Microbial Diversity of the northern star coral, *Astrangia poculata*

Molly A Moynihan

Microbial Diversity 2017

INTRODUCTION

Scleractinian (reef-building) corals grow at rates of approximately 3-15cm per year in tropical, low-nutrient waters (Lesser et al. 2004), creating habitats of complex topography. The high biodiversity of reef environments is attributed to this heterogeneous habitat, which favors niche diversification (Birkeland, 1997a; Paulay, 1997). In addition, coral reefs provide important ecosystem services, such as coastal protection, tourism, and fisheries (Moberg & Folke 1999). The success of coral in oligotrophic conditions is largely due to tight nutrient coupling between the coral host and their symbiotic dinoflagellates, known as zooxanthellae (*Symbiodinium sp*). Zooxanthellae transfer up to 95% of their photosynthetic products (sugars, amino acids, carbohydrates) to the host, providing energy for respiration, growth and $CaCO_3$ deposition (Muscatine et al. 1989). In turn, the host's metabolic waste provides zooxanthellae with ammonia, phosphate and carbon dioxide.

However, in addition to dinoflagellates, corals possess a diverse range of bacterial associates (Ainsworth et al. 2015; Apprill et al. 2016; Neave et al. 2016), with individual coral colonies having up to several thousand associated, distinct bacterial phylotypes (Bayer et al. 2013). While early work on coral and microbes focused on pathogens, recent findings suggest that coral have both core and transient microbial communities that may play important roles in coral physiology (Ainsworth et al. 2010 and references therein). The community of the coral host, bacteria, archaea, fungi, and viruses are now viewed as a metaorganism, referred to as the coral holobiont (Rohwer et al. 2001; Ainsowrth et al. 2010; Neave et al. 2016).

Molecular studies of coral-associated microbes have revealed a wide range of metabolic function and potential, including nitrogen fixation (Lesser et al. 2004; Lema et al. 2015; Rädecker et al. 2015), ammonium oxidation (Wegley et al. 2007), antibiotic production (Ritchie 2006), carbon metabolism and transport (Neave et al. 2016), and sulfur metabolism (Raina et al. 2009). Nevertheless, the localization of these organisms within the holobiont and the stability of their associations remains largely uncharacterized. Moreover, abiotic environmental changes (e.g. pH, temperature) can alter coral microbial community composition and metabolism, which may in turn impact the coral health and decrease the organism's resilience (Thurber et al. 2009). Consequently, understanding the diversity and metabolic function of coral-associated microbial communities is not only crucial for a better understanding of coral physiology, but is also crucial to predict how reefs respond to stress.

In order to learn how to characterize coral-associated microbial communities, a species readily available at the MBL Marine Resource Center, *Astrangia poculata* (Ellis & Solander, 1786), was studied throughout the course of this project. *A. poculata*, a Scleractinian coral, has a wide thermal distribution, and is found in the Western Atlantic Ocean from Cape Cod to the Gulf of

Mexico. Moreover, it has a facultative symbiosis with zooxanthellae. When water temperatures and light levels are low, *A. poculata* can expel its algal symbionts and rely on heterotrophic feeding for nutrition (Dimond & Carrington, 2007; 2008). To best of my knowledge, no study on bacterial associates of *A. poculata* exists in the scientific literature to date. The goals of this study were to (1) characterize the microbial diversity of *Astrangia poculata*, (2) investigate spatial distribution microbes in *A. poculata* tissue, and (3) compare microbial communities in *A. poculata* colonies with different densities of zooxanthellae. It was hypothesized that *A. poculata* with low and high densities of zooxanthellae would have different microbial populations.

METHODS

Sample Collection

Astrangia poculata colonies were collected from the Marine Biological Laboratory's Marine Resource Center. All *A. poculata* colonies were collected using SCUBA in the vicinity of Woods Hole, MA. The color of extended polyps was used to determine relative abundance of symbiotic dinoflagellates, known as zooxanthellae (*Symbiodinium sp*). Colonies with translucent polyps were categorized as low zooxanthellate (LZ) and colonies with brown polyps were categorized as high zooxanthellate (HZ). Individual colonies were split into smaller pieces for experimentation using a mallet and chisel, and rinsed thoroughly with 0.22µm filtered seawater to remove any loosely associated seawater microbes.

Enrichment, Isolate Culturing, & 16S sequencing

Low zooxanthellate (LZ) and high zooxanthellate (HZ) colonies crushed using a mortar and pestle. The homogenate was vortexed for 1 minute, and then centrifuged at 150xg for 30 seconds to concentrate calcium carbonate particles. The supernatant was removed and centrifuged at 16,000xg for 5 minutes to pellet cells. The supernatant was removed and the pellet was resuspended in 0.22µm filtered seawater. The resulting homogenate was used both for DNA extraction and for isolate culturing. DNA from the homogenate was extracted using the DNeasy PowerSoil kit (Qiagen).

100µL coral homogenate aliquots were spread onto petri dishes containing seawater complete medium. Plates were left to incubate at room temperature by a window, and one plate from the LZ samples was placed at 30°C in the dark. Individual colonies from these enrichments were spread onto new plates until pure isolates were achieved. Seven isolate colonies from the LZ sample were selected for 16S rRNA sequencing (5x room temperature, ambient light and 2x 30°C and dark). A small amount of each pure colony was transferred into 20µL of an alkaline phosphatase reagent (alkaline PEG200) and lysed by boiling. Samples were then briefly vortexed and cellular debris was pelleted. The supernatant, containing sample DNA, was transferred for downstream polymerase chain reaction (PCR) amplification.

The 16S rRNA gene was amplified in DNA from the total coral homogenate DNA extraction and cultured isolates. 50-100ng of sample DNA was combined with 1µL 16S_8F (15pmol), 1µL

16S_1391R (15pmol), 25µL Promega GoTaq® G2 Hot Start Green Master Mix, and 18 µL Nuclease-free water. PCR was performed with an initial temperature of 95°C for 2 minutes to start denaturation and activate hot start polymerase. Samples were then cycled 30 times with denaturation at 95°C for 30 seconds, annealing at of 55°C for 30 seconds, and extension at 75°C for 1.5 minutes. A final extension was performed at 72°C for 10 minutes. Gel electrophoresis was used to confirm 16S rRNA amplification.

For microbial community analysis, PCR products from total coral homogenate samples were cloned into a plasmid vector by combining 3µL of PCR product with 5µL 2x rapid ligation buffer, 1µL pGEM T-easy Vector, and 1µL T4 DNA ligase. The ligation reaction was incubated for 5 minutes at room temperature and then placed on ice for transformation. *E. coli* (JM109) were inoculated with 2µL of the ligation reaction and kept on ice for 30 minutes. Cells were then heat-shocked at 42°C water for 30 seconds and transferred to ice for 2 minutes. 300µL of S.O.C. medium was added to each sample, and vials were placed on a shaker at 37°C for 1 hour. Samples were gently pelleted using a table-top centrifuge and the supernatant was removed. The pellet was spread on LB-Amp plates and incubated at 37°C overnight. 43 colonies from the LZ sample and 53 colonies from the HZ sample were selected and placed in a 96-well plate with 1mL of LB-Amp media, generating two clone libraries (LZ, HZ).

Clone library and coral isolate samples were submitted for Sanger sequencing. Sequences were aligned and analyzed using the NCBI Nucleotide BLAST database (NCBI), Mega7 (Kumar et al. 2016), and iTOL (Letunic and Bork, 2016).

FISH, CARD-FISH & Imaging

Samples for FISH were simultaneously fixed and decalcified by immersion and incubation in Cal-Ex II (Fisher) (weight percent: 10.6% Formic Acid, 7.4% Formaldehyde, <1% Methyl alcohol) at room temperature for 2-3 days. (Various attempted dissolution solutions can be found in Table i, appendix). Coral tissue was then dissected using forceps and a scalpel into subsample containing 1-5 polyps per sample. Samples were hybridized in 1µL of probe working solution (50ng/µL) and 9 volumes of hybridization buffer. The percentage of formamide (FA) in the hybridization buffer corresponded with each probe working solution. Probes used include Alphaproteobacteria (Cy3, 35% FA), Deltaproteobacteria (Cy3, 30% FA), Gammaproteobacteria (Cy3, 35% FA), Cyanobacteria (Cy3, 0% FA), Eubacteria (Cy3, 35% FA), and Eubacteria (FITC, 35% FA) (Table ii, appendix). Samples were incubated on a parafilm-covered glass slide with probes in humic chambers at 46°C for 2 hours. Chamber FA concentrations corresponded to that of the hybridization buffer. Samples were then transferred to washing buffer and placed in a 48°C water bath for 15 minutes. Samples were rinsed sequentially in deionized water and 96% ethanol, placed in MatTex dishes, immersed in 0.22µm PBS, and stored at 4°C until analysis. A Zeiss LSM 880 Confocal with FAST Airyscan used to image FISH samples. Four lasers (405nm, 488nm, 561nm and 640nm) were used for excitation and emission was measured in the following ranges: 646-680nm (Chlorophyll *a*), 543-589nm (Cy3 probe), 490-562nm (FITC probe), 410-515nm (DAPI/coral tissue auto-fluorescence).

CARD-FISH samples were prepared from the concentrated coral homogenate described above for isolate cultures. 100µL of homogenate were fixed for 1 hour in 2% formaldehyde, filtered onto a white polycarbonate membrane filter, and rinsed in deionized water. The filter was cut into two smaller subsamples and embedded in 0.1% agarose. Samples were permeabilized by incubation in lysozyme (10mg/mL) for 30 minutes at 37°C. Samples were then incubated at 0.01M HCl with 3% H_2O_2 for 10-30 minutes to inactivate endogenous peroxidases, washed with deionized water, and then washed in 96% ethanol. Hybridization buffer was mixed with a probe (*Oceanospirillales*-Cy3 & Eubacteria-Cy3; Table ii) working solution (50ng DNA/µL) in a 300:1 ratio. Filters and hybridization solution were combined on a parafilm-covered glass slide and placed in a humic chamber (35% FA) and incubated for 3 hours at 46°C. After hybridization, filters were washed in washing buffer for 10 minutes at 48°C and then transferred to 1xPBS and incubated for 15 minutes at room temperature. Amplification buffer was mixed with hydrogen peroxide (0.15% H_2O_2 in PBS) in a ratio of 100:1. Fluorescently labeled tyramide (1 mg dye /mL) was diluted 1:300 in this buffer. This amplification mix was combined with filters on a parafilm-covered glass slide, and samples were incubated in 35% FA humic chambers for 40 minutes at 46°C. After amplification, filters were transferred to 1xPBS and incubated for 10 minutes at room temperature in the dark, washed with deionized water, and then washed in 96% ethanol. A Zeiss Axioplan Imager A2 microscope was used to image CARD-FISH filters.

RESULTS & DISCUSSION

Enrichment, Isolate Culturing, & 16S sequencing

Table 1. Closest BLAST hits (NCBI) of 16S rRNA sequences from low zooxanthellate *Astrangia poculata* isolates cultured on seawater medium.

Class	Order	Family	Genus	Query Cover %
Gammaproteobacteria	Alteromonadales	Pseudoalteromonadaceae	Pseudoalteromonas sp.	99%, 98%
Gammaproteobacteria	Alteromonadales	Pseudoalteromonadaceae	Pseudoalteromonas luteoviolacea	99%
Gammaproteobactera	Pseudomonadales	Pseudomonadaceae	Pseudomonas parafulva	99%
Gammaproteobactera	Vibrionales	Vibrionaceae	Vibrio coralliiytius	98%
Flavobacteria	Flavobacteriales	Flavobacteriaceae	Tenacibaculum	99%
Sphingobacteria	Sphingobacteriales	Flammeovirgaceae	Flammeovirga kamogawensis	99%

Seven bacterial strains were isolated and sequenced from enrichment cultures of LZ, representing three classes: Gammaproteobacteria, Flavobacteria, and Sphingobacteria (Table 1). Isolation from the HZ sample was not successful due to dense microbial growth on the plate. Isolates were highly pigmented. A *Pseudoalteromonas* isolate, which matched closely to *P. luteoviolacea* (99%, NCBI BLAST), produced dark violet colored colonies, likely containing violacein. The remaining two *Pseudoaltermonas* isolates had orange pigments. The isolate closely matching *Flammeovirga* (99% NCBI BLAST) was pink and "sunk" into the agar. Members of the *Flammeovirga* genus are typically aerobic, chemoorganotrophs that produce saproxanthin pigments, which result in orange to reddish colors. Strains are known to dissolve agar by producing gelase fields (Nakagawa, 2015). The *Tenacibaculum* isolate had green iridescence.

Tenacibaculum have also been found in association with jellyfish, where it suggested that their proteolytic enzymes could play a role in digestive metabolism (Dinasquet et al. 2012). As scleractinian corals and jellyfish descent from the same phylum (Cnidaria), it is possible that *Tenacibaculum* could have a similar metabolic role in the coral gut. Lastly, an isolate closely resembled *Vibrio coralliilyticus* (99%), a known lysogenic pathogen of coral. *V. coralliilyticus* has been isolated globally from various marine organisms and is considered an endemic member of coral reefs. *V. coralliilyticus*' virulence is linked to sea surface temperature, and as global temperatures rise, it is predicted to increase rates of coral disease (Kimes et al. 2011).

Table 2. Closest BLAST hits (NCBI) of 16S rRNA sequences from HZ and LZ *Astrangia poculata* homogenate clone libraries.

Class	Order	Family	Genus	Query Cover %
Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	Pelagibius litoralis	92%, 92%, 86%
Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Stappia carboxidovorans	98%, 100%, 98%
Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Pseudovibrio ascidiaceicola	88%, 98%
Alphaproteobacteria	Rhizobiales	Rhodobiaceae	Tepidamorphus gemmatus	94%
Betaproteobacteria	Burkolderiales	Comamonadaceae	Comamonas testosteroni	98%
Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	Desulfonatronum cooperativum	82%, 85%
Gammaproteobacteria	Legionellales	Coxiellaceae	Coxiella burnetii	98%
Gammaproteobacteria	Chromatiales	Chromatiaceae	Thiohalobacter thiocyanaticus	95%
Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas plecoglossicida	99%
Gammaproteobacteria	Legionellales	Legionellaceae	Legionella drancourtii	88%
Cyanophyceae	Chroococcales	Aphanothecaceae	Crocosphaera watsonii	92%
Cyanophyceae	Synechococcales	Synechococcaceae	Synechococcus rubescens	99%
Planctomycetia	Planctomycetales	Planctomycetaceae	Bythopirellula goksoyri	93%

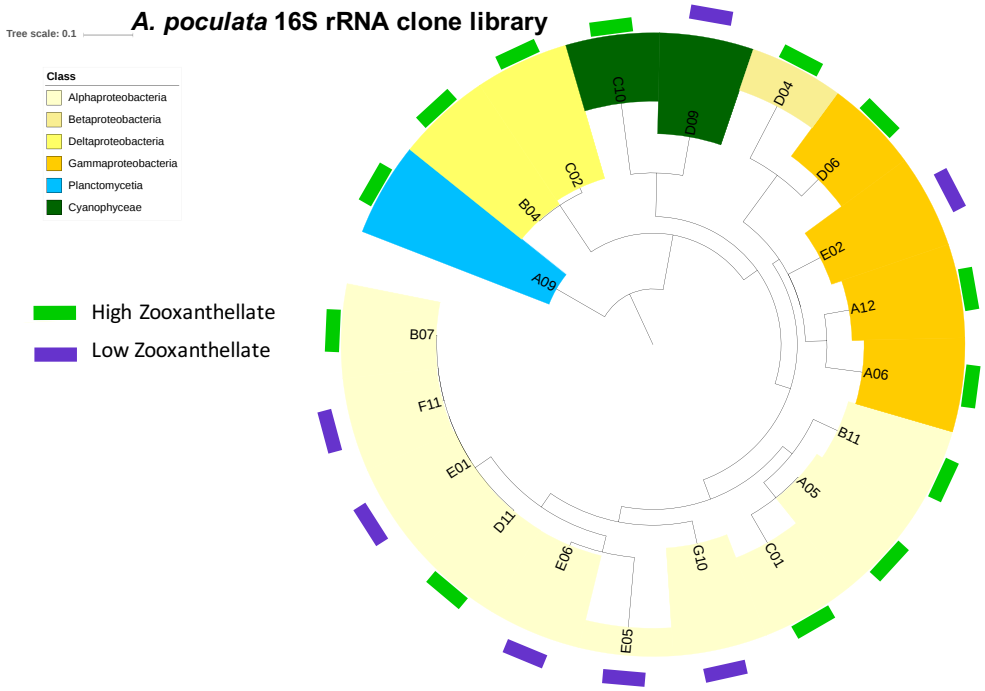

Figure 1. Phyogentic tree of 16S rRNA clone libraries. Classes are represented in colors on the inner circle. The outer circle color corresponds to sample origin (LZ or HZ *Astrangia poculata*), with high zooxanthellate (HZ) sequences in green and low zooxanthellate (LZ) sequences in purple.

From clone library samples, 24% of the HZ clones and 16% of the LZ clones were successfully classified (Table 2). Betaproteobacteria, Deltaproteobacteria, and Planctomycetia classes were unique to the HZ sample (Figure 1). Results from NCBI BLAST display a wide range of metabolic potential. Families of two close matches (Aphanothecaceae and Rhodobiaceae) are known nitrogen fixers, and some Synechococcaceae fix nitrogen. Synechococcaceae possess *chlorophyll a*, as well as a light harvesting antennae (Phycobilisome), which is composed of highly pigmented protein complexes. Pigments associated with phycobilisomes can absorb (A) and emit (E) a wide range of wavelengths: 488-561nm_A-578nm_E (phycoerythrin), 580nm_A-630nm_E (phycocyanin), 594nm_A-660nm_E (allophycocyanin) (Six et al. 2007). Their ability to change relative pigment abundances allows them to rapidly adapt to changing light regimes (Six et al. 2007).

In addition to photosynthetic nitrogen fixers, clone library results also suggest the presence of sulfate reducing bacteria (Desulfovibrionaceae) and sulfur oxidizing bacteria (Chromatiaceae) (Table 2). These bacteria could be associated with coral sulfur cycling. Dimethlysulfonionpropionate (DMSP, $C_5H_{10}O_2S$), an organic sulfur compound, is produced by *Symbiodinium* (zooxanthellae) in coral tissue (Raina et al. 2009; Rania et al. 2010). DMSP is catabolized by bacteria via two pathways: cleavage into DMS (CH_3SCH_3) and demethylation into methanethiol (CH_3SH). Desulfovibrionaceae possess DMSP lyase genes and are known DMSP degraders (Raina et al. 2009). Moreover, methanethiol is a source of reduced sulfur and can be rapidly consumed by sulfur oxidizing microbes (Reisch et al. 2011), such as Chromatiaceae. Nevertheless, the identification of these bacteria is only based on closest matches from NCBI BLAST results, and further study would be necessary to prove both their presence and metabolic role in the *A. poculata* holobiont.

Lastly, clone library data also indicate that anaerobic bacteria might found within *A. poculata.* The family Rhodospirillaceae (Table 2) is comprised of purple non-sulfur bacteria. These bacteria are phototrophic, often capable of nitrogen fixation, and consume simple organic compounds anaerobically; however, they may also grow aerobically. Rhodospirillaceae have been found in relatively low abundances in a wide range of corals (Bourne et al. 2014; Ng et al. 2015; Ziegler et al. 2016). They are often considered opportunistic pathogens and are associated with coral disease (Ng et al. 2015). Rhodospirillaceae have also been found in higher abundances in corals living in environments of high temperature variability (Ziegler et al. 2016). While their exact role and location in the coral holobiont remain unknown, it is possible that they are ubiquitous low-abundance residents of anaerobic coral microniches, whose abundance and potential virulence is increases with temperature.

FISH, CARD-FISH & Imaging

FISH using the eubacteria probe (EUB338) reveals a high degree of autofluorescence in LZ coral tissue (Figure 2). Potential coccoid, autofluorescenct bacterial aggregates were visualized, along with symbiotic dinoflagellates and large filamentous structures. Filaments emitted in orange (eubacteria) and green (autofluorescence), and faintly in blue (DAPI) channels. These filaments were ubiquitous on a DAPI stained filter of coral homogenate (Figure 3), and the staining pattern suggests that filaments are comprised of a chain of cells. However, due to bright

fluorescence of blue fluorophores in the coral host tissue, the signal from these filaments is often overwhelmed.

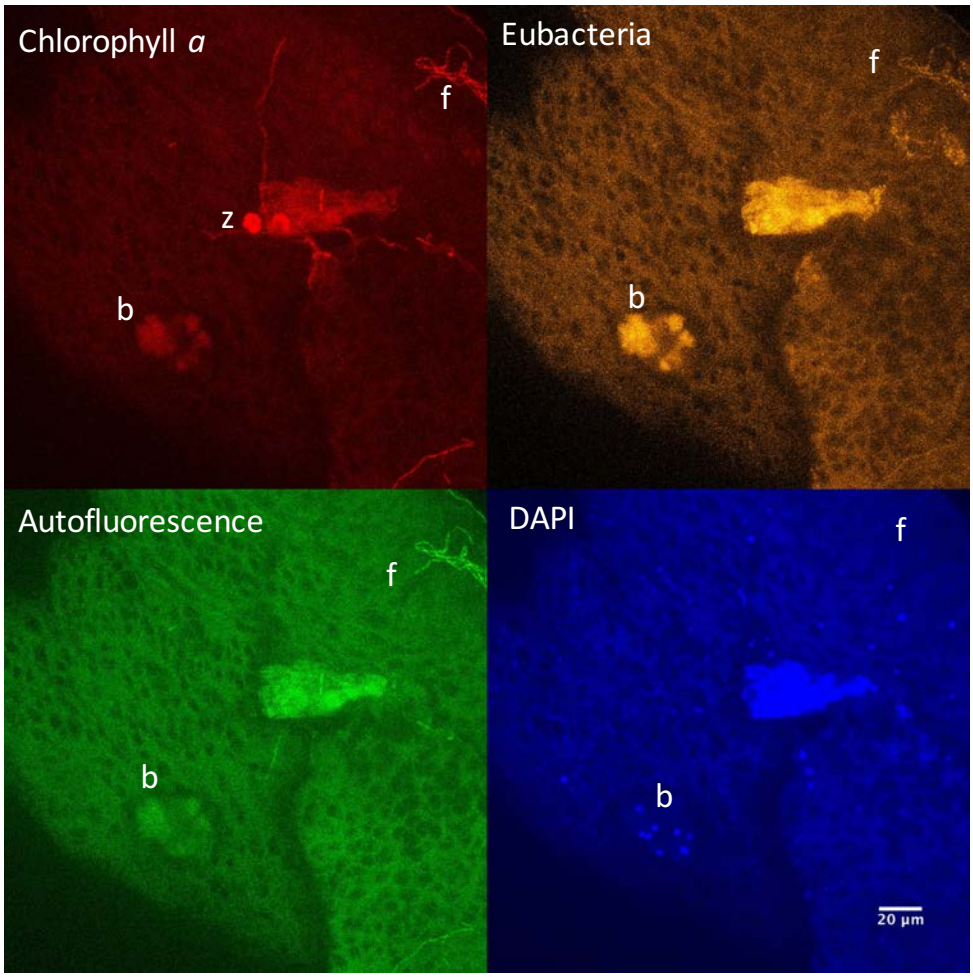

Figure 3. DAPI staining of *Astrangia poculata* homogenate on a polycarbonate filter; (f) filament

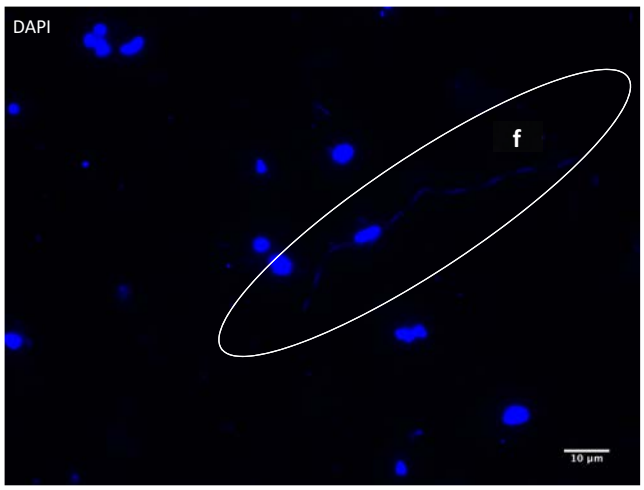

Figure 2. FISH of *Astrangia poculata* with eubacteria probes (orange) and DAPI staining (blue). (z) zooxanthellate (b) potential bacterial aggregate (f) filaments.

Similar to FISH eubacteria probes, all specific probes (Alphaproteobacteria, Deltaproteobacteria, Gammaproteobacteria, Cyanobacteria) showed signs of autofluorescence, as well as some successful and specific binding to bacterial cells (Figures 4-7). Large filaments were observed in all samples, fluorescencing in red, blue, orange, and green.

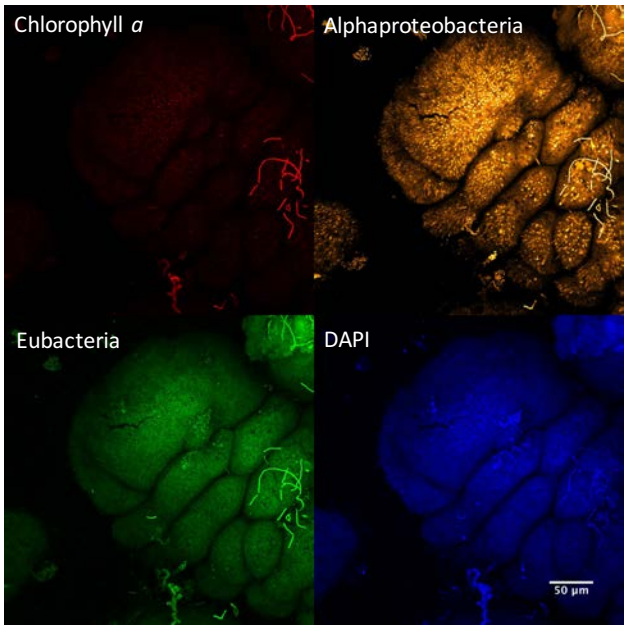

Figure 4. FISH of *Astrangia poculata* with alphaproteobacteria probes (orange), eubacteria probe (green), and DAPI staining (blue).

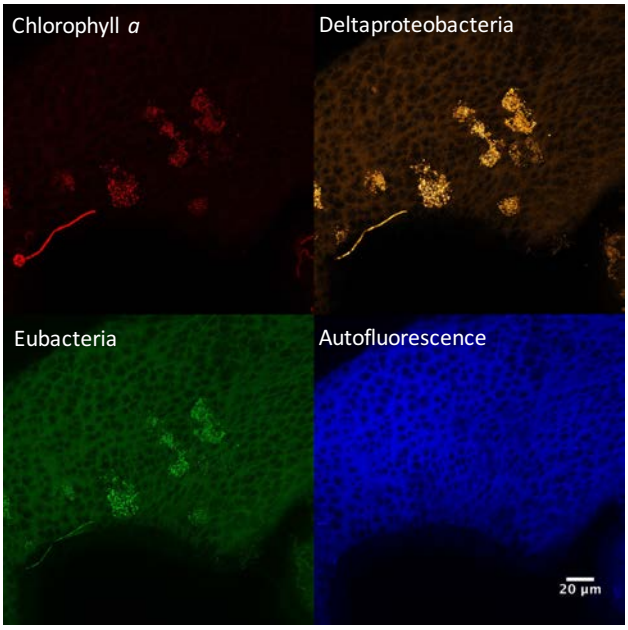

Figure 5. FISH of *Astrangia poculata* with deltaproteobacteria probes (orange) and eubacteria probe (green).

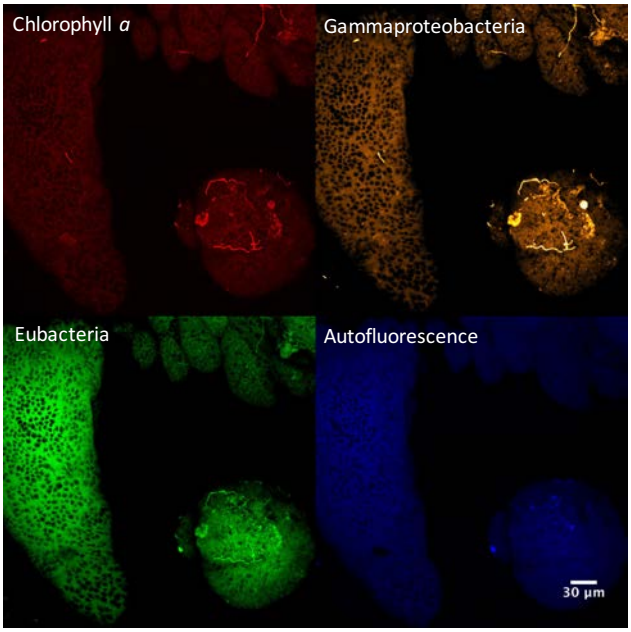

Figure 6. FISH of *Astrangia poculata* with gammaproteobacteria probes (orange) and eubacteria probe (green).

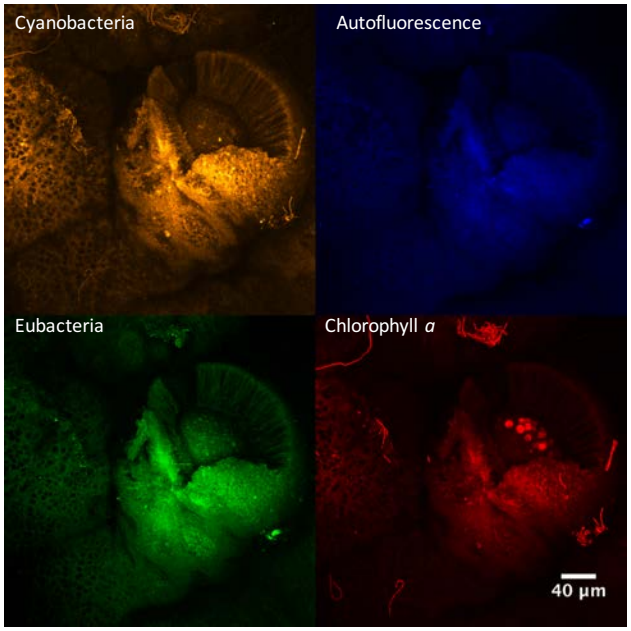

Figure 7. FISH of *Astrangia poculata* with cyanobacteria probes (orange) and eubacteria probe (green).

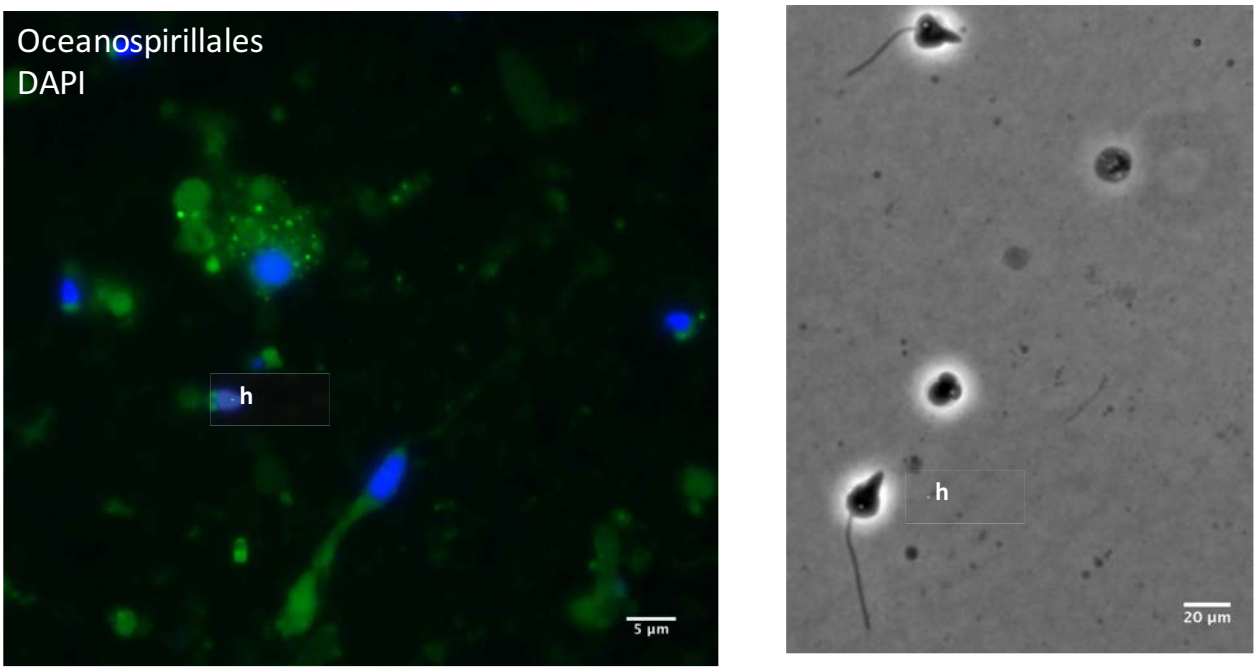

Figure 8. a) CARD-FISH of *Astrangia poculata* homogenate on a polycarbonate filter with Oceanospirillales probes (green) and DAPI staining (blue). **b)** Host cells (h) visualized under phase contrast microscopes are also see in the CARD-FISH sample.

CARD-FISH using the Oceanospirillales probe was inconclusive due to high autofluorescence (Figure 8a). However, flagellated cells viewed under phase contrast microscopy (Figure 8b) were also found DAPI stained on CARD-FISH filters, and appear to have either bacterial symbionts or a storage granule that fluoresces in green.

The large filamentous cells found on *A. poculata* surfaces in all FISH samples are likely cyanobacteria, which were also observed in sequencing data (Table 2). As discussed above, cyanobacteria possess phycobilipigments, which absorb light in the green-orange portion of the visible spectrum (Figure 9). Cyanobacteria are also rich in carotenoids, and some species contain

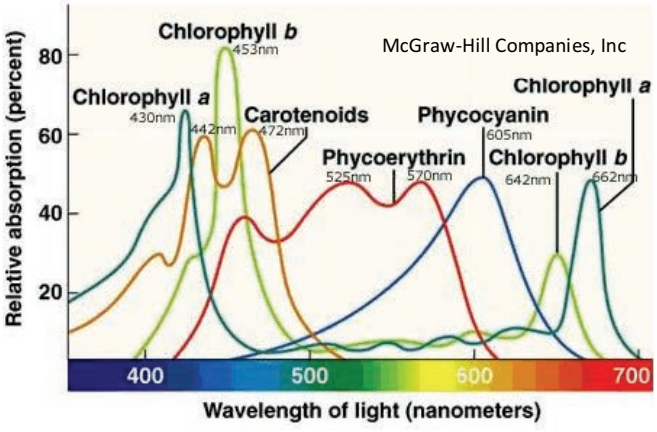

Figure 9. Absorption spectra of cyanobacteria pigments.

chlorophyll *b*. Thus, given the abundance of autofluorescent filamentous cells, it is possible that cyanobacteria are associated with *A. poculata* mucus or surface tissue.

Potential aggregates of unicellular cyanobacteria were also observed (Figures 2, 4, 5). Cyanobacteria have varying roles in the coral holobiont. For example, nitrogen fixing cyanobacteria are considered endosymbionts in the Caribbean coral (*Montastraea cavernosa*) (Lesser et al. 2004), and coral mucus has been shown to contain densities of *Synechococcus,* over two orders of magnitude greater than in the overlaying water column (Naumann et al. 2009). However, black band disease, a tissue degradation disuse of coral, is associated with a microbial consortium of cyanobacteria, sulfide-oxidizing and sulfate-reducing bacteria (Gantar et al. 2011). To test the hypothesis that cyanobacteria are associated with *A. poculata* and to determine their metabolic role, replicate samples should be analyzed, along with additional molecular work to further characterize the cyanobacterial community function.

Previous studies combining FISH and molecular tools to characterize and localize coral-associated microbes have found dense microbial aggregates (Ainsworth et al. 2006; Neave et al. 2016). Using CARD-FISH, *Endozoicomonas* (Gammaproteobacteria) aggregates over 20µm in diameter were observed in gastrodermal tissues of *P. verrucosa* and within tentacles of *S. pistillata* (Neave et al. 2016). *Endozoicomonas* is often found in association with coral and may play a role in the nitrogen cycle (nitrate reduction), as well as scavenging of unwanted components from *Symbiodinium* cell walls (Neave et al. 2016). Ainsworth et al. (2015) found *Actinobacter* co-localized within zooxanthellae, and *Ralstonia* (Betaproteobacteria) in host cells adjacent to zooxanthellae (Ainsworth et al 2015). Moreover, uptake and establishment of *Vibrio* in the coral aboral epidermis has also been observed (Lema et al. 2015)

While such conclusive bacteria localization results were not able to be drawn from this study, molecular data suggests that *A. poculata* harbors a diverse array of microbial-associates. Improvements in sample preparation and microscopy techniques may help to resolve issues associated with imaging depth and autofluorescence. Without sectioning the coral sample, imaging depth within the tissue was limited. Future studies should attempt to make thick or thin sections of either coral tissue samples (after dissolution) or of the coral while still attached to its skeleton. Imaging deeper into the tissue will allow different microniches, such as the gastrodermis, to be investigated. Additionally, background interference from autofluorescence can be reduce by soaking samples in 6% H_2O_2 overnight (14-16h) (Fukatsu et al. 2007; Neave et al. 2016). Lastly, linear unmixing algorithms in the Zeiss software can be used during confocal imaging to help process autofluorescence.

CONCLUSIONS

- Betaproteobacteria, Deltaproteobacteria, and Planctomycetia were unique classes of HZ *Astrangia poculata* samples.
- Autofluorescent filamentous and coccoid cells observed are likely cyanobacteria, and may be common associates of *A. poculata* mucus and surface tissue.
- Future work should aim to further characterize the metabolic potential of *A. poculata* associated-microbes through molecular work and imagery. FISH results could be improved by thin or thick sectioning coral, treating the sample to reduce autofluorescence, and using linear unmixing to process results.

ACKNOWLEDGEMENTS

Thank you to all Microbial Diversity 2017 instructors and teaching assistants for an intellectually stimulating summer, and for giving us the freedom to explore and experiment. I would like to particularly thank Viola and Georgia for their assistance with FISH, microscopy, and image processing, as well as Matthew and Scott for sequencing help. Thanks to MBL's Marine Resource Center and Loretta Roberson for *A. poculata* specimens. And lastly, I am very grateful to all funding sources that made it possible for me to attend this program and conduct this research: Selman A. Waksman Endowed Scholarship, Earth Observatory of Singapore, Simons Foundation, NSF, NASA, DOE, and Promega.

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APPENDIX

Table i. Various attempted dissolution solutions. Aside from Cal-ex ii (Fisher), 10% ascorbic acid also dissolved the skeleton rapidly, while preserving tissue.

Anesthetic	Fixative	Dissolution	pH
-	2% Formaldehyde (1 hour)	1uM EDTA, acetic acid (pH buffer)	7
-	2% Formaldehyde (1 hour)	3M Sodium Acetate, 0.05M EDTA, acetic acid (pH buffer)	6
-	2% Formaldehyde (1 hour)	3M Sodium Acetate, 0.05M EDTA, acetic acid (pH buffer)	5.5
-	2% Formaldehyde (1 hour)	1.5M Sodium Acetate, 0.5M EDTA, acetic acid (pH buffer)	6
4% $MgCl_2$ (1 hour)	-	10% Ascorbic Acid, 4% Formaldehyde	3
4% $MgCl_2$ (3 hours)	4% Formaldehyde	10% Ascorbic Acid	3
-	-	2.1M Formic Acid 6.8% Formaldehyde 1.2% Methanol	3.5, 5
4% $MgCl_2$ (1 hour)	5% w/v $ZnCl_2$	3M Sodium Acetate, acetic acid (pH buffer)	3
4% $MgCl_2$ (1 hour)	2% agarose embedding		
-	-	Cal-ex ii (Fisher)	2

Table ii. Sequences, label, and formamide (FA) percentage of fluorescent probes used in FISH and CARD-FISH.

Probe Name	Specificity	Sequence 5'-3'	Label	FA %
EUB338	most Bacteria	GCTGCCTCCCGTAGGAGT	Cy3	35
EUB338	most Bacteria	GCTGCCTCCCGTAGGAGT	FITC	35
CYA664	Cyanobacteria	GGAATTCCCTCTGCCCC	Cy3	0
DELTA495a	Deltaproteobacteria	AGTTAGCCGGTGCTTCCT	Cy3	30
DELTA495b	Deltaproteobacteria	AGTTAGCCGGCGCTTCCT	Cy3	30
DELTA495c	Deltaproteobacteria	AATTAGCCGGTGCTTCCT	Cy3	30
GAM42a	Gammaproteobacteria	GCCTTCCCACATCGTTT	Cy3	35
BET42a_unlab	Betaproteobacteria (used as competitor together with Gam42a probe)	GCCTTCCCACTTCGTTT	no label	35
OCE232	Oceanospirillum	AGC TAA TCT CAC GCA GGC	HRP	40