) to which the viruses belong. The finding of occasional relatively large viruses in the sponges supports this view (data not shown). The presence of anti-microbial compounds in specific sponge layers can also not be discounted (Galeano and Martínez 2009).

Increase in coral disease outbreaks is documented during environmental perturbations, which can provide conditions where complex microbial interactions are altered. Potential host shifts may occur, in which existing or previously "benign" microorganisms infect new hosts and become opportunistic pathogens (Harvell et al. 1999). For instance, *Vibrio mediterranei* is virulent only during periods of increased water temperature in the summer (Israely et al. 2001). Furthermore, the prevalence of *V. mediterranei* within corals diminishes with lower winter temperatures (16°C), indicating that incidences of coral bleaching may require a new infection each year (Sussman et al. 2003). Various *Vibrio* spp. can provide antibiotic protection to their coral hosts, which is lost during bleaching (Ritchie 2006). In support of this has been the observation that an increase in temperature appears to allow *Aurantimonas coralicida* to colonize the otherwise inhospitable low pH coral surface layer (Remily and Richardson 2006). Having a potential reservoir for CDAB and other microbes to reside until favorable environmental conditions occur (e.g. weakened host immunity) may be crucial to the disease process in facilitating infection. Bacteria that can cause coral diseases are present in several marine habitats, such as sediment (Richardson 1997), fire worms (Sussman et al. 2003), algae (Nugues et al. 2004), and sponges (this study). Therefore,

Fig. 4 FISH images illustrating different bacterial spatial arrangements found within the two target sponges. **a** FISH of *Amphimedon compressa* with *Cytophaga* sp. probe. Bacteria appeared dispersed across sponge mesohyl; **b** FISH of *Agelas tubulata*, with *Vibrio mediterranei* probe. This bacterium was commonly found concentrated with one area of the sponge; **c** and **d** FISH of *A. compressa* sponge with *Serratia marcescans* and *Desulfovibrio* spp. probes, respectively. Both bacteria were found as aggregates
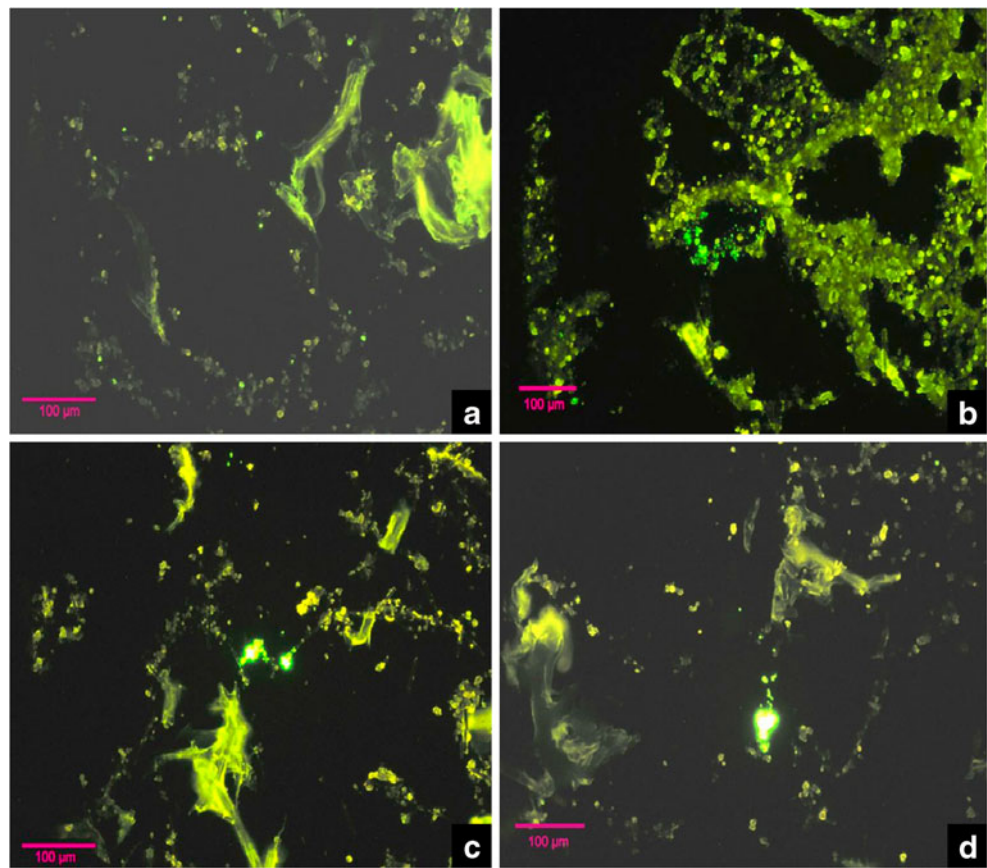
the possibility of multiple marine organisms should be evaluated as reservoirs for CDAB.

4.2 Microbial spatial distributions within sponges

TEM results (Fig. 5) show different bacterial spatial distributions in the choanosome and ectosome. These variations in spatial distribution suggest that FISH quantification of bacteria utilizing histologically sectioned tissue may be problematic, but are useful for comparing overall abundance and in identification of the bacteria. In part this is due to the ubiquitous siliceous spicules in most demosponges, which may cause considerable disruption of tissue during sectioning for light microscopy. In contrast,
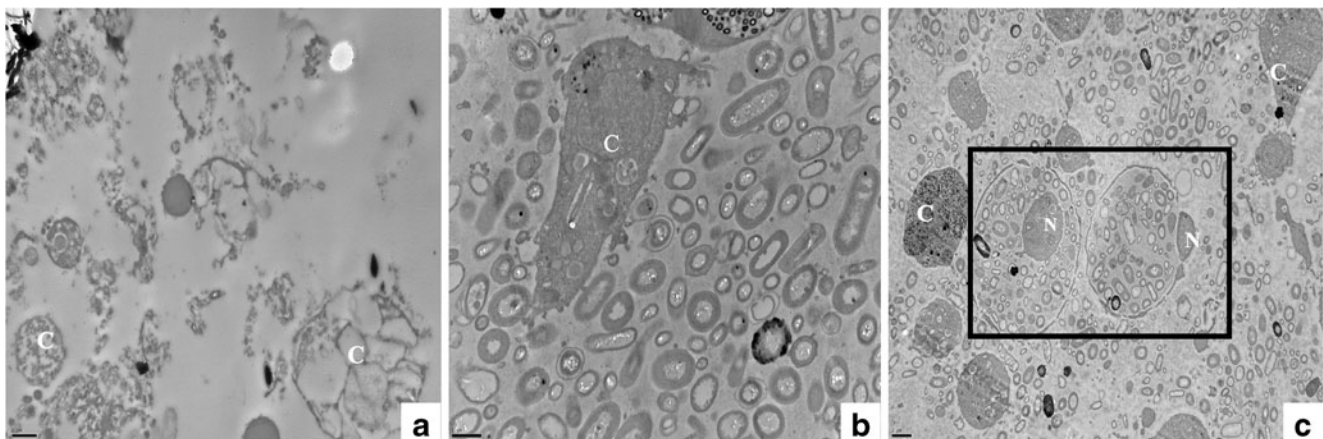

Fig. 5 Distinct spatial organization of bacteria in transmission electron micrographs of *Aplysina fistularis* and *Amphimedon compressa*. **a** mesohyl of an ectosome in *A. fistularis* illustrating the presence of very few bacteria (scale bar 2 μm); **b** mesohyl of a choanosome in *A. compressa* with abundant bacterial morphotypes (scale bar 1 μm); **c** mesohyl of a choanosome in *A. fistularis* with two bacteriocytes indicated by the box (scale bar 2 μm). The nucleus (N), without nucleolus is oval (1.6×3 μm) and situated either at the cell periphery, or inside of the cell in the peripheral cytoplasm internal protrusion Abbreviation: *C*—sponge cells of mesohyl

the use of a low viscosity resin (Spurr™) and a diamond knife in the TEM methodology retained the siliceous spicules without disruption of the surrounding tissue. However, utilizing FISH for microbial distributions in sponges provides a greater surface area of the tissue to be imaged and assessed, and most importantly, the bacteria were identified taxonomically. Therefore the use of TEM with FISH provided complementary information on bacteria location within specific areas of the sponge.

Since higher concentrations of bacteria were observed in the interior of the sponge compared to the ectosome, it is important to question why this particular internal environment may facilitate bacterial growth. One possible explanation is that the ectosome layer is more prone to artifacts from sampling collection. For example, simply squeezing the sponge may cause loss of bacteria. However, another process that may influence the internal concentration of bacteria is sponge filtration, the velocity of which changes with a variety of parameters such as temperature, turbidity, storms and diurnal cycles (Reiswig 1974). Therefore, microhabitats within the sponge may be ephemeral environments, in which bacterial concentration may fluctuate on relatively short time scales.

Moreover, an interesting future study of this phenomenon would entail conducting sampling and fixation of the ectosome and choanocome along a temporal (seasonal) gradient, because of the possibility that different spatial distributions within the sponge may be found during different reproductive stages, temperature or salinity regimes.

Desulfovibrio spp. and *S. marcescans* were the only CDAB found in large aggregates (Fig. 3c and d). As bacterial aggregates have been observed in such diverse environments as in the epidermis of coral tissue and coral larvae (Renegar et al. 2008) and plant leaves (Monier and Lindow 2003), the same phenomenon may apply to sponges. The presence of aggregates may provide a mechanism to maintain low oxygen conditions during fluctuating levels of ambient oxygen (Krekeler et al. 1997; Cypionka 2000). Support for this hypothesis is that peaks of oxygen in anoxic zones occur within the sponge due to the sponge pumping oxygenated water (Hoffman et al. 2005). Therefore due to a fluctuating internal sponge environment, spatial arrangements and abundances of CDAB within sponges may change accordingly.

Amphimedon compressa is classified as a Low Microbial Abundance (LMA) sponge (Hentschel et al. 2003), while the data in Table 3 indicates that *Agelas tubulata* is also a LMA sponge. LMA sponges are described as having a microbial concentration similar to that of seawater (Hentschel et al. 2003). However in this study, the TEM images indicate localization of bacteria in the choanosome layer in two sponge species. Therefore, ultrastructural examination of inta-sponge spatial relationships in bacterial distribution is important. This study documents spatial relationships between the sponge and its microbial community. Other species of sponges, which are described as having a High Microbial Abundance (HMA), may not exhibit this localization, and would result in a seemingly higher bacterial concentration due to the presence of bacteria throughout the sponge layers. It is likely that HMA sponges may exhibit different distributions of bacteria within the sponge and be reservoirs for CDAB as well. Additionally, *Amphimedon compressa* tends to produce more mucus than *Agelas tubulata*, which could also be a factor in bacterial distributions and overall microbial diversity.

In conclusion, FISH showed that coral disease associated bacteria (*A. coralicida*, *Cytophaga* sp., *Desulfovibrio* spp., *S. marcescans*, and *Vibrio mediterranei*) are found within sponges. Therefore, the hypothesis that sponges can act as a reservoir for CDAB appears acceptable. However, all species of CDAB were not found in each individual sponge specimen, suggesting that location may play a role and/or more exhaustive screening and sampling is needed due to the patchy distribution observed. Sporadic appearance of some bacterial targets and the autofluorescence of some sponge hosts proved to be problematic for quantification of bacteria. Nonetheless, internal bacterial niches within the mesohyl were observed in the aggregation of two anaerobic CDAB (*Desulfovibrio* spp. and *S. marcescans*) with higher concentration of bacteria in *Aplysina fistularis* and *Amphimedon compressa* choanosome relative to the ectosome. With the increasing impacts of climate change on habitats, including sensitive coral reefs, a need within conservation biology circles for reliable and diagnostic monitoring of baseline and dynamic fluctuations of normal and pathogenic bacterial communities has been demonstrated. This study has demonstrated that the marine sponge lifestyle of filtration and harboring diverse bacterial communities includes bacteria that can be pathogenic to corals (CDAB).

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