Targeted culturing of putative pathogens associated with stony coral tissue loss disease

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Final Report

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I would like to thank Kalie Januszkiewicz for her help in getting anaerobic culturing set up, along with her tireless efforts as lab manager to keep the lab running smoothly.

Management Summary

Identification of the pathogens that cause stony coral tissue loss disease (SCTLD) is critical to understanding the disease outbreak and reducing its impact and further spread. This project isolated strains of anaerobic bacteria from SCTLD lesions, sequenced their genomes, and tested whether anaerobic bacteria could initiate disease signs in coral or in their algal symbionts. None of the tested bacteria induced disease signs but it is possible that toxin production was not elicited under the growth conditions tested. Continued investigation of the genomes of these potential pathogens will continue with alternative funding sources which may further uncover the role of these bacteria in disease progression. This project produced a unique dataset of publicly available genomes for coral-associated anaerobic bacteria, including members of the class Clostridia that are implicated in SCTLD.

Executive Summary

Stony coral tissue loss disease (SCTLD) has impacted Florida's Coral Reef for nearly a decade. Despite years of research, a causative agent has not been identified. Identification of potential pathogens requires cultivation in the lab to demonstrate damage to the host. Previous attempts to cultivate potential SCTLD pathogens have not produced obvious or consistent pathogenic strains. We propose that anaerobic bacteria may be an overlooked source of potential pathogens in coral disease. Characterization of coral microbiomes through both taxonomic and functional surveys have implicated bacteria in the class Clostridia as potential pathogens in SCTLD. Clostridia would have been missed with standard cultivation methods because they are strict anaerobes, growing only in the absence of oxygen. In the coral host, Clostridia may inhabit anoxic niches deep within coral tissues where the disease is thought to begin. Thus, we used a targeted approach to cultivate anaerobic bacteria like Clostridia and to test for virulence in both corals and cultures of *Durisdinium* algal symbionts. We isolated and sequenced the genomes of more than 20 anaerobic strains from SCTLD-affected corals, including *Orbicella faveolata* exhibiting fast lesion progression. We identified four strains of Clostridia from the genera *Wukongibacter* and *Vallitalea*. Anaerobic bacteria were tested for virulence against both corals and cultures of the algal symbiont *Durisdinium*, but no signs of disease were observed. While we were not able to initiate disease with these strains under the conditions tested, ongoing analysis of the genome content of these bacteria may reveal clues about their potential virulence and aid our understanding of the disease.

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List of Acronyms

SCTLD: stony coral tissue loss disease
FLP: fast lesion progression
SMSFP: Smithsonian Marine Station at Fort Pierce

1. BACKGROUND

Florida's Coral Reef is currently experiencing a multi-year disease-related mortality event, that has resulted in massive die-offs in multiple coral species. Approximately 21 species of coral, including both Endangered Species Act-listed and the primary reef-building species, have displayed tissue loss lesions which often result in whole colony mortality. First observed near Virginia Key in late 2014, the disease has since spread to the northernmost extent of Florida's Coral Reef, and southwest past the Marquesas in the Lower Florida Keys. The best available information indicates that the disease outbreak is continuing to spread west and throughout the Caribbean.

Nine years after this disease was first observed off the coast of South Florida, the causative agent of stony coral tissue loss disease (SCTLD) is still unknown. We have learned that antibiotic treatment with amoxicillin can stop some disease lesions from progressing (Aeby et al., 2019, Neely et al., 2020) and that coinfections with the pathogen *Vibrio coralliilyticus* can cause lesions to progress more rapidly, indicating that bacteria are important in SCTLD etiology (Ushijima et al. 2020). A recent meta-analysis of SCTLD microbial community studies identified the need to characterize the role of bacteria within the Clostridia order Peptostreptococcales-Tissierellales in disease progression due to their potential for toxin production (Rosales et al., 2023). In addition, our work characterizing the functions in bacteria in newly infected corals following SCTLD transmission in the lab has shown that SCTLD-associated Clostridia are genetically capable of producing bacterial toxins (Meyer et al., in prep). While other bacterial groups like Alphaproteobacteria and Bacteroidia were also recovered, the Clostridia genomes contained the most bacterial toxin-producing genes per genome, including those most likely to induce host tissue damage such as alpha toxin, exfoliative toxin, and thiol-activated cytolysin.

We performed preliminary trials to isolate anaerobic bacteria from a slurry of coral surface mucus and tissue collected from SCTLD-impacted Montastraea cavernosa at Hen-and-Chickens reef in July 2022. This resulted in the isolation of anaerobic strains identified as *Marinifilum* (Bacteroidia), *Halodesulfovibrio* (Deltaproteobacteria), and *Acidaminobacter* (Clostridia order Peptostreptococcales). While growth of these cultures was not vigorous, we are confident that we will be able to optimize our culturing media and techniques to recover anaerobic bacteria including Clostridia strains for future experiments.

Identification of the pathogens that cause SCTLD is critical to stopping the outbreak. Previous attempts to cultivate potential SCTLD pathogens have not produced obvious or consistent pathogenic strains. Clostridia would have been missed with standard cultivation methods because they are strict anaerobes, growing only in the absence of oxygen. In the coral host, Clostridia may inhabit anoxic niches deep within coral tissues where the disease is thought to begin. Thus, we propose here to use a targeted approach to cultivate anaerobic bacteria like Clostridia and to test for virulence among strains.

The overall goal of this project is to determine if anaerobic bacteria like Clostridia are potential pathogenic agents of SCTLD. The specific tasks of this project are to 1) grow and isolate anaerobic bacteria from SCTLD-infected corals, 2) challenge Symbiodinaceae strains with potential pathogenic bacteria, and 3) challenge healthy corals with potential pathogenic bacteria. In addition, samples of fast lesion progression-affected colonies of *Orbicella faveolata* will be used as a source of potential anaerobic pathogens and the microbiome composition of these corals will be determined for comparison to microbiome composition of classic stony coral tissue loss disease.

The outcomes of this project will be incorporated into an on-going coral disease response effort which seeks to improve understanding about the scale and severity of the coral disease outbreak on Florida's Coral Reef, identify primary and secondary causes, identify management actions to remediate disease impacts, restore affected resources, and ultimately prevent future outbreaks. As such, collaboration among partners is encouraged when appropriate to avoid duplication of efforts and ensure alignment of needs. Coordination with other Principal Investigators is recommended and required, as appropriate. This project involves collaboration among partners at three different institutions, including the University of Florida, University of North Carolina Wilmington, and the Smithsonian Marine Station.

2. METHODS

2.1. Isolation and genome sequencing of anaerobic bacteria from SCTLD lesions

Small samples (~1 ml of surface mucus/tissue slurry) from active SCTLD lesions were collected from disease transmission experiments with *Montastraea cavernosa* and field-collected samples from *O. faveolata* affected by fast lesion progression. These sample collections were conducted in coordination with sampling of diseased corals for the projects "Development of alternative in situ treatments for stony coral tissue loss disease"

and “Initial assessments of a potentially novel coral disease: fast lesion progression (FLP)”, minimizing the cost and effort of obtaining new disease samples.

Culturing and isolation were conducted under anoxic conditions using several approaches including the use of a Coy anaerobic chamber, a miniature anaerobic chamber, sodium thioglycolate media, and Balch tubes. The purity of isolated cultures and the identity of strains was confirmed by Sanger sequencing of nearly full-length 16S rRNA genes. Strains were selected for genome sequencing to confirm the presence of bacterial toxin-producing genes that could cause tissue damage in corals.

Potential pathogens were sent as either glycerol stocks in cryotubes (facultative anaerobes) or as live cultures in Balch tubes. These potential pathogens were used to challenge cultures of Symbiodiniaceae at the Ushijima lab and live corals at the Paul lab to determine if SCTLD-like disease symptoms could be initiated.

2.2. Challenge cultures of Symbiodiniaceae with putative pathogens

2.2.1. Growth conditions

Algal cultures were grown for 1.5 weeks before experimental set up to approximately 5x 10^5 cells/mL. *Vibrio alginolyticus* strains AN2, AN3, AN5, AN7, AN8, *Qipengyuania* strain AN20, and *Mesoflavibacter* strain AN21 were revived from glycerol on GASW and incubated at 27°C until single colonies formed. Bacterial cultures were inoculated from 3 colonies for a mixed bacterial community and incubated until turbid at 28°C, approximately 1-5 days.

2.2.2. Experiment setup

Cultures of potential pathogens were spun down at 6,000 rpm for 3 minutes to separate cells from spent media. Aliquots of 1 to 10 ml of culture were spun down depending on the density of growth. Cells were washed twice with sterile artificial seawater, resuspended in sterile artificial seawater, and diluted to an OD600 of 0.9 - 1.0. Challenge experiments included 9 ml *Durisidinium* and 1 mL of potential bacterial pathogen at densities of 10^5, 10^6, 10^7, and 10^8 colony forming units per ml. A control treatment used 1mL of sterile artificial seawater in place of washed bacterial cells. Each treatment was replicated five times. Treatments were mixed by pipetting and incubated in an algal incubator at 27° C.

2.2.3. Sampling

Samples were collected for cell counts on days 2, 5, 10, and 14. Culture tubes were vortexed lightly on a 3-4 speed and 100uL of sample was aliquoted into microcentrifuge tubes with 1uL paraformaldehyde, vortexed, and stored at 4°C. Algal cells were counted for each time point, and photos were captured of replicate 1.

2.3. Challenge healthy corals with putative pathogens

2.3.1. Coral collection, maintenance, and cutting

Montastraea cavernosa and *Siderastrea siderea* corals used in this experiment were collected from the Florida Keys in 2018 and 2019 following guidelines set forth by permits issued by the Florida Keys National Marine Sanctuary (permit numbers FKNMS-2017-128, FKNMS-2019-160). *Orbicella faveolata* corals in this experiment were picked up from the FKNMS Coral Nursery at the NOAA facility in Key West. Corals with disease lesions were maintained in 5 L aquaria that were held in water tables outside, and apparently healthy corals were kept in recirculating tanks inside a wet lab at the Smithsonian Marine Station at Fort Pierce (SMSFP). Specific information on coral housing at SMSFP is detailed in a previous study (Ushijima et al., 2023), with the exception that all tanks were held at 26.5 °C prior to starting this experiment and Marine Snow is no longer used as a feeding supplement.

Coral fragments assigned in this study were cut using a Gryphon Diamond Band Saw Model C-40 Tall fitted with a 42-inch stainless steel Gryphon Diamond Band Saw blade. Fragments were cut into at least two approximately 2 cm^2 pieces and trimmed of any dead skeleton. They were then placed in 5 L aquaria, separated by genotype. 50% water changes occurred at the end of the day after the corals had halted their excess mucus production. For the following week leading up to the start of the experiment, the fragments were fed twice a week with Reef Roids followed by partial water changes the next day. For the following 3 trials of this experiment, pre-cut fragments were used as experimental fragments.

To set up each experiment trial, fragments were separated and placed into individual 5 L aquaria with new filtered seawater (FSW) in a table set to 27 °C. One fragment of each genotype was labeled as control while the remaining fragments in each genotype were assigned one bacterial treatment (**Table 1**).

***Table 1**. Challenge experiments testing potential pathogens with live coral fragments. Coral genotypes beginning with "Mc" indicates* Montastraea cavernosa*, "Ofav" indicates* Orbicella faveolata*, "BBSS" and "Ssid" indicate* Siderastrea sideraea.

Trial	Challenge Strain(s)	Coral Genotypes
Trial 1- Feb. 2024	Control (no bacteria)	McD-111, McH-102, McD-104, McH-106, McH-unknown
Trial 1- Feb. 2024	*Vibrio mediterranei* strains McD51-1 + McD53-9 + McD53-10	McD-111, McH-102, McD-104, McH-106, McH-unknown
Trial 1- Feb. 2024	*Mesoflavibacter* strain AN21	McD-111, McH-102, McD-104, McH-106
Trial 1- Feb. 2024	*Vibrio coralliilyticus* strains OfT6-21 + OfT7-21	McD-111, McH-102

Trial 2 - Mar 2024	Control (no bacteria)	Ofav-A, Ofav-C, Ofav-D, BBSS1, Ssid-C, Ssid-E
Trial 2 - Mar 2024	*Vibrio alginolyticus* strains AN2 + AN3 + AN5 + AN7 + AN8	Ofav-A, Ofav-C, Ofav-D, BBSS1, Ssid-C, Ssid-E
Trial 2 - Mar 2024	*Vibrio* sp. AN10	Ofav-A, Ofav-C, Ofav-D, BBSS1, Ssid-C, Ssid-E
Trial 2 - Mar 2024	*Qipengyuania* strain AN20	Ofav-A, Ofav-C, Ofav-D, BBSS1, Ssid-C, Ssid-E
Trial 3 - Apr/May 2024	Control (no bacteria)	Ofav-A, Ofav-C, Ofav-D, BBSS1, McH-104
Trial 3 - Apr/May 2024	*Wukongibacter* strain AN31	Ofav-A, Ofav-C, Ofav-D, BBSS1, McH-104
Trial 4 - May 2024	Control (no bacteria)	Ofav-A, Ofav-C, Ofav-D, BBSS1, McH-104
Trial 4 - May 2024	*Wukongibacter* strain AN30	Ofav-A, Ofav-C, Ofav-D, BBSS1, McH-104
Trial 4 - May 2024	*Vallitalea* strains FLP68 and FLP75	Ofav-A, Ofav-C, Ofav-D, BBSS1, McH-104

Dosing corals at SMSFP

Three treatments were prepared for Trial 1 of this experiment. Treatment 1 consisted of a combination of three *Vibrio mediterranei* strains: McD51-1, McD53-3, and McD53-10; Treatment 2 consisted of *Mesoflavibacter sp.* AN21; and Treatment 3 consisted of two strains of the known coral pathogen *Vibrio coralliilyticus*: OfT6-21 and OfT7-21. The *V. mediterranei* and *V. coralliilyticus* strains were grown from cryovials on seawater agar (SWA) plates, and AN21 was grown from a cryovial on marine broth agar (MBA). All plates were incubated at 28 °C in a Thermo Scientific Heratherm incubator. Liquid cultures were made by adding a single colony of bacteria to 2 mL of seawater broth (SWB) for the strains grown on SWA and 2 mL of marine broth (MB) for AN21 to remain consistent with growing media. The cultures were placed in a Benchmark Incu-Shaker 10LR at 200 RPM and 28 °C and left overnight to grow. Optical density (OD_{600}) measurements were taken on a Thermo Scientific Genesys 180 UV-Visible Spectrophotometer at 600 nm the next morning to ensure growth occurred before inoculating flasks containing additional growth media and an aliquot of 50% sterile glycerol with the 2 mL liquid cultures. Treatment doses consisted of 60 mL of bacterial strains, and treatments were divided evenly among bacterial strains for those treatments comprised of multiple strains; therefore, media volumes and glycerol aliquots differed across treatments. Treatment 1 was divided into three flasks, each with 110 mL SWB (100 mL to allow for 20 mL of each strain for each of the five coral genotypes included in Treatment 1, and an additional 10 mL to use for checking OD_{600}) and 440 µL of 50% glycerol. Treatment 2 was divided into two flasks with 70 mL of SWB and 280 µL of 50% glycerol to account for the two genotypes dosed with that treatment. Inoculated

flasks were placed in the Incu-Shaker with the same settings until the OD_{600} reached approximately 1.0. Once an OD_{600} of 1.0 was reached, 30 mL of evenly combined bacteria strains (10 mL of each strain for Treatment 1 and 15 mL of both strains for Treatment 3), or 30 mL of AN21 for Treatment 2, were added to 50-mL Falcon tubes. Samples were spun down on either an Avanti JXN-26 centrifuge fitted with a JA-14.50 rotor set to 8000 RPM and 25 °C for 10 minutes or an Eppendorf 5810 R centrifuge at 5000 RPM and 25°C for 15 minutes. After centrifuging, the supernatant was poured off and the remaining 30 mL of bacteria strains to achieve a 60 mL dose was added to the same 50 mL Falcon tube and placed back into the centrifuge to spin down again. After the second round of centrifuging, the supernatant was poured off again, leaving a cell pellet for dosing. Trial 2 of this experiment followed the same protocol.

For Trials 3 and 4, strict anaerobic bacteria were grown in Balch tubes at Univ. of Florida and sent to SMSFP. Upon arrival in the morning, tubes were placed in the refrigerator until the afternoon. Tubes were brought to room temperature prior to centrifuging. The volume of doses for these trials were 50 mL, so 2-25mL Balch tubes were combined into 1-50mL Falcon tube. Tubes were spun down in the Avanti JXN-26 centrifuge fitted with a JA-14.50 rotor at 8,000 RPM and 25 ℃ for 15 minutes in attempts to solidify the softer pellets that formed from these bacteria. After centrifuging, the supernatant was poured off, leaving a pellet for dosing.

To dose the coral fragments, the airlines were first disconnected from the air source on the table for the treatment coral and its respective control. A 3 mL sterile transfer pipette was used to uptake ambient seawater from the treatment coral's aquaria. The cell pellet was then resuspended in the seawater in the Falcon tube before being dispersed over the entire surface area of coral tissue using the same transfer pipette. This process was repeated for each coral, ensuring that a new sterile transfer pipette was used for each coral. The air was left off for two hours to allow the bacteria to settle on the coral without being displaced by bubbles.

Partial water changes took place three times a week and all treatment corals were administered a second and third dose one week after the prior dose. During this 3-week trial, corals were not fed. Photographs were taken of the corals prior to dosing and daily afterwards to track any signs of emerging disease.

2.4. Characterize the microbiome composition of Fast Lesion Progression (FLP)-affected colonies

As part of the Florida Keys strike team's regular monitoring and treatment of SCTLD-affected corals, observers began noticing lesions on *Orbicella faveolata* colonies that progress across the colony more rapidly than classic SCTLD. Additionally, these lesions did not appear to respond to antibiotic applications. Tentatively termed FLP – fast lesion progression – this potentially novel disease may have implications for some of the largest reef-building corals in Florida. In collaboration with Dr. Karen Neely's project titled "Initial assessments of a potentially novel coral disease: fast lesion progression (FLP)",

the Meyer lab received samples for analysis of microbiome composition and samples of FLP lesions for anaerobic culturing. *O. faveolata* colonies with FLP have been identified at Looe Key, Grecian Rocks, and Carysfort Reef. At each site, a slurry of surface mucus/tissue from 10 colonies was sampled from the FLP lesion and from apparently healthy tissue on the diseased colony. In addition, mucus slurries were sampled from 10 colonies without signs of disease. The microbiomes of these 90 samples were characterized by sequencing the V4 region of the 16S rRNA gene on an Illumina MiSeq with paired 150-bp reads. Community structure will be analyzed in FY25 for changes between healthy tissue, apparently healthy tissue, and diseased tissue. Samples of mucus slurries were received for isolation and culturing efforts of anaerobic bacteria as described above. Extra biomass from these mucus slurries is being stored frozen for additional future isolation efforts.

3. RESULTS

3.1. Isolation and genome sequencing of anaerobic bacteria from SCTLD lesions

3.1.1. Isolation of anaerobic bacteria

Syringe samples of SCTLD lesions/surface mucus and a fragment of SCTLD-affected from *Montastraea cavernosa* were received from the Smithsonian Marine station at the end of July 2023. Culturing of bacteria under anoxic conditions was initiated in a Coy anaerobic chamber on the UF campus. The chamber failed to hold air (a repair that could cost a few thousand dollars), so we also employed small boxes to which we added packets for the generation of anaerobic conditions. However, opening the boxes for transfer to fresh media for isolation results in temporary exposure to oxygen. From this first culturing attempt, we successfully isolated eight bacterial strains, five of which were >99% similar to *Vibrio alginolyticus* and one of which was > 99% similar to *Photobacterium rosenbergii*. *V. alginolyticus* is a common marine bacterium that can be an opportunistic human pathogen by infecting open wounds. The type species of *P. rosenbergii* was isolated from bleached corals in Australia. Each of these cultures was very fast-growing, indicating they are likely facultative anaerobes (grow with and without oxygen). In addition to the six *Vibrio* strains, one strain of *Photobacterium* and one strain of *Alteromonas* were isolated with anaerobic boxes.

Since we were targeting Clostridia as potential pathogens, we modified our protocol for the second culturing attempt. First, we purchased a miniature chamber with hard sides that is less susceptible to leaks and can be purged with nitrogen gas but lacks the ability to scrub residual oxygen (a feature that would cost a few thousand dollars). We used the anaerobic boxes within the miniature chamber to keep conditions as low oxygen for as long as possible. In addition, we added ciprofloxacin to the media in the second culturing attempt. This antibiotic is primarily effective against Gram-negative bacteria like *Vibrio* and *Photobacterium*. Coral-associated Clostridia have previously shown resistance to ciprofloxacin. Growth under these conditions is much slower, consistent with the stricter growth conditions of low oxygen and antibiotics. This method resulted in the successful isolation of one strain of *Qipengyuania* (class Alphaproteobacteria) and one strain of

Mesoflavibacter (Class Bacteroidia). Both strains form bright yellow colonies and exhibit slower growth than the vibrios.

The third culturing attempt used liquid culturing under strictly anaerobic conditions using sealed Balch tubes. We received 24 fresh disease samples from Karen Neely as part of the "Fast Lesion Progression" project in January 2024 for isolation of new strains under strictly anaerobic conditions using Balch tubes.

Culturing under strictly anaerobic conditions using Balch tubes has resulted in four confirmed Clostridial isolates: two in the genus *Wukongibacter* and two in the genus *Vallitalea*. Excitingly, we previously recovered genomes of *Vallitalea* from our metagenomic dataset from SCTLD transmission experiments for comparison to the genomes sequenced by this project. In addition, five additional cultures were grown with Balch tubes including isolated cultures of *Pseudovibrio* (class Alphaproteobacteria), *Pantoea* (class Gammaproteobacteria), and *Latilactobacillus* (class Bacilli). Two cultures (FLP49 and FLP83) were not fully isolated (may contain a few strains) so their taxonomic classification is unknown.

We also tested sodium thioglycolate media in plastic Falcon tubes as a potential way to grow and transfer anaerobes for testing by collaborators as an alternative to shipping glass Balch tubes. Inoculation of Clostridia strains AN30, AN31, and FLP75 in sodium thioglycolate media promoted growth of minor "hitchhikers" in the cultures including *Oceanicaulus* (class Alphaproteobacteria) and *Pseudoalteromonas* (class Gammaproteobacteria). The use of sodium thioglycolate media was not used further. Shipments of glass Balch tubes were successfully sent to SMSFP, but Balch tubes sent to the University of North Carolina Wilmington often leaked in transit, likely due to pressure changes during air travel that altered the rubber stoppers and allowed oxygen to enter.

3.1.1. Genome sequencing of anaerobic bacteria

A total of 22 anaerobic cultures were prepared for genome sequencing (**Table 2**). Extracted DNA from these cultures were submitted to the Interdisciplinary Center for Biotechnology Research on June 3, 2024. Data from the sequencing center has not yet been received. Quality-filtering of the raw sequencing reads, assembly of the genomes, and annotation of the 22 genomes can be completed in less than one workday once the sequencing data is received from the sequencing center. Genome data will immediately be made publicly available through Zenodo and genomes will also submitted to NCBI (where genome submissions can take months to become public).

Table 2. *Summary of strains isolated with anaerobic culturing. All strains beginning with "AN" were sourced from diseased* Montastraea cavernosa *corals and strains beginning with "FLP" were sourced from diseased* Orbicella faveolata *corals. Some of the FLP cultures submitted for genome sequencing may contain two strains, as indicated.*

Strain	Genus	Isolation Technique	Submitted for genome sequencing
AN2	*Vibrio alginolyticus*	Coy chamber + anaerobic box	yes
AN3	*Vibrio alginolyticus*	Coy chamber + anaerobic box	yes
AN5	*Vibrio alginolyticus*	Coy chamber + anaerobic box	yes
AN6	*Photobacterium rosenbergii*	Coy chamber + anaerobic box	no
AN7	*Vibrio alginolyticus*	Coy chamber + anaerobic box	yes
AN8	*Vibrio alginolyticus*	Coy chamber + anaerobic box	yes
AN10	*Vibrio* sp.	Coy chamber + anaerobic box	no
AN11	*Alteromonas*	Coy chamber + anaerobic box	no
AN20	*Qipengyuania*	anaerobic box + mini chamber	yes
AN21	*Mesoflavibacter*	anaerobic box + mini chamber	yes
AN30	*Wukongibacter*	Balch tube	yes
AN30T	*Oceanicaulus*	thioglycolate tube	yes
AN31	*Wukongibacter*	Balch tube	no
AN31T	*Pseudoalteromonas*	thioglycolate tube	yes
FLP24	*Pseudovibrio*	Balch tube	no
FLP34	*Pantoea*	Balch tube	yes (FLP34 + FLP34.1)
FLP34.1	*Latilactobacillus*	Balch tube	yes (FLP34 + FLP34.1)
FLP49	not determined	Balch tube	yes
FLP68	*Vallitalea*	Balch tube	yes (FLP68 + FLP68.1)
FLP68.1	*Zunogwangia*	Balch tube	yes (FLP68 + FLP68.1)
FLP75	*Vallitalea*	Balch tube	yes (FLP75 + FLP75.1)
FLP75.1	*Zunogwangia*	Balch tube	yes (FLP75 + FLP75.1)
FLP75T	*Pseudoalteromonas*	thioglycolate tube	yes

FLP83	not determined	Balch tube	yes
McD51-1	*Vibrio mediterranei*	anaerobic box	yes
McD53-10	*Vibrio mediterranei*	anaerobic box	yes
McD53-9	*Vibrio mediterranei*	anaerobic box	yes

3.2. Challenge cultures of Symbiodiniaceae with putative pathogens

Cultures of *Durisdinium* algal symbionts were challenged with a total of 11 putative pathogens in two trials. In the first trial, *Durisdinium* cultures were challenged with one of three strains (McD53-9, McD53-10, McD51-1) of *Vibrio mediterranei* which were isolated a few years prior to this project using an anaerobic box. This experiment also included a seawater control with no bacteria and a treatment with the probiotic strain *Pseudoalteromonas* McH1-7. Applications of the bacteria included four different concentrations: 10^5, 10^6, 10^7, and 10^8 cells per ml. In all treatments *Durisdinium* cells increased over the 14-day observation period (**Figure 1**). Growth of *Durisdinium* exposed to the *Vibrio mediterranei* strains was not significantly different from growth in the untreated seawater control.

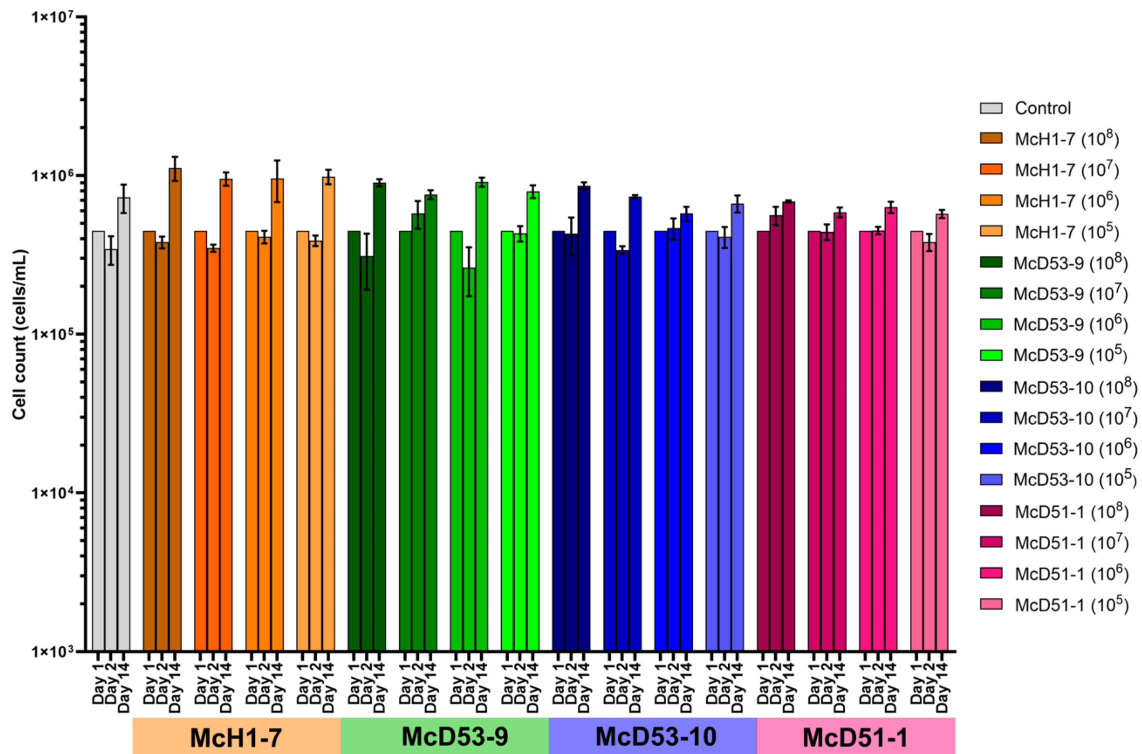

***Figure 1**. Counts of* Durisdinium *cells over time when challenged with Vibrio mediterranei strains McD53-9, McD53-10, and McD51-1. Probiotic strain McH1-7 used*

as a non-pathogenic control. Each bacterial strain was added at concentrations ranging from 10^5 to 10^8 cells per ml. Control Durisdinium *cultures received no bacterial inoculum.*

In the second trial, *Durisdinium* cultures were challenged with one of three treaments: 1) the *Vallitalea* (class Clostridia) strain FLP68, 2) a mix of *Qipengyuania* AN20 and *Mesoflavibacter* AN21, or 3) a pool of five *Vibrio alginolyticus* strains (AN2, AN3, AN5, AN7, AN8). The challenge bacteria were again added at concentrations of 10^5, 10^6, 10^7, or 10^8 cells per ml. Again, we saw no significant differences in the growth of *Durisdinium* over the 14-day observation period and growth in the presence of the putative pathogens was not significantly different than growth in the seawater control (**Figure 2**).

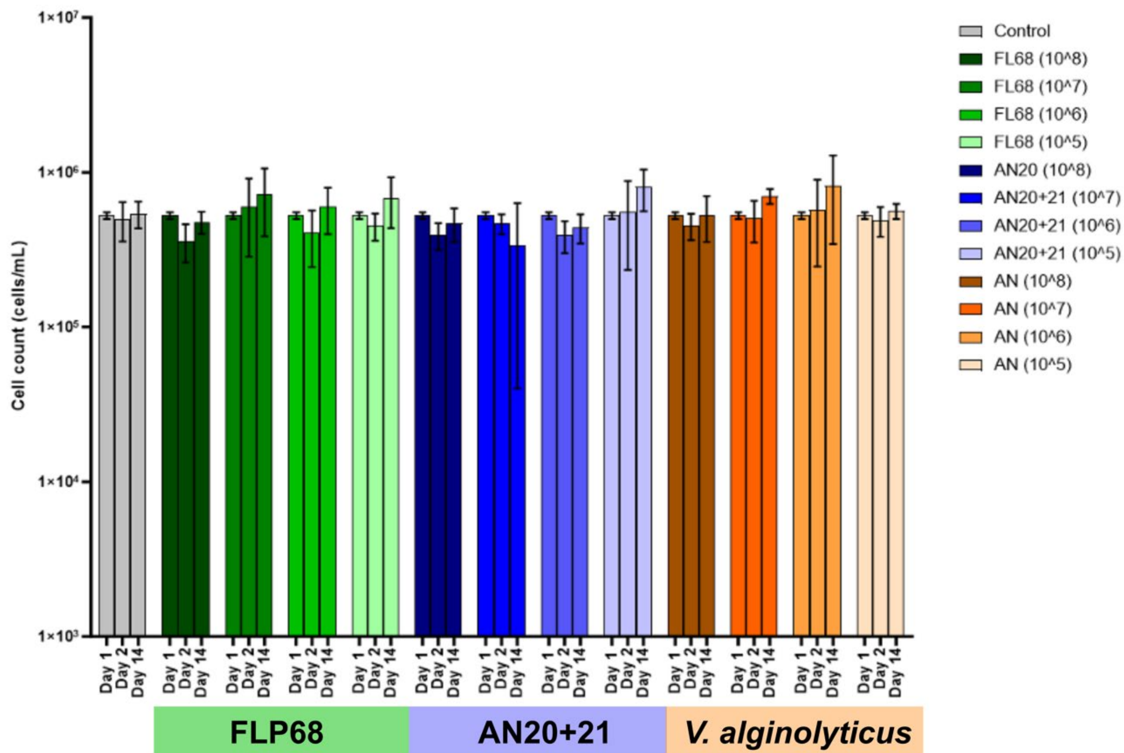

Figure 2*. Counts of* Durisdinium *cells over time when challenged with 1)* Vallitalea *FLP68, 2)* Qipengyuania *AN20/* Mesoflavibacter *AN21, or 3) a pool of 5* Vibrio alginolyticus *strains (designated AN). Each bacterial strain was added at concentrations ranging from 10^5 to 10^8 cells per ml. Control* Durisdinium *cultures received no bacterial inoculum.*

3.1. Challenge healthy corals with putative pathogens

Healthy colonies of *Montastraea cavernosa*, *Orbicella faveolata*, and *Siderastrea siderea* were challenged with a total of 14 anaerobic bacteria in a series of four trials (**Table 1**). In each trial, the corals were dosed with the challenge bacteria once a week and observed over three weeks, receiving a total of three doses of challenge bacteria over the trial. In the first trial, corals were challenged with 1) a pool of three *Vibrio mediterranei* strains (McD51-1, McD53-9, McD53-10), 2) *Mesoflavibacter* strain AN21, or 3) a pool of two *Vibrio coralliilyticus* strains (OfT6-21, OfT7-21) that are known coral pathogens. Untreated control corals that received no bacteria were also observed for the 3-week trial. While a few coral fragments displayed tissue loss over the course of the trial, including an untreated control fragment, most coral fragments exhibited no change overall.

In the second trial, corals were challenged with 1) a pool of five *Vibrio alginolyticus* strains (AN2, AN3, AN5, AN7, AN8), 2) *Vibrio* sp. strain AN10, or 3) *Qipengyuania* strain AN20. No changes were observed in coral appearance over the course of this trial.

In the third and fourth trials, corals were challenged with Clostridia strains including: 1) *Wukongibacter* strain AN31, 2) *Wukongibacter* strain AN30, 3) *Vallitalea* strain FLP68 and 4) *Vallitalea* strain FLP75. No changes were observed in coral appearance over the course of these trials.

3.2. Characterize the microbiome composition of Fast Lesion Progression (FLP)-affected colonies

Samples of disease lesions from *Orbicella faveolata* corals exhibiting Fast Lesion Progression (FLP) were received for microbiome characterization. Samples included apparently healthy coral colonies, active lesions on diseased colonies, and apparently healthy tissue on diseased colonies. We successfully characterized the bacterial community through 16S rRNA gene libraries (V4 region) for 77 FLP samples. After quality-filtering, there were 625 - 112,101 sequencing reads per sample (average = 11,059 reads per sample). Raw sequencing reads are publicly available in NCBI under BioProject PRJNA1120359 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1120359). A total of 5,522 microbial taxa were detected from these 77 samples. Analysis of the microbiome libraries will continue in the next fiscal year.

4. DISCUSSION AND MANAGEMENT RECOMMENDATIONS

Identification of the pathogens that cause SCTLD is critical to understanding the disease outbreak and reducing its impact. Traditional pathogen identification relies on the culturing of bacteria in lab in order to induce disease in a new host. Previous attempts to cultivate potential SCTLD pathogens have not produced obvious or consistently pathogenic strains. Bacteria in the class Clostridia have been detected in multiple studies of SCTLD disease lesions and they possess genes that can produce toxins that may lead to tissue loss in the host. We successfully cultivated four strains of Clostridia from coral disease lesions through this project, as well as several potentially pathogenic *Vibrio*

strains. None of the tested bacteria induced disease signs in corals or in cultures of the algal symbiont *Durisdinium*. It is possible that toxin production was not elicited under the growth conditions tested, therefore we cannot completely rule them out as potential pathogens. Analysis of the genomes of these potential pathogens will continue with alternative funding sources which may provide additional insights regarding the role of these bacteria in disease progression. Nonetheless, this collection of genomes represents a unique dataset of publicly available genomes for coral-associated anaerobic bacteria, including members of the class Clostridia.

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