Bacteria along for the ride: Exploring microbial interactions from copepods to *Anabaena*

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INTRODUCTION

In aquatic ecosystems there has been growing recognition of the abundance and ecological significance of bacteria that associate with living and/or non-living organic particles. Dynamic interactions with living prokaryotic and eukaryotic hosts can provide bacterial associates with numerous benefits including a steady supply of nutrients (e.g. Muller and Overmann, 2011; Carman, 1994; Moller, 2005) and protection from environmental stresses (Tang et al., 2010) leading to increased growth rates (Tang, 2005) and production (Griffith, 1990) relative to those 'free-living' bacteria in the surrounding seawater. Positive benefits of these associations for the host/partner can include chemical protection against biofouling (Gil-Turnes et al., 1989), expansion of utilizable substrates via syntrophy (Muller et al., 2008), and an increase in growth or metabolic activity (Lupton and Marshall, 1981).

Copepods, which are extremely abundant planktonic crustaceans, represent unique microenvironments that enrich for bacterial communities that would not otherwise persist in the marine environment (i.e. strict anaerobic bacteria inhabiting the guts of zooplankton) (Braun et al., 1999; Proctor, 1997) and permit widespread dispersal of bacteria via the zooplankton's diurnal vertical migrations (Grossart et al., 2010). A few previous studies have done general surveys of the bacterial communities found on various species of copepods (reviewed by Carman and Dobbs, 1997), but hardly any have attempted high-throughput sequencing of the bacterial communities on copepods to better capture a deeper understanding of the microbial-host interactions in this system. My project aims to answer the question of "who's there?" in copepod microbial communities by taking several approaches, including 1) enrichments 2) 454 sequencing 3) clone libraries and 4) FISH on copepod sections. This project will also explore the copepod microbial community in an ecological context by seeing how these communities change by water depth and after copepods have cleared their gut.

Another part of my mini-project will probe the symbiosis of longstanding interest to the MBL Microbial Diversity course – the *Anabaena*-epibiont interaction. *Hoeflea anabaena* sp. nov was isolated from School Street Marsh in 2000 and was found to specifically associate with the heterocysts (nitrogen-fixing cells) of the cyanobacteria *Anabaena.* This association appears to increase the nitrogenase activity of the *Anabaena* (Laura Meredith, Microbial Diversity reports 2010), while the epibionts appear to benefit by uptaking carbon and potentially nitrogen from the *Anabaena* (Behrens et al., 2008). It is not yet known how the epibiont increases the nitrogenase activity of the *Anabaena,* although it is hypothesized that they may be decreasing the oxygen

concentration (oxygen poisons nitrogenase) or the hydrogen concentration (hydrogen is produced when nitrogen is fixed) around the heterocyst. It is also not known what carbon substrates the *Anabaena* is providing to the epibiont. With the genome of the epibiont recently sequenced, my project sought to make use of this available information about the completeness of metabolic pathways complemented with growth experiments to explore what motivates the association of the epibiont with *Anabaena* and what potential carbon sources might be exchanged between the host and epibiont.

METHODS

Copepod sampling: The *Calanus finmarchicus* specimens used for the molecular analyses (454 sequencing and clone libraries) were collected in 2010 off of Georges Bank, Cape Cod Bay on the NOAA vessel the 'Delaware II'. All samples were collected with a plankton tow, with "shallow" samples collected from the surface and the "deep" samples collected around 100 m. A subset of the shallow samples were incubated in cold, dark buckets filled with freshly filter-sterilized (0.2 µm) seawater for 3 hours to allow them to clear their guts of recently ingest phytoplankton. Copepod samples were concentrated with a 500 µm sieve and rinsed with filter-sterilized (0.2 µm) seawater before preserving bulk samples (~15 individuals/tube) in 1.5 mL of RNAlater (Qiagen) at -20 °C.

Fresh copepod samples were collected by the Marine Resources Center, MBL off the coast of Woods Hole for the culture experiments. (I attempted sampling for copepods in local Salt Pond, Falmouth but the ctenophores were voraciously consuming them so it became necessary to attempt collection further from shore where the ctenophores were not as abundant). Copepods were sorted by pre-filtering the water collected from the plankton tow with a 500 µm sieve placed above a 300 µm sieve. From the 300 µm fraction, copepods were sorted to the taxonomic order *Calanoida* (Figure 1), rinsed with sterile seawater, and placed into 1.5 mL microcentrifuge tubes.

Copepod Enrichments: Approximately 15 copepods were homogenized with a sterile plastic dart and placed into replicate bottles with 10 mL chitin liquid media (10 mM NH_4Cl, 1 mM Na_2SO_4, 1 mM KH_2PO_4/K_2HPO_4, 1 mL trace mineral solution, 4.0 g unbleached chitin in 500 mL of autoclaved seawater adjusted to pH 8.0 ± 0.2 with 10 M NaOH) or sea water complete liquid media (250 ml distilloed water, 5 g Bacto tryptone, 1 g yeast extract, 3 ml glycerol in 750 ml of seawater adjusted to pH 7). After 4 d incubation at 30 °C on a shaker, aliquots of the liquid media were spread on the solid media. To enrich for methanogens living in the copepod gut, ~50 copepods were homogenized in the anaerobic chamber and placed in 5 mL of basal anaerobic modular medium supplemented with 5 mM acetate (see Lab Manual for media recipe) and one of the following 1) trimethylamine (1 M); 2) formate (1 M); or 3) H_2/CO_2 (7 psi) in the headspace. All treatments were incubated in anaerobic containers at 25°C anaerobically.

To isolate aerobic chitin-degraders, plates comprised of autoclaved seawater agar (1 L freshly collected seawater plus 15 g agar) with a chitin overlay (4.0 g unbleached chitin, 7 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H20$, 0.3 g KH_2PO_4 and 1.0 mL trace mineral solution per 500 mL MilliQ water adjusted to pH 8.0 ± 0.2). After the chitin overlay was autoclaved and cooled to approximately 50°C, it was poured in a thin layer over the hardened basal media and allowed to harden overnight to allow a gradient to form between the basal media and the chitin overlay. Organisms enriched in the SWC liquid media were plated on SWC plates (SWC liquid media + 30 g of agar). The solid media were inoculated with 100 µl and 10 µl of their respective liquid media enrichments. The Sea Water Complete plates were incubated at room temperature

overnight and two colonies of different morphology were re-streaked for isolation on new SWC plates. The chitin plates were incubated at 30°C to attempt to speed up colony development.
Colony PCR: To characterize colonies isolated from the copepod enrichments (chitin and sea water complete media), colony PCR was performed using bacterial 16S primers 8F (5'- AGAGTTTGATCCTGGCTCAG-3') and 1492 R (5'-GGTTACCTTGTTACGACTT-3') at 0.4 µM. PCR reactions included 12.5 µl of Promega Master Mix 2x, 1 uL of 8F, 1 µl of 1492R, and 9.5 µl of nuclease-free water). The PCR reaction was performed with a 5 min "boil" at 95°C followed by 95°C for 2 minutes, 30 cycles of 95°C for 30 seconds, 46°C for 30 seconds, 72°C for 1 minute 30 seconds and a final 72°C elongation for 15 minutes. The PCR products were visualized on a 1% agarose gel with ethidium bromide and purified with a MinElute purification kit.
Community analysis: DNA was extracted from three *C. finmarchicus* sub-samples taken from the deep, shallow, and shallow with gut cleared treatments to survey diversity via 454 sequencing and clone libraries. DNA was extracted using the MoBio Power Soil kit following the experienced user protocol with a few slight modifications: 1) copepods were either homogenized within the 1.5 mL tubes using an autoclaved plastic dart and transferred to the PowerBead tubes (shallow sample), added as whole organisms, or crushed with the plastic darts in the cap of the PowerBead tube to decrease the loss of sample due to tube transfer; 2) instead of vortexing the PowerBead tubes containing the sample and 60 µl of Solution C1, the tubes were bead-beaten for 1 minute; 3) 50 µl of Solution C6 was added instead of 100 µl in order to concentrate the sample. The extracted DNA was NanoDropped to determine the approximate concentrations and a test PCR was run with 8f and 1492 primers and visualized on a 1% gel to see if any bacterial sequences could be amplified from the samples detected.

Metagenomic sequencing by 454 Life Sciences technology was performed using 20 µl of template DNA from each extracted *C. finmarchicus* sample. SSU rRNA genes were amplified with barcoded primers that also incorporated the Roche 454 Ti adapter sequences (see below). The 9 nucleotide barcode was on the forward primer. The primer targets are 515F and 907R on the *E. Coli* 16S gene. Forward primer (X denotes barcode, lowercase is the linker between barcode and 16S primer),
5'-CGTATCGCCTCCCTCGCGCCATCAGXXXXXXXXgaGTGYCAGCMGCCGCGGTAA-3'
Reverse primer (lowercase denotes linker between adapter and 16S primer),
5'-CTATGCGCCTTGCCAGCCCGCTCAGggCCGYCAATTCMTTTRAGTTT-3'

The PCR program utilized a touchdown annealing temp for the first 10 cycles from 68-58C. Then there were 12 cycles of three-step PCR (denaturation, annealing, elongation) followed y 10-14 cycles of tow-step PCR (annealing and elongation at same temp). Chuck used Phusion HF polymerase (2X mastermix) to amplify the gene with 8% DMSO and 0.5 uM primers in the final reaction volume. Chuck normalized our template to 15 ng/uL (for DNA above 15 ng/uL) and used 2 uL of each template for PCR. Post amplification Chuck quantified our DNA using the PicoGreen assay and then pooled ~125 ng of each PCR product. That pool was concentrated down to 100 uL using the vacufuge and gel purifed using the Montage Kit (Millipore). The gel-purified pool was then shipped to Penn St. for sequencing (The above protocol for the 454 sequencing was graciously written by Chuck). The 454 amplicon data was analysed (library splitting, clustering at a 97% cutoff, defining OTUs, and assigning taxonomy with RDP) using Qiime on quality sequences between 400 and 600 bp in length. Rarefaction curves were generated using Qiime at a 97% OTU cutoff.

Clone libraries on the three *C. finmarchicus* samples were also performed to investigate the diversity of the bacterial communities associated with the different copepods. Bacterial 16S sequences were amplified using the universal bacterial primers 8F and 1492R (PCR reactions included 12.5 µl of Promega Master Mix 2x, 1 uL of 10 µm 8F, 1 µl of 1492R, 1 µl DNA, and 9.5 µl of nuclease-free water). The PCR reaction was performed at 95°C for 2 minutes followed by 30 cycles of 95°C for 30 seconds, 46°C for 30 seconds, 72°C for 1 minute 30 seconds and a final 72°C elongation for 15 minutes. PCR products were gel purified, cloned using the TOP10 electrocompetent "One Shot Cells", and plated on LB/AMP plates for 12-15 hours at 37 °C. For each clone library 96 colonies were picked and prepared for the capillary sequencer at the Josephine Bay Paul Center (Applied Biosystems model 3730).

The best matches to each sequence were determined by using "Seqmatch" on RDP. Sequences were aligned using MUSCLE, were formatted into a tree using Fasttree in Qiime, and exported to iTOL to visualize the clustering patterns of the relative environmental samples. RDP classifier was used to compare the relative proportion of the taxonomic phyla and orders represented between the three environmental samples with 95% confidence.

Fixation, embedding, and slicing of individual copepods: Copepods were placed in sterile SW in small Petri dishes (60 mm diameter) and were washed several times with sterile filtrated sea water (water was pipetted in and out of the petri dish with a sterile Pasteur pipette). The water was removed and the copepods were washed with sterile 1x PBS for 10 min on a slowly moving oscillator. After careful removal of the PBS with a Pasteur pipette, the copepods were fixed (4% PFA, 0.5 % glutaraldehyde in 1x PBS) in parafilm sealed Petri dishes for 1 hour at room temperature. The fixative was removed and the copepods were washed three times with 1x PBS for 10 min on a slow moving oscillator. The copepods were then incubated in a 30% sucrose-1x PBS solution for 1.5 hours, then a 15% sucrose solution for 30 minutes on a slowly moving oscillator. The sucrose was carefully removed and OCT (Optical Cutting Solution, Tissue-Tek, Sakura) was added to the Petri dish so that the copepods were completely covered. The petri dishes with the copepods were incubated overnight on a slowly moving oscillator at room temperature. The copepods were moved into cryo-freezing molds and snap-frozen in liquid nitrogen. The molds were directly moved to -80°C for an hour and then -20°C for an hour before being transferred to a cryotome for slicing. (This procedure is a modification of Verena's berry FISH procedure, with a slight modification in the time and concentration of the sucrose incubation by Leah's suggestion).

During my first attempt at CARD-FISH on copepod sections, the samples were lost during the inactivation step (methanol plus hydrogen peroxide). This may have been due to the thickness of the sections (most were 20-35 µm) and/or the fact that I used glass microscope slides as opposed to Polysine® slides which seem to work better with finicky tissue sections. In my second attempt at CARD-FISH, I re-sectioned my copepod samples to attain thinner slices and modified the lab manual protocol by 1) using 0.4% low-melt agarose for the initial embedding and 2) instead of moving slides in and out of Falcon tubes during the MilliQ and Ethanol wash steps, I drizzled the liquids on the surface of the slides to prevent the sections from slipping off.

CARD-FISH: After embedding the copepod sections on the slide using 0.4% low gelling point agarose, I permeabilized the sections with lysozyme buffer (0.1 g lysozyme, 1 mL EDTA, 1 mL Tris in 5 mL MiliQ) soaking the top of the slide and incubated for 60 min at 37 °C. The slides were washed in MilliQ water and then the endogeneous peroxidases were inactivated by incubating the slides in a solution of 5 mL methanol and 25 ul of 30% hydrogen peroxide for 30

minutes under the hood. The slides were individually placed into a Falcon tube hybridization chamber (1/2 a Kimwipe folded and placed on the side of a Falcon tube and drizzled with 500 ul of 35% formamide-MilliQ solution) and incubated with 30-50 ul of hybridization buffer on each copepod section for 2 h 45 minutes at 46 °C. The tops of the Falcon tubes were wrapped with Parafilm and placed in a Styrofoam holder to prevent the Falcon tubes from moving around. After the incubation the slides were washed well in MilliQ and 96% ethanol 2x.

The slides were then left in Falcon tubes filled with pre-warmed washing buffer (0.5 mL EDTA, 1mL Tris, 700 ul of 5M NaCl in 50 mL MilliQ with 25 ul SDS) at 48°C for 10 minutes. The slides were then transferred to 1x PBS for 10 minutes at room temperature. For each color dye, 1000 ul of sterile-filtrated amplification buffer, 10 ul of diluted hydrogen peroxide (0.15%), and 1 ul of the Alexa dye (594- red; 488- green). The tubes were vortexed, pippetted onto the slides, and incubated at 46°C for 30 minutes in the dark. The slides were dried at room temperature in the dark, and a drop of the DAPI-Citifluor/Vectashield mounting medium (1 ug/ml) was added to each section before a coverslip was added and the slides were stored at -20°C until viewed under the Zeiss fluorescent microscope.

***Hoeflea anabaenae* growth experiments:** The genome sequence of the *Hoeflea* epibiont was submitted for flux balance analysis (FBA) on the RAST server. FBA is a mathematical method used to analyze metabolism of organisms by considering the completeness of metabolic networks and predicting what substrates are likely transported into the cell versus those that can be synthesized de novo or from the degradation of other substrates. In choosing substrates for my epibiont growth experiments, I was interested in exploring potential substrates that the FBA suggested needed to be transported into the cell (ribose and potentially maltose) as well as some substrates that have been previously been found to be important plant products (glycolic acid) and substrates exchanged in prokaryotic symbioses (branched chain fatty acids). I was also interested in testing the hypothesis that the terminal residues (xylose, galactose) and the backbone (mannose, glucosyl) of polysaccharides unique to *Anabaena* heterocysts compared to those of neighboring vegetative cells may be an important cue or stimulus for growth of the epibionts, given that epibionts specifically attach to heterocysts even when heterocysts are detached from the vegetative cells.

For the growth experiments a concentrated stock of epibionts provided by the Brad Stevenson lab at University of Oklahoma were grown at 1:5 dilution with PY media (3.0 g peptone, 0.5 g yeast extract, 15 g sea salts in 1 L MilliQ) for 4 days at 30 °C on a shaker. Eight different carbon sources were tested at a concentration of 1 g $l^{-1}$to see if they enhanced growth of the epibiont: D-(+) mannose (U.S. Biochemical Corp., Cleveland, OH), D-(+) xylose (U.S. Biochemical Corp., Cleveland, OH), D-(+) galactose (Sigma-Aldrich, St. Louis, MO), Glycolic acid (Fluka/Sigma-Aldrich, Germany), L-leucine (Sigma-Aldrich, St. Louis, MO), L-isoleucine (Sigma-Aldrich, St. Louis, MO), D-(+) maltose (Sigma-Aldrich, St. Louis, MO), and D-(-) ribose (U.S. Biochemical Corp., Cleveland, OH). In 5 mL of media, 100 µl of epibiont culture were inoculated into small glass test tubes. After two days the tubes were reinnoculated with 200 µl of epibiont culture to attempt to speed up the growth of the epibiont cultures. Growth in the tubes was also monitored via microscopy to spot-check that growth was due to that of epibiont cultures rather than that of contaminants. Carbon substrates were considered to support growth if they resulted in a significant increase in optical density at 600 nm (Thermospectronic machine) relative to those cultures grown on minimal sea-water media [20 l^{-1} and 0.1 g l^{-1} yeast extract for required growth factors (Stevenson et al., 2010)] alone.

RESULTS and DISCUSSION:

Enrichments: The SWC and chitin enrichments quickly became turbid and after 4 days were re-inoculated into new liquid media and also plated. There was no apparent growth on the chitin plates after 1.5 weeks, but microscopy images from the liquid culture revealed a mixture of highly motile coccoid and rod-shaped bacteria. It was also observed that many rod-shaped bacteria were physically attached to chitin particles while their tail ends were furiously wriggling (Figure 2). The SWC plates grew overnight and two colonies were streaked for isolation and prepared for colony PCR for classification. The methanogen enrichments had not become turbid or yet produced any detectable methane within the two weeks of the project, which isn't all together surprising given that in previous papers (Marty, 1993) it took 30 d at 25 °C for methanogens to be detected from their 50 homogenized copepods.

Colony PCR: The colony PCR for the two isolates obtained from the SWC-copepod enrichment did not yield good sequence information despite the observation of very strong bands. The sequences were both less than 200 bp reads and did not give informative hits from the blastn database.

454 Sequencing: Of the three samples, only the deep sample had sufficient quality reads that were further analyzed. From the approximately 100 OTU counts in the deep sample, 79 were classified as *Deferribacterales*, genus *Caldithrix*. *Gammaproteobacteria* (*Halomonas*) and *Flavobacteria* (*Tenacibaculum*) were the next most abundant OTUs although they each represented only about 5% of the OTU counts (Table 1). Therefore the rank abundance curve of the deep sample (Figure 3A) expresses that there are a few highly abundant OTUs with a number of low abundance organisms. The rarefaction curve at a 97% OTU cutoff (Figure 3B) approaches saturation suggesting that the diversity has been highly sampled for these deep copepods.

Clone libraries: DNA extraction from the three *C. finmarchicus* samples gave yields of 76.7 ng/ul (shallow), 40.9 mg/ul (shallow with gut cleared) and 95.1 ng/ul (deep), suggesting that the MoBio PowerSoil kit works best on homogenized copepods. A preliminary PCR reaction with 8F and 1492R 16S bacterial primers gave a strong, specific product for all three samples, so I proceeded to make clone libraries for these three groups. The "shallow" and "shallow with cleared gut" transformations were problematic with the minimal and infrequent growth of colonies on the LB/AMP plates. I and others in the class found that the colonies seemed to grow better when 2 µl vs. 4 µl of gel purified PCR products were added to the TOPO vector (final volume of 6 µl with 2 µl of water, 1 µl of salt, and 1 µl of TOPO). Also, sometimes cells yielded more colonies when re-plated after a night in the fridge.

After eliminating sequences that were under 400 bp or hit eukaryotic sequences in blastn, approximately 85 sequences from each clone library were further analyzed. A tree of all three clone libraries generated in iTOL demonstrated that the sequences present do not seem to cluster by environmental sample, but rather all samples are relatively interspersed throughout the tree (Fig. 4). This suggests that the represented sequences are not unique to any particular environmental sample. The one small branch that is a cluster of the deep sample represents "unclassified *Gammaproteobacteria"*.

The shallow and shallow with cleared gut samples demonstrated a higher phyla-level diversity than the deep samples, with *Planctomycetes*, *Proteobacteria*, *Bacteroidetes*, *Verrumicrobia* (shallow with cleared gut only), *Firmicutes* (shallow only), and *Cyanobacteria* represented across both samples (Fig. 5A). The deep samples did not have detectable *Planctomycetes* sequences present. The "library compare" function from RDP with 95% confidence showed that the major significant difference between the shallow/shallow with cleared gut samples and the deep samples derived from differences in the relative representation

of the families *Alteromonadaceae* and *Colwelliaceae* within the order *Alteromonadales* (class *Gammaproteobacteria*) (Figure 5B; Table 2B,C). The shallow and shallow with cleared gut samples were enriched with *Alteromonadaceae* (genus *Haliea*) while the deep samples had more *Collwelliaceae* dominating the *Alteromonadales.* Other differences between the samples include an enrichment of the order *Flavobacteriales* (*Alphaproteobacteria*) in the shallow with gut cleared sample relative to the deep samples and the representation of the order *Piscirickettsiaceae* (family *Thiotrachales*; *Gammaproteobacteria)* in the deep samples. In comparing the two shallow samples there was a significant difference due to the absence of the family *Rhodobacteraceae* (*Bacteriodetes*) in the shallow library but the moderate abundance in the shallow with cleared gut library. Interestingly, there was no difference in the relative abundance of cyanobacteria between the shallow with cleared gut library and the other two libraries. In fact, the relative proportion of the library represented by cyanobacteria actually went up in the shallow with gut cleared library relative to the other two libraries (from 30% to approximately 50% of all represented phyla). This suggests that the copepod guts were not in fact fully cleared or that there were cyanobacteria still attached to the copepod after rinsing.

Fixation, Embedding, Sectioning and CARD-FISH: The protocol used for fixing and embedding copepods seemed to work fairly well given that only a few copepods within the group seemed to shrink over the course of the procedure (maybe the shrinking was specific to the copepod species type?). The sectioning of the copepods also went quite well and I was able to attain 10 um, 20 um, and 35 um thick slices of individual copepods. Using the Polysine® slides helped keep the copepod sections from washing away during the FISH procedure although I was also careful to do all of the wash steps using a pipette without dunking the slides within Falcon tubes for the various washes as an extra precaution. I noticed though that when the slices were washed with a lot of 96% denatured ethanol that some of the agarose embedding appeared to start coming off. Therefore, it will be important in the future to closely monitor the amount of ethanol that is used to rinse the slides. Unfortunately the FISH Eubacteria and Roseobacter probes did not seem to work on the copepod slices, although the DAPI stain showed up nicely. In the future, it would probably be best to work with the red dye given that the copepod seems to autofluoresce much more intensely in the green than in the red fluorescence, so it would likely be easier to detect red FISH probes. I did not have time to thoroughly examine the DAPI images and attempt to do bacterial counts because it was often unclear what cells were of microbial origin given the strong staining of the copepod cells and the chitin autofluorescence (Fig. 6).

***Hoeflea anabaena sp. nov.* growth experiments:** The growth of the epibiont was overall low over the time course of the growth experiment despite even re-inoculating the tubes with 200 ul of the epibiont on the night of day 2. By 118 hours after the initial inoculation, it seemed that maltose and galactose had stimulated the most growth relative to those cultures in the minimal media (Figure 7). Interestingly, galactose is an important terminal residue in the polysaccharides of the *Anabaena* heterocysts and its addition seemed to stimulate growth even though the FBA predicted that galactose is represented in the metabolism of the epibiont and is not required to be transported into the cell. Maltose is a substrate that is predicted to be transported into the cell and it does appear to begin to enhance growth of the epibiont. However, substrates predicted by FBA to be transported into the cell are not always observed to actually stimulate growth (Figure 7; Table 3). Growth in mannose seemed to be approximate to that in minimal media, and the remaining substrates had lower growth than that in minimal media. It is important to note that the values on the y-axis for the growth curve are extremely low and that some of the growth results that I have just mentioned may still be within the error of the spectrophotometer readings.

However, the observations at 118 hours suggest a trend for further enhanced growth of the maltose and galactose treatments. For future growth experiments I would recommend beginning with higher amounts of epibiont innocula (~300 ul of relatively concentrated epibiont growth/5 mL of media) and running the experiment for a longer period of time.
Due to time constraints I was not able to fully explore the epibiont genome as much as I had wanted, but this presents an interesting opportunity for future Microbial Diversity students. Whole-genome comparisons with *Sinorhizobium melioti* and *Ahrensia* may provide interesting information regarding the status of the epibiont's nitrogen metabolism (*Sinorhizobium* is a nitrogen fixer) and partially conserved photosynthetic pathways (the epibiont seems to have some conserved photosystem elements and *Ahrensia* does not).

CONCLUSION

Although there was not adequate time to utilize enrichments and colony PCR as a strategy to find what can grow on coastal calanoid copepods, sequence data from 454 sequencing and clone libraries on previously collected *C. finmarchicus* samples revealed some interesting stories about the bacterial communities on copepods. The 16S 454 sequencing of the deep copepod sample suggested a predominance of *Deferribacterales* which are obligate anaerobes that can use a diverse number of electron acceptors (including Fe^{3+} and Mn^{4+}) and degrade complex organic compounds. The described members of the genus *Caldithrix* (*C. abyssi* and *C. palaeochoryensis*) are strictly anaerobic, hyperthermophilic, and chemo-organotrophic (Miroshnichenko et al., 2003; 2010) making it seem curious that they were found to be so abundant in copepods! Yet perhaps *Caldithrix* is more broadly distributed in deep waters and seeks refuge in the anaerobic microenvironment of copepod guts. It would be interesting to see if the presence of *Caldithrix* with deep copepod samples is stable and re-occurs over several years. The next step in this analysis is to compare the bacterial communities on copepods to those from concurrently collected water samples to explore whether copepods may be enriched in particular bacterial types and how this may vary by water depth.

It was curious that the results from the 454 sequencing were so different from that of the clone library from the deep sample, namely that *Deferribacterales* was not at all represented in the clone library and that the *Alteromondales* were not detected in the 454 sequencing. This demonstrates that comparing next-generation sequencing and clone library results can sometimes be like comparing apples and oranges in light of the differences in primers and reagents that are used for the two procedures. Nevertheless, these two techniques demonstrate the relative importance of *Gammaproteobacteria* and *Flavobacteria* in the microbial communities of copepods in both shallow and deep environments.

In comparing the three clone libraries it became clear that the major difference between the shallow and the deep communities lay within the order *Altermondalas*, specifically the relative representation of the genus *Haliea* and *Colwellia*. Most prevalent in the deep copepod samples, *Colwellia* are facultative anaerobes that can degrade starch and chitin under aerobic conditions, are psychrophilic (optimum growth at < 15°C), sometimes barophilic, and require high amounts of salt ions in order to maintain membrane integrity. Many species of *Colwellia* are found in continually cold marine environments including Arctic sediments and sea ice (e.g. *Colwellia psychrerythraea)* (Bowman et al., 1998). C. *psychrerythraea* also has adapted to form extracellular polymers to enhance biofilm production and cryopreservation. The other *Alteromondales* genus that was more frequently found in the shallow environments was *Haliea* which includes aerobes that have been isolated from the surface of coastal waters in the

Mediterranean (Urios et al., 2008; 2009). The characteristics of *Colwellia* and *Haliea* provide rational context to their discovery on deep and shallow copepods, respectively.

Future FISH analysis of copepod sections will provide an informative examination of the relative distribution of the bacterial communities and how relative abundances may change from the exterior to the interior of the copepod. CARD-FISH has been previously successful on paraffin copepod sections (Peter and Sommaruga, 2008), so it would definitely be worthwhile to attempt FISH with on copepod sections embedded with Tissue Tek and fixed in paraformaldehyde.

Finally, in my exploration of the factors that may drive the association of the *Hoeflea anabaenae* with *Anabaena*, primarily potential C-sources, I was interested in exploring potential substrates that were suggested to be needed to be transported (ribose and potentially maltose) according to a flux balance analysis of the epibiont genome on RAST as well as other *a priori* carbon substrates of interest (i.e. glycolic acid, branched chain fatty acids, xylose, galactose, mannose). Maltose and galactose were the only two susbtrates that appeared to enhance growth of the epibiont relative to the minimal media control group, although a longer time course is required in order to make any definitive conclusions given that the change in OD at 600 nm is still quite low. It is interesting though that galactose is an important terminal residue in the heterocyst polysaccharide and a substrate that appears to be synthesized by the epibiont as well based on flux balance analysis. Maltose is a substrate that FBA predicts needs to be transported to the organism and it does seem to enhance the growth as the epibiont as one might expect. However, I would have also xpected the growth of the epibiont to be much higher in treatments such as ribose and xylose which are predicted by flux balance analysis to need to be transported into the organism but perhaps this is a factor of the time course not being run for long enough, that these substrates are not required for growth or that the organism doesn't have the physical capacity to transport the substrates into the cell. It is curious that some of the substrates seem to be causing even lower growth than in the control, but it is not clear whether this could be due to inhibition of growth, inadequate substrate concentrations, or just initial slower growth. As a whole, flux balance analysis was a helpful hypothesis generator for potential substrates that might stimulate epibiont growth and give clues into the possible C-sources that the *Anabaena* might be supplying to the epibiont in their specific association. However, this project also supports the notion that FBA and genomic information in itself is not always enough to understand the functioning of an organism in an ecological context. Further exploration of C-sources and other nutrients would be a worthwhile addition to the *Anabaena*-epibiont story to try to better understand what factors are really driving these associations.

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Fig. 1: A calanoid copepod collected in Martha's Vineyard Sound

Fig. 2: The bacterial assemblage enriched from ~15 homogenized calanoid copepods after incubation in chitin liquid media for 4 days at 30 °C in an oscillator. The arrows are pointing to the rod-shaped bacteria that were observed attached (and wriggling!) to the chitin particles.

Table 1: 16S 454 sequencing results for the deep *C. finmarchicus* samples. This OTU table was produced by Qiime considering only those sequences between 400-600 bp in length and 97% identity clusters.

Number of OTU counts	Consensus Lineage
78	Bacteria;Deferribacteres;Deferribacteres;Deferribacterales; Caldithrix
5	Bacteria;Proteobacteria;Gammaproteobacteria;Oceanospirillales;Halomonadaceae;Halomonas
5	Bacteria;Bacteroidetes;Flavobacteria;Flavobacteriales;Flavobacteriaceae;Tenacibaculum
5	Bacteria;Proteobacteria;Gammaproteobacteria
3	Bacteria;Bacteroidetes
2	Bacteria;Bacteroidetes;Flavobacteria;Flavobacteriales;Cryomorphaceae
2	Bacteria;Proteobacteria
2	Bacteria;Proteobacteria;Gammaproteobacteria;Alteromonadales;Colwelliaceae
1	Bacteria;Proteobacteria;Alphaproteobacteria;Rhodospirillales;Acetobacteraceae
1	Bacteria;Proteobacteria;Gammaproteobacteria;Vibrionales;Vibrionaceae;Listonella
1	Bacteria;Cyanobacteria;Cyanobacteria;Chloroplast;Bacillariophyta
1	Bacteria
1	Bacteria;Firmicutes;Bacilli;Bacillales;Bacillaceae;Tumebacillus
1	Bacteria
1	Bacteria;Deferribacteres;Deferribacteres;Deferribacterales;Caldithrix
1	Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales;Rhizobiaceae;Rhizobium
1	Bacteria
1	Bacteria
1	Bacteria;Bacteroidetes;Flavobacteria;Flavobacteriales;Cryomorphaceae
1	Bacteria;Proteobacteria;Deltaproteobacteria

A.

B.

Fig. 3A: Rank abundance curve of the OTUs counted from the 454 sequencing of the deep copepod sample. 3B: Rarefaction curve with 97% ID cutoff for the deep sample 454 sequencing results. The blue curve represents a line with a slope of 1, demonstrating that deep sample (red curve) was deeply sampled in the 454 run.

Fig. 4: iTOL tree generated by Fasttree of the three copepod clone library sequences. The white gaps in the color scheme represent the closest match sequences to the copepod clone library sequences as determined by RDP SeqMatch.

A.

B.

Fig. 5A: The relative proportion of phyla represented by the three copepod clone libraries as classified by RDP classifier. The phyla counts were normalized by the total number of sequences present in each library. 5B: The relative proportion of different orders within the class *Gammaproteobacteria* that were represented in the three clone libraries.

Tables 2A,B,C: The significant results from RDP's pairwise "Library compare", highlighting the taxonomic level at which there are significant differences in the microbial community composition between copepod 16s clone libraries.

A.

Rank	*Name*	Shallow-cleared gut Library	Shallow Library	Significance
family	*Rhodobacteraceae*	7	0	8.55E-03

B.

Rank	Name	Shallow Library	Deep Library	Significance
family	*Alteromonadaceae*	23	2	1.36E-05
genus	*Haliea*	23	2	1.36E-05

family	*Piscirickettsiaceae*	0	8	3.52E-03
order	*Thiotrichales*	0	8	3.52E-03
family	*Colwelliaceae*	6	15	3.16E-02

C.

Rank	Name	Deep Library	Shallow-Cleared Gut Library	Significance
family	*Alteromonadaceae*	2	23	1.73E-05
genus	*Haliea*	2	23	1.73E-05
class	*Gammaproteobacteria*	53	31	2.80E-04
family	*Colwelliaceae*	15	2	9.42E-04
phylum	*"Proteobacteria"*	59	40	1.12E-03
order	*"Flavobacteriales"*	9	21	2.32E-02
class	*Flavobacteria*	9	21	2.32E-02
family	*Flavobacteriaceae*	9	21	2.32E-02

Fig. 6: 20 um slice of a calanoid copepod collected from Martha's Vineyard sound and stained with DAPI

Figure 7: Growth curves of the *Anabaena* epibiont cultured in 8 different carbon substrates. Absorbance is measured at 600 nm. The arrow is highlighting the time (night of the third day) at which the cultures were re-inoculated with 200 ul of epibiont to try to stimulate epibiont growth.

Table 3: Summary of tested carbon substrates for *Hoeflea anabaena sp. nov.* growth experiments. Flux balance analysis of the epibiont's genomic sequence was used to predict the completeness of relevant metabolic pathways and therefore whether a particular substrate might enhance growth. Growth experiments were performed to see how these genome predictions matched up with actual observations. Growth on a particular carbon substrate is always

considered in reference to the observed growth on minimal media. The *substrates represent those studied in Stevenson et al. 2010

Substrate	Pathway complete?	Growth?
acetate*	Yes	Yes
pyruvate*	Yes	Yes
malate*	Yes	Yes
fumarate*	No	Yes
lactate*	Yes	No
fructose*	Yes	No
L-arabinose*	Yes	No
D-glucose*	Yes	No
mannose	Not known	Not yet
Xylose	No. Requires transport	Not yet
galactose	Yes	Maybe
L-leucine	Yes/Transport required	Not yet
L-isoleucine	Yes/Transport required	Not yet
glycolate	Yes	Not yet
ribose	No. Requires transport	Not yet
maltose	No. Requires transport	Maybe