INTRODUCTION

Coral-associated microbial communities, also known as coral holobiont, consisting of bacteria, archaea, fungi, viruses, and protists, have been shown to be functionally highly diverse (Ainsworth et al., 2010; Thompson et al., 2014). The endosymbiotic dinoflagellates were known to provide most of the carbon source for the coral host (Muscatine et al. 1989); photosynthetic cyanobacteria are shown to be responsible for nitrogen fixation (Lesser et al., 2004), anaerobic nitrogen-fixing bacteria have also been isolated from coral (Chimetto et al., 2008). However, the roles played by many other microbes are still unknown. Studying the diversity and function of these microbes is not only crucial for a better understanding of coral physiology, but also important for our understanding of host-microbe interaction in general.

The northern star corals, the only coral species which can be found around Woods Hole, Astrangia poculata, were readily available at the MBL Marine Resource Center. No studies were reported about the microbial associates of A. poculata until last year, a student in this course developed a mini-project investigating the bacterial diversity (Moynihan, 2017). She isolated several aerobic chemoorganoheterotrophs and constructed a 16S rRNA gene library. Some interesting findings were revealed and discussed in her report. Among them, I am interested in the discovery of coral-associated sulfate-reducing bacteria (SRB) belonging to the family Desulfovibrioaceae.

SRBs have been implicated in the well-known coral polymicrobial black band disease (BBD): a cyanobacteria dominated microbial consortium, characterized as a thick black band with sulfidic anaerobic environment where SRB thrives, migrates across the living coral colonies and lyse the host cells (Richardson, 2004). However, the virulence factors of BBD are not identified yet since cyanobacteria and SRBs are also found in healthy corals (Sato et al, 2016). To my best knowledge, only SRB from BBD infected corals have been enriched. Therefore, one goal for this project is to enrich and isolate the SRBs from living healthy corals. Pure cultures of SRB from coral could provide opportunity to study the complete genome and metabolism of SRB, which can be very useful for future syntrophy study with SB, as well as construction of
community for understanding black band disease virulence factors. Since acetogens and methanogens are common competitors with SRB, enrichment cultures were also set up for comparison.

The second part of this project aims to investigate the archaea community from coral. The approach was mainly culture-independent: 16S rRNA amplicon analysis and in situ fluorescence imaging on coral tissue.

METHODS

Sample Collection

*Astrangia poculata* colonies were originally collected from the Great Harbor near Woods Hole by the Marine Resource Center (MRC), Marine Biological Laboratory. All colonies were incubated at sea water table for a month at MRC, and thoroughly washed with 0.22µm filtered to remove any adventitious microbes. As a control sample, 12.5 m deep sea water was collected from Buzzard’s Bay, and kept at 4 °C for a month before use.

Culture and metabolite analysis

Hydrogenotrophic medium were prepared essentially according to the recipe listed in Chapter 6, student manual with slight modification. Briefly, 1x sea water base was used instead of 1x fresh water base; MOPS buffer (pH 7.2) was used instead of MES buffer (pH 6.15). For inoculation, *Astrangia poculata* colonies were crushed using a mortar and pestle. The crude homogenate was vortexed and filtered through a 40 µm cell strainer. The resulting homogenate was used for inoculation. Cells were passaged after 10 days or based on the appearance of turbidity. For SRB, solid medium was prepared in Balch tubes. Briefly, 6 mL of liquid SRB medium in a Balch tube was inoculated with 2nd passage SRB enrichments under N₂/CO₂ flow, 3% agar in SRB medium (maintained at 55 °C in water bath) was quickly mixed with inoculated SRB medium and sealed with serum stopper. After the tube was crimped, the head space gas was exchanged into H₂/CO₂. Wet-mount was taken to examine the cell morphology and heterogeneity by using epifluorescence scope Imager A1 and A2 (Zeiss). Hydrogen sulfide was assayed by using a colorimetric assay (Kline, 1969). Acetate ion accumulation was monitored by using ion chromatography systems (Thermal-Fisher). Methane production was monitored by using GC-FID (Shimazu).
16S rRNA gene amplicon analysis

Genomic DNA extraction from coral homogenate was aided by using the QIAamp PowerFecal DNA Kit. For control sea water sample, 1 L of sea water was filtered through a 0.22 µm filter and the genomic DNA was extracted from the filter. DNA concentration was measured by using the Qubit 4 fluorometer. 16s amplicon was amplified by PCR reaction. Briefly, 1 µL of gDNA was combined with 1 µL 515F-EMP primer (15 pmol), 1 µL ARCH958R primer (15 pmol), 25 µL GoTaq Hot Start Green Master Mix, and 22 µL of Nuclease-free water. PCR reaction was performed with an initial denaturation at 95 °C for 2 min, and then cycled 25 times with denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1.5 min. A final extension was performed at 72 °C for 10 min. Gel electrophoresis was used to confirm purity and DNA concentration was measured with Qubit 4 fluorometer. 16S amplicon data was analyzed by using Qiime2.

FISH, CARD-FISH IMAGING

Mono-FISH imaging on planktonic enrichment cells was performed according to the protocol in chapter 14, student manual. For coral tissue imaging, coral colonies were fixed and decalcified by immersion in Cal-Ex II (Fisher) for 24 hours. Coral tissue was then dissected under a ZEISS Axio Zoom.V16 stereo microscope. The tissue was dried on poly-L-lysine coated glass slides, and permeabilization was performed with lysozyme and proteinase K. The endogenous peroxidase was inactivated with methanol containing 0.15% H2O2. Hybridization and amplification were performed according to the protocol in chapter 14, student manual. For all the probes, hybridization was performed at 46 °C in the presence of 35% formamide for 4 hrs.

RESULTS & DISCUSSION

Enrichment

Growth of SRB under hydrogenotrophic condition was observed 10 days after first inoculation as indicated by increase of turbidity. After the 2nd passage, hydrogen sulfide production was detected, and cell morphology was documented by phase-contrast light microscope (Figure 1). To confirm the identity of the enriched sulfate reducer, mono-FISH imaging was performed by hybridizing fixed enrichment with different probes. Best result was obtained after hybridization with Cy3-labeled DSR651 probe (5'- CCC CCT CCA GTA CTC AAG -3'). In contrast, probes targeting other class of proteobacteria failed to show any signals (Figure 2.
and 3). Furthermore, comparing the result between DSR651 probe with EUB I-III probes mix suggests that the enrichment is “phylogenetically” pure since all bacteria cells were hybridized with DSR651 probe. Liquid enrichment culture was transferred to solid medium in order to obtain single colonies. However, pure isolates did not grow yet before the course ended.

Figure 1. Enrichment of sulfate reducing bacteria under hydrogenotrophic condition. (left) Serum bottles containing enrichment. (middle) Phase contrast image of the enrichment. (right) Sulfide production was assayed calorimetrically.

Figure 2. Mono-FISH imaging of SRB enrichment with Cy3-DSR651 probe and 6-FAM-EUB I-III. (a) Cy3 channel (b) 6-FAM channel (c) DAPI channel (d) merged all channels
Attempt to enrich methanogenic archaea by using similar hydrogenotrophic condition failed. No methane production was observed by GC (data not shown). It is worthy to note that failure of methanogen enrichment cannot prove the absence of methanogen in corals.

Figure 3. Mono-FISH imaging of SRB enrichment with Cy3-DSR651 probe and FITC-EUB I-III. (a) Cy3 channel (b) FITC channel (c) DAPI channel (d) merge all channels.

Figure 4. Enrichment of hydrogenotrophic acetogen like bacteria. (left) Serum bottle containing enrichment. (middle) Bright-field image of enrichment. (right) DAPI stain.
Interestingly, bacteria cells were enriched under “acetogen” enrichment condition (Figure 4), despite that “homoacetogenesis” is theoretically energetically less favorable compared to methanogenesis under the standard condition. Surprisingly, quantifying the accumulation of acetate by ion chromatography showed that there was no measurable acetate in the culture (Figure 6). Further mono-FISH imaging on the enrichment revealed phylogenetic heterogeneity. As shown in Figure 6, none of the probes studied can hybridize all the cells from the enrichment when the images were overlaid with DAPI stain. Based on the positive result, β-proteobacteria or/and γ-proteobacteria were present in the culture. The ambiguity cannot be resolved due to high sequence similarity between these two probes. To further confirm the identity of these cells, 16S rRNA gene sequencing is necessary. Furthermore, the heterogeneity of enrichment is likely the reason for the absence of acetate in the culture, since acetate may be utilized as energy/carbon source by other nonacetogenic bacteria.

Figure 5. Ion chromatography analysis of acetogen enrichment. An unknown peak was consistently detected with retention time 2.23 min. Note that acetate standard was eluted at 2.06 min.

Figure 6. Mono-FISH imaging of acetogen enrichment (a) Cy3-labeled Bet42a probe + DAPI + cGam42a (b) Cy3-labeled Gam42a probe + DAPI + cBet42a (c) Cy3-labeled EPSY404 probe + DAPI + cBet42a
Analysis of 16S rRNA gene amplicon showed that there is tremendous diversity of coral-associated archaea (Figure 7). On the contrary, no archaea reads were retrieved from sea water sample. Interestingly, more than half archaea population were dominated by Parvarchaeota. This data could be very helpful to hunt for archaea from coral.

Figure 7. Coral archaea 16S rRNA amplicon analysis. The taxonomy was assigned by Qiime 2.

Figure 8. CARD-FISH imaging of coral tissues. a) EUB-HRP probe hybridization followed by Cy3-labeled tyramide. b) DAPI stain. c) tissue autofluorescence (Ex = 470 ± 20 nm, Em = 525 ± 25 nm).

Directed imaging of archaea from coral is not successful. First of all, coral tissue autofluorescence makes it difficult to image any nonautofluorescent bacteria and archaea. Secondly, even with the help from CARD-FISH technique, it is still extremely difficult to image microbes that are not abundant in coral tissues, such as archaea. As shown in Figure 8, after painstakingly trials, bacteria were successfully imaged by using EUB-HRP probe followed by Cy3-labeled tyramide. Notably, the coral tissues cells also show increased fluorescence signal,
probably due to endogenous peroxidase activity. For future optimization, the current “inactivation condition”, MeOH + 0.15% H₂O₂, should not be used.

Conclusion

The culture-dependent and culture-independent approaches reported here revealed great microbial diversity from coral. More importantly, it was a vivid demonstration that many kinds of microbes from different environmental niches can be isolated from coral. Future work should aim to isolate more unique microbes and reconstruct the coral holobiont.

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