# *Development of probiotics and alternative treatments for stony coral tissue loss disease*

Final Report

Prepared By:

Julie Meyer

University of Florida
2033 Mowry Rd.
Gainesville, FL 32611

August 31, 2020

***Introduction***

The ongoing stony coral tissue loss disease (SCTLD) was first observed in Florida in late 2014 and has since spread through nearly the entirety of the Florida Reef Tract. More than half of the 45 coral species in Florida are impacted by this disease and once infected, colonies typically dies within weeks or months. The geographic scale, the duration of the outbreak, the number of impacted coral species, and the speed with which the disease kills coral, all make this disease outbreak both unprecedented and devastating. The disease is now spreading around the Caribbean, with confirmed cases in Jamaica, Mexico, St. Maarten, US Virgin Islands, the Dominican Republic, Turks and Caicos, and Belize.

Current intervention efforts to curb the spread of this disease include both antibiotic treatments and the development of probiotic treatments. An investigation of beneficial microorganisms that inhibit targeted bacteria cultured from diseased corals was conducted in 2018-2019 at the Smithsonian Marine Station. Preliminary results demonstrated the effectiveness of probiotic bacterial strain McH1-7 at stopping SCTLD progression and preventing infection in laboratory aquaria. Additionally, the examination of the relationship of *Vibrio coralliilyticus* to SCTLD suggested that this bacterial species may be involved in coinfections, which may be causing the variability seen in disease lesions.

The overall goal of this project is to **identify potential factors preventing effective probiotic treatments** that are in development by colleagues at the Smithsonian Marine Station.

**Task #1: To evaluate the colonization efficiency by probiotics and the identification of factors that may reduce treatment efficacy.**

***Task 1a. To evaluate the colonization efficiency of probiotic treatments on different coral species (using digital PCR) to certify host-treatment compatibility (laboratory and field-treated corals).***

The first step to developing droplet digital PCR (ddPCR) assays to track probiotic strains was to sequence the genomes of promising probiotic bacteria. We sequenced the genomes of four *Pseudoalteromonas* genomes, including strains McH1-7, McH1-42, and SMS1 isolated from *Montastraea cavernosa* and strain Of7M-16 from *Orbicella faveolata*.

The most promising probiotic strain so far has been McH1-7. In experiments conducted by the Smithsonian Marine Station in 2018-2019, McH1-7 slowed or stopped SCTLD progression in *M. cavernosa* corals. Sequencing the genome of this probiotic strain has provided insight on the antibiotics that this strain can potentially produce. The assembled genome of *Pseudoalteromonas* sp. McH1-7 contained 59 contigs over 1,000 bp in length. The longest contig was 313,663 bp and the total length of assembled contigs was 5,134,549 bp. The genome quality is excellent, with an estimated 100% completeness and 1.9% contamination, as assessed by MiGA Online. Raw sequencing reads and the assembled genome are available through NCBI Bioproject PRJNA639770. The assembled genome is also publicly available through the Joint Genome Institute's Integrated Microbial Genomes and Microbiomes database under IMG Genome ID # 2881214049.

Fourteen biosynthetic gene clusters were identified by antiSMASH, including the biosynthetic gene cluster for korormicins. Of these 14 biosynthetic gene clusters, six were hybrid clusters with both non-ribosomal peptide synthases (NRPS) and type I polyketide synthases (T1PKS), seven others were NRPS or NRPS-like (likely incompletely assembled), and two were bacteriocins. In addition to the biosynthetic gene clusters identified through antiSMASH, the genes necessary for the production of marinocin (*lodAB*) were identified through annotation and the tetrabromopyrrole gene cluster was located through similarity to the previously published gene cluster in *Pseudoalteromonas* sp. PS5 (GenBank Accession #KR011923.1). Overall, the genomes of *Pseudoalteromonas* sp. McH1-7 and *Pseudoalteromonas* sp. PS5 had an average nucleotide identity of 98.91% in shared genes. The gene clusters for marinocin and tetrabromopyrrole were 97.9% and 98.7% similar between strains McH1-7 and PS5, respectively.

We designed ddPCR primers for the identification and quantification of McH1-7 from the second gene of the korormicin biosynthetic gene cluster (Figure 1, Table 1). We confirmed that this primer set did not amplify any gene products in the other *Pseudoalteromonas* strains that were available in the lab and therefore this primer set appears to be unique to strain McH1-7.

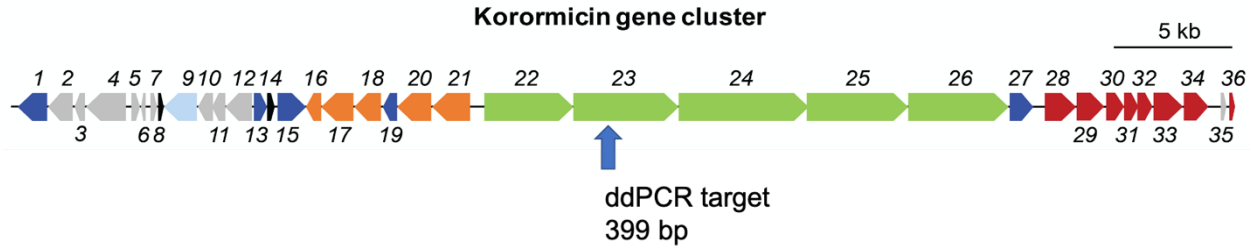



**Figure 1.** The biosynthetic gene cluster encoding korormicins is shown in green (genes 22-26). The 399-bp ddPCR product is encoded by gene 23.

**Table 1.** Biosynthetic gene targets used for development of unique markers for probiotic *Pseudoalteromonas* strains (McH1-7, McH1-42, SMS1, Of7M-16) in digital PCR.

| Isolate | Product | Primer sequences | Product size |
|---|---|---|---|
| *Pseudoalteromonas* McH1-7 | korormycin (antibiotic) | McH17_KOR F:<br>5'- ACGTTACCCGCTATCTGTGG-3'<br>McH17_KOR R:<br>5'- CGCTTTCCTAAAGCACTTGG-3' | 399 bp |
| *Pseudoalteromonas* McH1-42 | part of chalcone/stilbene biosynthesis (T3PKS) | McH142_bact F:<br>5'- GTTGATCGTCTGCGTTGAGA-3'<br>McH142_bact R:<br>5'- CCGGTATGAAAAATCGTGCT-3' | 342 bp |
| *Pseudoalteromonas* SMS1 | N-acyl homoserine lactone (Quorum sensing) | SMS1_hsl F2:<br>5'-GACTCATCACAAGCCCCATT-3'<br>SMS1_hsl R2:<br>5'- GCCAATTTAGGCGAGACAAG-3' | 283 bp |
| *Pseudoalteromonas* Of7M-16 | thiomarinol (*tmlY*) (antibiotic) | tmlY F:<br>5'- CAGTTTTGGCATCCCTTCAT-3'<br>tmlY R:<br>5'- AGAGACCAATGCCAAACGAC-3' | 371 bp |

Experimental application of probiotic strain McH1-7 were conducted in aquaria. Four separate tanks with fragments of *M. cavernosa* corals were dosed with live cultures of McH1-7 and maintained for 28 days. Samples of coral tissue and aquarium water were collected immediately before probiotic treatment, one hour after treatment, and then 1 day, 3 days, 7 days, 21 days, and 28 days after treatment (Figure 2). McH1-7 was detectable in the water 1 hour and 1 day after treatment in all four tanks. McH1-7 was detectable in coral tissue at very low levels 1, 7, 21, and 28 days after treatment in half of the corals (2/4).

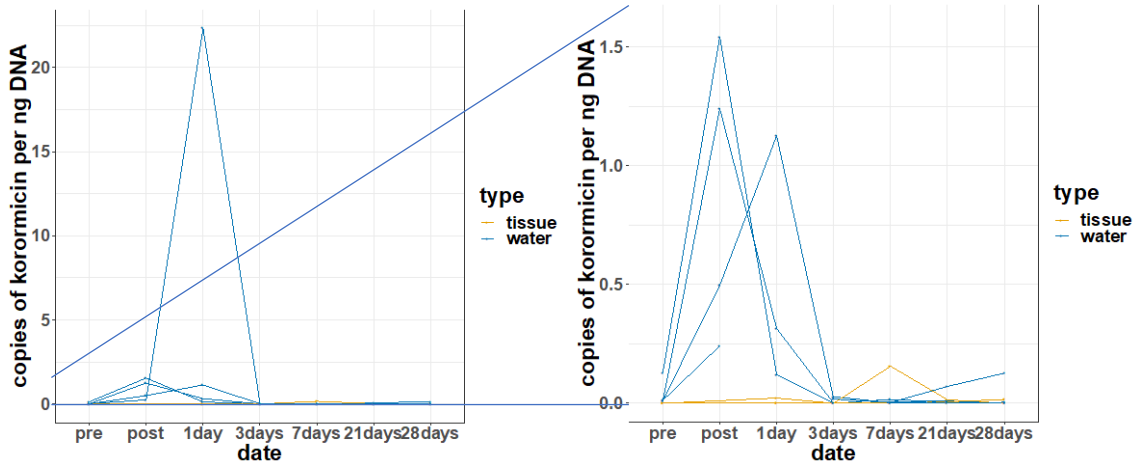
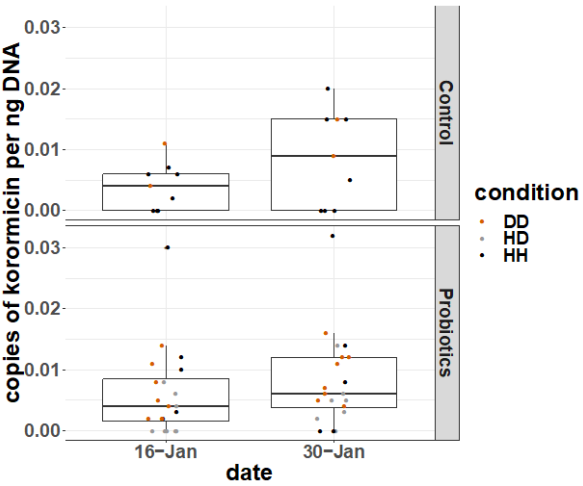



**Figure 2.** Detection of probiotic strain McH1-7 in aquarium trial of probiotic treatment. McH1-7 was detected in the water of all replicates 1 hour and 1 day after treatment. McH1-7 was detected at very low levels in coral #25 on 21 and 28 days after treatment and in coral #26 on days 1, 7, and 21 days after treatment.

Experimental application of probiotic strain McH1-7 was also conducted in field trials in January 2020. Tagged corals, both healthy and diseased, were sampled before treatment and 2 weeks after probiotic treatment. Corals were temporarily enclosed with a plastic bag to which live cultures of McH1-7 was added. Control corals were also sampled where a bag was placed over the coral, but no probiotic was added to the bag.




The korormicin gene from McH1-7 was detected at very low levels, with no statistical difference between treated and control corals and no statistical difference between the sample dates. While this application of probiotic treatments inside a bag did not appear to increase the amount of McH1-7 on corals, the strain was detectable both before and after treatment. Future probiotic treatments will be more targeted by using a paste applied directly to disease lesions.

**Figure 3.** Detection of korormicin gene in field trials of probiotic treatments.

***Task 1b. To compare the microbiomes of probiotic-treated corals that did and did not respond to treatments to identify potential pathogens interfering with probiotic colonization and protection as well as the effect of treatment on the coral.***

All of the samples used in the aquarium-based and field-based probiotic treatment trials have been sent for sequencing of the 16S rRNA gene at the University of Florida's Interdisciplinary Center for Biotechnology Research. Data have not yet been received to include in this report but will be included in upcoming reports for the next fiscal year.

***Task 1c. To determine what effect Vibrio coralliilyticus has on the coral microbiome by comparing colonies that are negative and positive for this pathogen.***

In addition to sequencing probiotic strains of bacteria, we also sequenced eight strains of the coral pathogen *Vibrio coralliilyticus* in the search for potential SCTLD pathogens. We conducted a comparative genomics of the Atlantic strains of *V. coralliilyticus* newly isolated by Dr. Blake Ushijima (Smithsonian Marine Station) with previously published genomes of *V. coralliilyticus* from the Pacific and Indian Oceans. This analysis revealed that the genomes of the Atlantic strains did not have a geographically isolated signature (Figure 4).

Pairwise comparison of the average nucleotide identity of shared genes for eight newly isolated strains of *V. coralliilyticus* ranged from 96.8% to 100%. All three strains isolated from diseased *O. faveolata* corals, strains OfT6-17, OfT6-21, and OfT7-21, had 100% sequence identity of shared genes. Likewise, the two strains isolated from healthy *M. cavernosa*, strains MCA25 and MCA32, had 100% sequence identity of shared genes. However, pangenome analysis showed that each of the eight genomes contained unique combinations of genes such that some genes present in one strain were not found in all strains, even when shared genes were identical (Figure 4). While most of these draft genomes are of excellent quality and more than 90% complete, it is possible that some of these genomes, if finished completely (i.e. a closed, circular genome was obtained), may be completely identical to each other.

The pangenome of all 14 strains of *V. coralliilyticus* with sequenced genomes contained a total of 9,547 genes, with 2,771 core genes (present in all strains), 2,881 shell genes (present in three to 13 of the strains), and 3,895 cloud genes (present in only one or two strains) (Figure 4). Overall, the phylogenetic tree based on the alignment of core genes did not cluster genomes based on geographic origin (Atlantic versus Indian or Pacific Oceans), nor by host type. For example, strains RE22 and RE98 were both isolated from oysters, but cluster with strains isolated from corals instead of with each other. In general, strains from the same coral species clustered together, for example the three strains from a diseased *M. cavernosa* infection of a healthy *O. faveolata* (OfT6-17, OfT6-21, OfT7-21) clustered together and the two strains from an apparently healthy *C. natans* (CN26H-1 and CN52H-1) clustered together. The only caveat was the strain MmMcT2-4, which was only 70% complete, so completion of its genome could change its results for this analysis.

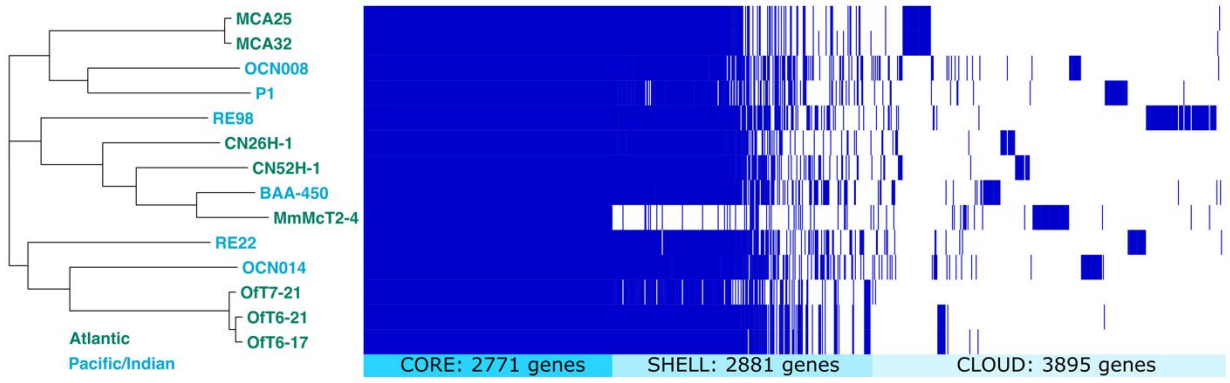



**Figure 4.** Comparative genomic analysis of *V. coralliilyticus* strains. All Atlantic strains of *V. coralliilyticus* were sequenced in this project and compared to existing sequenced genomes from *V. coralliilyticus* strains from the Pacific and Indian Oceans. The phylogenetic tree on the left shows the genetic relatedness of genomes based on all shared genes. The bar graph on the right shows the presence and absence of all genes in the pangenome of all 14 strains. Clustering of genomes is based on the alignment of 2,771 core genes present in all strains. Shell genes were present in three to 13 of the genomes. Cloud genes were present in only one or two of the genomes.

A comparison of 13 the 14 genomes of *V. coralliilyticus* with six other vibrio pathogens, including the marine invertebrate pathogens *V. proteolyticus*, *V. shilonii,* and *V. shiloi*, as well as the human pathogens *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*, revealed diverse genes for the production of toxins and secondary metabolites in *V. coralliilyticus* (Figure 5). *V. coralliilyticus* strain MmMcT2-4 was excluded from the results of this analysis for clarity because of the incompleteness of the genome. Overall, the *V. coralliilyticus* genomes have the potential to make more kinds of toxins and secondary metabolites than any of the other six *Vibrio* species. First, all the *V. coralliilyticus* genomes have the vibriolysin-like zinc-metalloprotease. Vibriolysin metalloproteases identified in *V. proteolyticus*, *V. chloerae*, and *V. vulnificus* were not closely related to vibriolysin-like zinc metalloprotease in the *V. coralliilyticus* genomes. All the *V. coralliilyticus* genomes have genes to produce hydrogen cyanide, hemolysin/cytolysin, and areolysin/cytotoxic enterotoxin that were generally absent in the other pathogenic vibrios. In addition, all the *V. coralliilyticus* genomes have the gene for *V. cholerae* cytolysin that are not present in other pathogenic vibrios except for *V. cholerae*. Genes for the biosynthesis of siderophores were present in all *V. coralliilyticus* genomes, but inconsistently found in other pathogenic vibrios.

Perhaps most interesting is the observation that all the *V. coralliilyticus* genomes isolated from diseased corals and oysters have the genes to produce a thiopeptide which contains a linear azole/azoline-containing peptide (LAP), while the four strains of *V. coralliilyticus* isolated from healthy corals, do not. Thiopeptides are a diverse class of antibiotics that inhibit protein synthesis in Gram-positive bacteria, but generally have no effect on Gram-negative bacteria.

All the *V. coralliilyticus* genomes also have diverse genes for the secretion of these toxins and secondary metabolites, including Type I, Type II, Type III, Type IV, and Type VI secretion

systems. In particular, T3SS and T6SS are important secretion systems for pathogenesis, with their needle-like delivery of toxins and enzymes through host membranes. The presence of an active T6SS in *V. coralliilyticus* was confirmed in a previous study. Lastly, all the *V. coralliilyticus* genomes contained several genes for multidrug export proteins (*emrB*, *mepA*), multidrug resistance proteins (*mdtABCEGHKLMN*, *mexAB*, *norM*), multidrug transporter (*emrE*), and putative multidrug resistance protein (*emrK*), which may contribute to antibiotic resistance in *V. coralliilyticus*.

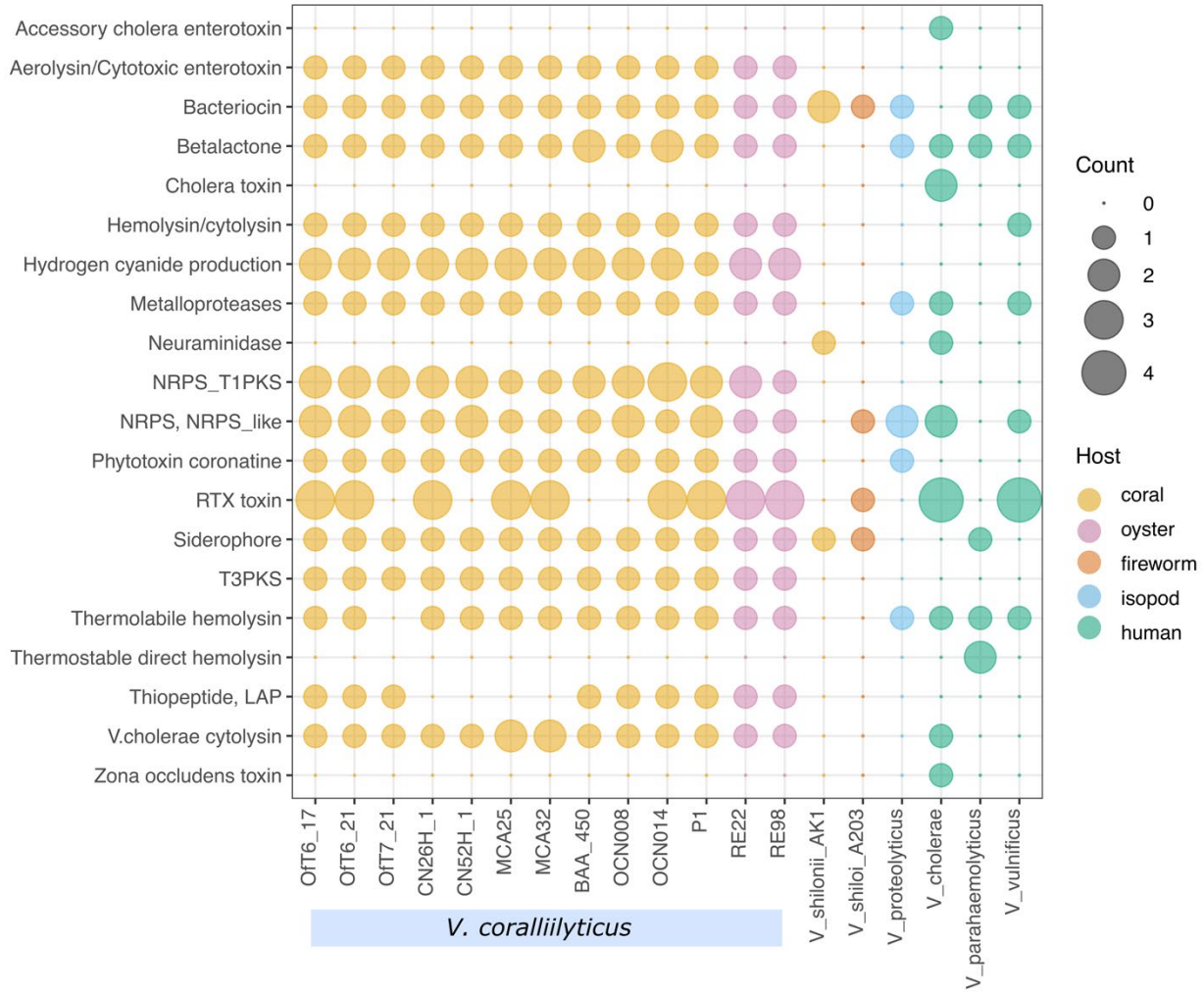



**Figure 5.** Comparison of secondary metabolites, antibiotics, and toxins produced by pathogenic *Vibrio* species.

From the sequenced *V. coralliilyticus* genomes, we also designed a ddPCR primer set (Table 2) to amplify part of the vibriolysin-like zinc metalloprotease gene (*vcpA*). We confirmed that this primer set did not amplify any gene products in the other *Vibrio* species (non-*coralliilyticus*) that were available in the lab and therefore this primer set appears to be unique to *V. coralliilyticus* strains, from both Atlantic and Pacific Oceans.

**Table 2.** Biosynthetic gene target used for development of unique markers for the coral pathogen *Vibrio coralliilyticus*

| Isolate | Product | Primer sequences | Product size |
|---|---|---|---|
| *Vibrio coralliilyticus* | Vibriolysin-like zinc metalloprotease (*vcpA*) | vibriolysin F:<br>5'- GGCGAACCAACTTTACTGGA-3'<br>vibriolysin R:<br>5'- GGTCAGTCACTGGCGTACCT-3' | 197 bp |

Using the ddPCR assay, we were able to compare the copies of the *vcpA* gene with the result of the immunoassay for the vibriolysin-like metalloprotease and the fate of the coral in SCTLD progression (Table 3). These results showed a strong correlation between gene presence and toxin presence, as well as a strong correlation with disease outcome. This ddPCR assay is therefore very promising for future studies.

**Table 3.** Comparison of disease progression and the detection of the *vcpA* gene by ddPCR and the vibriolysin-like zinc metalloprotease by immunoassay.

| Coral ID | copies of *vcpA*/ng DNA | VcpA immunoassy result | Outcome |
|---|---|---|---|
| McD-1 | 159 | + | Complete mortality |
| McD-2 | 119 | + | Complete mortality |
| McD-3 | 11 | - | Stopped |
| McD-4 | 922 | + | Complete mortality |
| McD-5 | 3 | - | Stopped |
| McD-6 | 1 | - | Stopped |
| McD-7 | 285 | + | Complete mortality |
| McD-8 | 155 | + | Complete mortality |
| McD-29 | 0 | - | Slow progression |
| McD-33 | 83 | + | Complete mortality |
| McD-35 | 1 | - | Slow progression |

***Task 1d. To identify the differences between the diseased corals with sub-acute/acute lesions with those that have comparatively slower disease progression.***

As described above, both ddPCR assays and immunoassays have shown that SCLTD progression is faster in corals with *V. coralliilyticus* (see final report by colleagues at the Smithsonian Marine Station for additional details). Additional differences in diseased corals with sub-acute/acute lesions versus those with slower disease progression may be revealed by sequencing the 16S rRNA genes to characterize differences in their microbial communities.

***Results summary and future directions:***

- Sequenced genomes of potential probiotic bacteria and coral pathogens
- Developed droplet digital PCR targets for 4 probiotic strains and *V. coralliilyticus*
- Quantified korormicin genes in aquarium and field trials with McH1-7
- Quantified vibriolysin-like metalloprotease genes in *V. coralliilyticus*
- Comparative genomics of *V. coralliilyticus* strains from Atlantic and Pacific Oceans
- Future work will include continued work to evaluate the colonization efficiency of new strains of probiotic bacteria
- Future work will also include quantification of the presence of *V. coralliilyticus* genes capable of producing the zinc-metalloprotease toxin known as vibriolysin through droplet digital PCR assays developed in FY20.