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Title: Bacterial Predation in Host Microbiomes

Abstract approved: _____

Rebecca L. Vega Thurber

Abstract

In host-associated microbiomes, the mechanisms that regulate community composition or the principles that govern dynamics remain far from clear. However, understanding how the structure of microbial communities shift as the system moves away from a healthy state is critical to assessing disease progression and to formulate any potential mitigation strategy. In this dissertation, I targeted a relatively understudied genus of predatory bacteria, *Halobacteriovorax*, capable of preying on known pathogens, and aimed to determine the ecological role of these unusual predators in the microbiome of their coral host.

Halobacteriovorax are a genus of delta proteobacteria which exhibit a biphasic lifestyle. In attack phase they are small (1 to 2 μm in length and 0.35μm in width), highly motile, single flagellated vibriod shaped bacteria that must attach to other bacteria before penetrating their periplasm where they undergo filamentous growth and genome replication without competition. As nutrients become exhausted from the prey cell, the

elongated *Halobacteriovorax* filament divides into multiple attack phase progeny that lyse the bdelloplast. The ecological role of these predators is still relatively understudied, but given their predatory lifestyle, high grazing rates, and broad prey range, *Halobacteriovorax* could play a major role in structuring microbial communities.

In order to study how cell-cell interactions impact microbial community structure and function, I employed a wide range of methods and technologies utilizing culture dependent and independent techniques. Using high throughput sequencing I detailed shifts in community structure of the microbiome of the mucosal surface layer of multiple coral species in their natural environment by repeatedly sampling individuals over a two-year time scale. *Halobacteriovorax* were a core microbiome component detected in over 78 percent of the 198 samples. Using network analysis I was able to obtain the temporal and spatial dynamics of *Halobacteriovorax,* and show that despite their predatory nature they predominately co-occur with their potential prey in our networks. I also isolated and cultured novel *Halobacteriovorax* strains from multiple coral species, and characterized these coral-associated predatory bacterial isolates using full-length 16S rRNA sequencing of cultures, phylogenetic analysis of the full length reads, prey range evaluation, and microscopic documentation of unique predatory lifecycle stages. In order to study cell-cell interactions I employed microfluidic devices and high-resolution video microscopy, image analysis, and cell tracking to observe individual predator-prey interactions utilizing predatory *Halobacteriovorax* and a common pathogen to a variety of aquatic host organisms, *Vibrio coralliilyticus*, as the prey. In this co-culture system, I captured striking microscale observations that demonstrate *Halobacteriovorax's* ability to effectively prey on and reduce pathogenic *V. coralliilyticus* populations.

To illuminate the role of *Halobacteriovorax* on the host microbiome, I challenged specimens of the important reef-building coral *Montastraea cavernosa* with *V. coralliilyticus* pathogens in the presence or absence of *Halobacteriovorax* predators, and then detailed the changes in the microbial communities over time using 16S rRNA amplicon sequencing. The pathogen challenge reshaped coral microbiomes by increasing richness and reducing stability (increased beta-diversity) of the rest of the microbiome, suggesting strong secondary effects of pathogen invasion on commensal and mutualistic coral bacteria. The addition of *Halobacteriovorax* alone had only minor effects on the microbiome, and no infiltration of *Halobacteriovorax* into coral tissues was detected in amplicon libraries. Simultaneous challenges with both pathogen and predator eliminated detectable *V. coralliilyticus* infiltration into coral tissue samples, ameliorated changes to the rest of the coral microbiome, and prevented secondary blooms of opportunistic bacteria. All together my results suggest predation by *Halobacteriovorax* may act as a mechanism to regulate population size of a wide range of opportunistic pathogens and illustrates the powerful role of these predatory bacteria in the marine microbiome.

Bacterial Predation in Host Microbiomes

by
Rory M. Welsh

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APPROVED:

Major Professor, representing Microbiology

Chair of the Department Microbiology

Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Rory M. Welsh, Author

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CONTRIBUTION OF AUTHORS

Chapter 2:

Rory M. Welsh collected the samples with contribution from scientific divers, isolated the *Halobacteriovorax* bacteria with help from Stephanie Rosales, conducted the data analysis, and prepared the manuscript. Drs. Rebecca Vega Thurber and Deron Burkepile contributed to the experimental design, data analysis, manuscript preparation, and funding of the project. Drs. Jerome Payet & Jesse Zaneveld contributed to data analysis and manuscript composition.

Chapter 3:

Rory M. Welsh conducted the experiments, contributed to experimental design, preparation of the manuscript, and data analysis. Dr. Rebecca Vega Thurber was involved in the experimental design, data analysis, manuscript composition, and funding of the project. Drs. Jerome Payet & Jesse Zaneveld contributed to data analysis and manuscript composition, Ryan McMinds and Stephanie Rosales contributed to manuscript composition.

Chapter 4:

Rory M. Welsh conducted the experiments, contributed to experimental design, preparation of the manuscript, and data analysis. Dr. Rebecca Vega Thurber contributed to funding of the project, experimental design, and manuscript composition; Dr. Roman Stocker was involved in the experimental design, and funding of the project; Dr. Melissa Garren contributed to experimental design and data analysis.

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Chapter 1

Introduction: Bacterial Predation in Host Microbiomes

Summary line: Halobacteriovorax are marine predatory bacteria that alter the dynamics of their host microbiomes, in turn, modulating the health of their animal hosts

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Introduction

In macroecology, predators play major roles in structuring ecosystem function and community diversity. Yet relatively little is understood about the roles of bacterial predators in the community ecology of microorganisms and host-associated microbiomes. Host-associated microbial community dynamics are affected by extrinsic environmental factors such as resource availability, temperature, and salinity, as well as host diet, genetic background, and health. Cooperative symbioses, antagonisms, competition, and predation also all occur within a microbiome (Rohwer & Kelley, 2004). Thus microbiomes are complex ecologies where intrinsic interactions among the members of the community may also significantly alter the structure and function of the microbiome, at the benefit or detriment to the host. For example, the normal flora on and within a host often act as effective barriers to pathogens by physically blocking attachment sites and consuming nutrients, depriving pathogens of habitat space and necessary resources (Round & Mazmanian, 2009). Thus, in these cases, microbiomes have the ability to prevent invasive pathogenic microbes from colonizing and infecting the host. Similarly, predatory bacteria likely play a significant role in controlling pathogenic bacterial populations. While the impacts of predation by viruses and protists on bacterial mortality, diversity, and evolution have been extensively studied (Fuhrman, Cram & Needham, 2015), less is understood about if or how microbial-mediated predation may shape communities or drive key alterations in host niches. Here we discuss recent evidence examining the hypothesis that predatory bacteria are keystone members of microbiomes that influence the health of their hosts through the consumption of pathogenic opportunists.

Bacterial Predators

Diverse modes of predation have evolved independently across taxonomically disparate bacteria (Jurkevitch, 2007). For example, the alphaproteobacteria *Micavibrio aeruginosavorus* and the Bacteroidetes *Cytophaga huntchinsonni* are epibiotic predators. The deltaproteobacteria *Myxococcus* employs a different "wolf pack" strategy that utilizes saprophytic swarming to consume prey. Both of these groups are typically facultative predators. However, another group of predators, the deltaproteobacteria *Bdellovibrio* and like organisms (BALOs), are primarily obligate periplasmic predators that consume a wide variety of bacteria (Box 1). This somewhat unique lifestyle has fascinated scientists and led to investigations of the use of BALOs as living antibiotics. Yet, as with all non-viral predators, these clades of predatory bacteria tend to be in low relative abundance (<1-10%) in their habitats, and thus have remained at the fringe of microbial ecology research.

In 1962 the Bdellovibrio were first discovered in terrestrial soils (Stolp & Petzold, 1962). Then in 1973 the application of BALOs as a biocontrol agent was first conducted to prevent the bacterial blight of soybeans caused by *Pseudomonas syringae* (formerly *P. glycinea*) (Scherff, 1973). Unfortunately, the considerable obstacles inherent to the co-culture requirements of growing predators and prey together has limited the rate of publications in the field for some time (mean ~10/year since their discovery). More recently, several significant advances in characterizing several aspects of BALO biology have occurred including research on their cell-cycle, physiology, biochemistry, taxonomy, and utility as probiotics. In 2004 the first genome was released (Rendulic et

al., 2004) and since then numerous freshwater and marine strains have been sequenced, providing additional tools for researchers interested in BALO biology (Hobley et al., 2012; Chen et al., 2015). For example, proteomic and transcriptomic analysis of the two phases of the life cycle, attack phase and growth phase, have revealed cell cycle dependent expression and functions in BALOs (Pan, Chanda & Chakrabarti, 2011). However, currently little is known about the ecology of these organisms and whether they truly act as the cheetahs of the microbiome.

Below we summarize research on the isolation, ecological characterization, and application of one group of predatory bacteria within the BALOs, the *Halobacteriovorax*, to demonstrate that these microbial predators play clear top-down roles (sometime referred to as sideways control) in microbial community ecology. We also present evidence that this top down control by *Halobacteriovorax* has major implications for the health of some animals by consuming opportunistic copiotrophs and preventing infection by pathogens.

Life cycle of the marine *Halobacteriovorax*

While the closely related genus *Bdellovibrio* are typically found in freshwater and terrestrial systems, *Halobacteriovorax* (formerly Bacteriovorax) are ubiquitous in saline environments such as the estuarine and marine systems that occupy 71 percent of the world's surface (Pineiro et al., 2007). Like their terrestrial cousins, *Halobacteriovorax* are a genus of small (1 to 2 μm in length and 0.35μm in width) highly motile delta-proteobacteria that exhibit a biphasic lifestyle that includes an attack phase and growth and replication stage. In the attack phase (a), *Halobacteriovorax* exist as single

flagellated vibriod shaped cell that prey exclusively on other gram-negative bacteria, including many known pathogens (b). When *Halobacteriovorax* encounters a potential prey they attach to and penetrate the outer membrane (c), enter the periplasm, seal their entry point in the prey's membrane, and quickly kill the prey cell by releasing an arsenal of hydrolytic enzymes. The prey is converted into the bdelloplast (d), a rounded structure that allows the *Halobacteriovorax* undergo filamentous growth and genome replication without competition (e). As nutrients become exhausted from the bdelloplast, the elongated *Halobacteriovorax* filament is triggered to divide into multiple (f) attack phase progeny that lyse the bdelloplast (g). In culture conditions the entire life cycle takes only ~3.5 hours.

Along with these main life cycles, some strains exhibit host independent stages, while others are capable of entering spore-like resting states in the bdelloplast, but whether these are broadly adopted strategies or whether they occur in the natural environment is unknown.

Isolating predatory bacteria from host-associated systems.

BALOs are among the smallest members of the rare microbiome and are primarily obligate predators; thus isolating and culturing these bacteria is challenging. To isolate individual strains, we have used techniques from Dr. Henry William's laboratory at Florida A&M which consists of an initial size-filtration step, followed by enrichment that uses the common double layer plate technique to identify potential isolates from characteristic plaques (Chauhan & Williams, 2008). For example, host samples are first homogenized and passed through a 0.45μm filter, which excludes larger cells and protozoan grazers, and thus separates out BALOs by exploiting their small size. The

filtrate can then be used directly for double layer plates and enrichment cultures. Because BALOs can sometimes preferentially attack and kill one prey more readily than others, enrichment cultures can either be made by adding nutrients to boost the levels of native prey when targeting a wide range of predators, or by simply adding in the target pathogen and then enriching for predators adapted to attack and kill the prey of choice. In ~2-3 days plaques begin to develop on double layer lawns of prey bacteria and these plaques continue to spread for a approximately a week. Plaques are excised and examined under phase contrast microscopy. Those found to contain small highly motile bacteria (these predators move at rates ~100-160 μm/sec) are further purified and characterized for downstream applications.

Using the methods described above, *Halobacteriovorax* have been isolated from a wide-range of organisms and habitats (Fig 1). Predatory bacteria have carved out niches in almost every environment on the planet. We ourselves have collected samples of *Halobacteriovorax* from marine environments ranging from the warm waters of Caribbean to the below-freezing waters and sea ice of Antarctica where the water column temperatures are a constant -2 °C year round (Fig. 1). We have also now isolated and characterized *Halobacteriovorax* strains from 4 different coral hosts in the Florida Keys (Fig. 1) and begun to use community based data and addition experiments to determine the roles of these predators in marine hosts. *Halobacteriovorax* are also routinely detected and in host-associated communities in gene amplicon and metagenome survey studies.

Ecological characterization of *Halobacteriovorax* interactions on a marine host

Using our *Halobacteriovorax* strains isolated from marine corals, we confirmed that these predators can prey upon known members of the coral microbiome in a cultured setting (Welsh et al., 2015b). However, whether they do so in nature and with what members of the host's microbiome were two unresolved questions. Considering the wealth of sequence data emanating from recent microbiome studies, a highly desirable next step beyond categorizing and cataloging the communities is to begin to assign roles to individual members. Exploring predator populations in amplicon and metagenomic datasets allows for clear hypothesis testing. Ideally predator-prey cycles in the microbiome would be studied by absolutely quantitative measures of abundance in future studies, but without *a priori* knowledge of which biological interactions to target out of the hundreds of taxa present, truly quantitative approaches are exceedingly costly and logistically infeasible. However network analysis now offers exciting possibilities for evaluating interactions in microbial ecology. Thus we used co-occurrence network analysis on a previously generated set of 16S amplicon data from 3 coral species to evaluate the potential interactions of *Halobacteriovorax* with other members of the microbiome (Fig 2). This three-year dataset of amplicon libraries revealed *Halobacteriovorax* were consistently associated with corals (79% prevalence) across the reef study site (Welsh et al., 2015b). Yet, as with all non-viral predators, these clades of predatory bacteria tend to be in low relative abundance, and their mean relative abundance across all libraries was quite low (0.40% ± 0.04 SEM). However, this three-year monthly sampling time series combined with network analysis revealed that corals harbor active bacterial predators that interact consistently with heterotrophic coral microbes (Fig 2). For example, on *Agaricia* spp. corals *Halobacteriovorax* were found to

positively co-occur with 8 members of the coral microbiome such as Vibrionales, Cytophagales and Atleramodadales, three known coral opportunists in Chapter 2 (Welsh et al. 2015). Using this approach we now had a condensed list of potential biological interactions to beta-test in our future studies using application experiments and truly quantitative methods.

Application of *Halobacteriovorax* to study the effects of predation and pathogenesis in hosts

The predatory nature, broad prey range, and high grazing rates of *Halobacteriovorax* all lead to the intriguing possibility of using these and other predatory bacteria as "living antibiotics," particularly against fast growing pathogens such as vibrios. An alternative to antibiotics is needed, given the rise in antibiotic resistance among pathogenic bacteria, however, the effectiveness of predatory bacteria as a viable alternative remains to be seen. Nevertheless, efforts to use these predators as probiotics have already begun. In the United States, the Defense Research Projects Agency recently initiated a program to study the effectiveness of predatory bacteria's ability to eliminate pathogens. And in aquaculture *Halobacteriovorax* has been shown to prey on a wide range of gram-negative bacteria including many known pathogens (Cao et al., 2013a, 2015). By controlling blooms of opportunistic pathogens, *Halobacteriovorax* has the potential to regulate opportunistic pathogens, reduce stress, and increase growth rates in aquaculture settings. For example, in a pair of recent studies the probiotic application of BALOs can significantly increased survival rates of shrimp when challenged with vibrio

pathogens in aquaculture systems (Cao et al., 2013b). Similarly, in a 2012 study of vibrios in seawater and shellfish, USDA researcher Gary Richards and others documented a rapid decrease of pathogenic vibrios accompanied by the simultaneous increase in native predatory bacteria populations, leading to the notion that predatory bacteria are important modulators of pathogenic vibrios in seawater and oysters (Richards et al., 2012).

Although these initial findings are encouraging, there is a wide gap in where we are now and where our understanding needs to be before predators of pathogens becomes a viable therapeutic or mitigation option. There are several important considerations and potential drawbacks to using any biological control and for predatory bacteria one must consider the following issues: i) incomplete removal of target prey (BALOs generally do not remove 100% of their prey), ii) unintended grazing upon beneficial or commensal bacteria, iii) unexpected side effects of predator persistence, and iv) evolution of resistant phenotypes or strains.

There are, however, tremendous opportunities beyond the therapeutic application of predatory bacteria as living antibiotics. Predatory and pathogenic bacteria, with their rapid growth and small size, represent a unique opportunity to study fundamental theories of ecology and evolution of predator-prey dynamics as well as the role of predators in structuring their communities. As we and others have found, the additions of pathogens to a microbiome can cause disproportionate changes to the microbial community and precisely predicting what causes a healthy microbiome to shift to a disease state or maintain a healthy steady state is one ultimate goal in the field of microbial ecology. For exampling, using our coral microbiome model, *Montastrea cavernosa*, we explored the

protective effects of using bacterial predators coupled with the time series microbiome data to discover how single members (i.e., pathogens) or combinations of interacting members (i.e., predators and potential prey) alter the structure of host microbiomes in Chapter 3 (Welsh et al., 2015a). We found that pathogens alter the microbiome in various ways and these changes and their resulting impacts on host health are somewhat alleviated by the addition of their predator. Yet we have only begun to scratch the surface and a better understanding of the cascading effects that drive shifts in microbiome structure and function during the therapeutic biocontrol of pathogen by predatory bacteria is clearly an area, which needs further exploration.

Figures

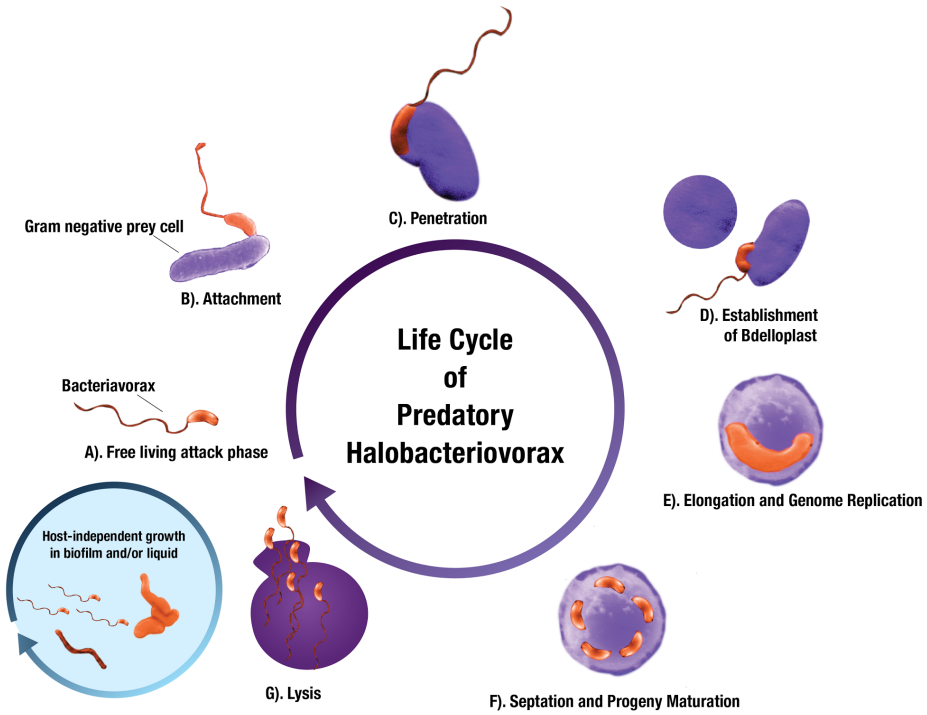

Figure 1.1 Life Cycle of Predatory Halobacteriovorax. A. Free Living Attack Phase *Haloacteriovorax* actively seek out prey. Once contact is made with a potential prey (B. Attachment) *Haloacteriovorax* determines whether it is an acceptable host and irreversibly enter the periplasm (C. Penetration). They quickly kill the prey, release hydrolytic enzymes (D. Establishment of Bdelloplast), and undergo filamentous growth and genome replication (E. Growth and Replication). As nutrients become exhausted from the prey cell the filament partitions (F. Septation and Maturation) and the progeny develops into highly motile flagellated predator cells. The progeny lyse their bdelloplast (G. Lysis) to repeat the process or enter host-independent (HI) growth. In laboratory controlled settings and in the presence of excess nutrient HI mutation rate is approximately 1 in 10^7 attack phase predators.

Figure 2.2 Map Illustratiing Global Range of Halobacteriovorax. Map illustrating the global distribution, variety of host, and range of environments where *Halobacteriovorax* has established a niche. The host-associated isolates and environments include (clockwise from top left) Pacific oysters, Caribbean coral, biofilms growing on the sea ice at the seawater ice interface in Antarctica, and the Great Salt Lake in Utah.

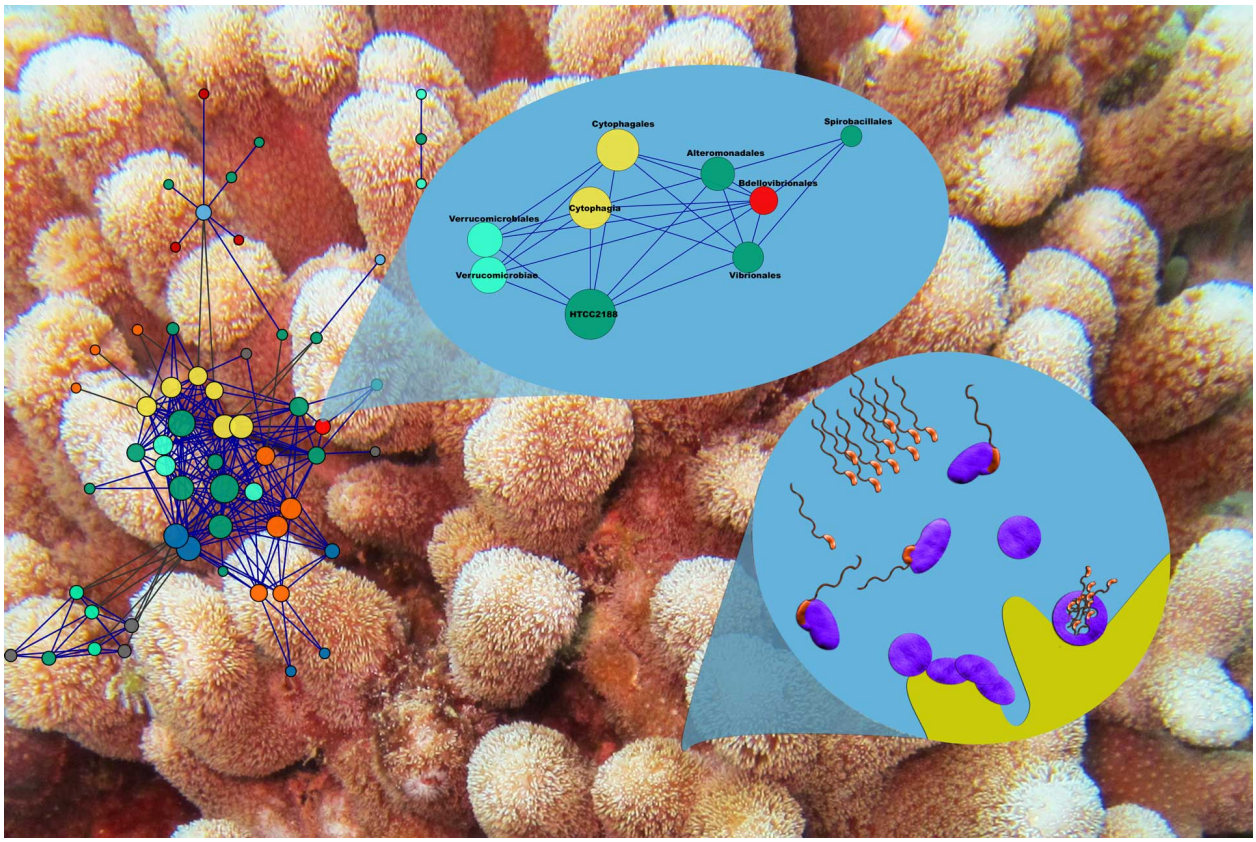

Figure 1.3 Schematic of Halobacteriovorax in Coral Host Microbiome and Co-occurrence Network Schematics of the interactions (left networks) and potential predation events (lower right diagram) of *Halobacteriovorax* on Caribbean coral microbiome members. Using longitudinal 16S rRNA amplicon data and network analysis we found that *Halobacteriovorax* (Bdellovibrionales, red node) positively interacted with 8 members of the coral microbiome. These data suggest that these predators likely enact top down control these gram-negative bacteria in the holobiont.

Chapter 2

Bacterial predation in a marine host-associated microbiome

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Abstract

In many ecological communities, predation plays a key role in regulating community structure or function. While predation has been extensively explored in animals and microbial eukaryotes, predation by bacteria is less well understood. Here we show that predatory bacteria of the genus *Halobacteriovorax* are prevalent and active predators on the surface of several genera of reef-building corals. Across a library of 198 16S rRNA samples spanning three coral genera, 79% were positive for carriage of *Halobacteriovorax*. Cultured *Halobacteriovorax* from *Porites asteroides* corals tested positive for predation on the putative coral pathogens *Vibrio coralliilyticus* and *Vibrio harveyii*. Co-occurrence network analysis showed that *Halobacteriovorax's* interactions with other bacteria are influenced by temperature and inorganic nutrient concentration, and further suggested that this bacterial predator's abundance may be driven by prey availability. Thus animal microbiomes can harbor active bacterial predators, which may regulate microbiome structure and protect the host by consuming potential pathogens.

Introduction

Host-microbe relationships are so important for the health of many plants and animals that the term "holobiont" has been coined to describe the sum of a host and its symbionts (Hosokawa et al. 2006). In addition to their interactions with an animal host, host-associated microbes simultaneously compete and cooperate with one another, and alterations to these interactions can affect holobiont function (De Boer et al. 2007). While top-down control via predation is known to structure many animal communities (Baum & Worm 2009), the role of predation by bacteria on the structure of microbial communities is less well studied. Both specialist and generalist predatory strategies impact ecological

communities by shifting community composition and abundance, but through different mechanisms. Specialists' predation via viral infections drives an evolutionary arms race within their specific prey population and can destroy entire clonal blooms of phytoplankton (Martínez et al. 2007). Conversely, generalist predators have more influence over the diversity and dynamics of their aggregate prey community.

Bdellovibrionales and like organisms (BALOs) are an order of predatory δ-proteobacteria that prey exclusively on other bacteria, including many known pathogens (Schoeffield & Williams 1990). Predation by BALOs also releases nutrients (Martínez et al. 2013), affecting biogeochemical cycling and production in nutrient limited environments, similar to the effects of phage (Brussaard et al. 2008; Fuhrman 1999). In contrast to viruses, which are the most abundant biological entity in the marine environment and maintain abundances approximately an order of magnitude greater than their hosts, BALOs do not need to be in high initial concentrations in the environment to drive significant bacterial mortality of their prey (Williams et al. 2015; Richards et al. 2012). One group of Bdellovibrionales, the marine *Halobacteriovorax*, are broadly distributed in marine waters across temperature, salinity, and pH gradients (Pineiro et al. 2007). *Halobacteriovorax* predation represents a regulatory mechanism that can alter the structure of bacterial communities (Li et al. 2014).

Scleractinian or hard corals are widely studied due to their fundamental role as ecosystem engineers that build tropical reefs. Previous metagenomic and 16S amplicon studies suggest that *Halobacteriovorax* are members of coral microbiomes, as they were detected in Porites (Wegley et al. 2007), and Acropora species (Sweet & Bythell 2015). However, active predation by coral-associated *Halobacteriovorax* strains and the

ecological interactions of this genus with other coral-associated microbes remain unexplored. Here we use a combination of cultivation, predation assays, microscopy, and 16S rRNA gene amplicon 454 pyrosequencing (Supplementary Methods) to assess *Halobacteriovorax's* role in the microbiomes of three corals (*Siderastrea siderea, Agaricia spp.* and *Porites spp*.) in a three-year dataset.

Results and Discussion

We confirmed that *Halobacteriovorax* are members of coral microbiomes by culturing isolates from the three coral genera sampled in the time series experiment and *Montastraea cavernosa* corals adjacent to the experimental plots (Fig. 2.1A-D; Table S2.1). Using full-length 16S analysis the phylogenetic relatedness of these isolates to other BALOs was inferred (Fig. 2.1E). Our *Halobacteriovorax* isolates were all identical at the 16S rRNA sequence level (Accession # KR493097), and grouped within the previously established *Halobacteriovorax* cluster XIII (Fig. 2.1E; Pineiro et al. 2007).

We then explored whether our strains targeted other members of the coral microbiome for predation by selecting one of our *Porites astreoides* isolates, *Halobacteriovorax sp.* PA1, for the plaque assay technique which determines prey range (Schoeffield & Williams 1990). *Halobacteriovorax sp*. PA1 successfully attacked and consumed the known coral pathogens *Vibrio coralliilyticus* and *Vibrio harveyi*, as well as a *Vibrio fortis* strain (accession no. AJ440005, CP009468.1, KT626460, respectively) we isolated from an apparently healthy *P. asteroides* colony. Like free-living isolates, host-associated *Halobacteriovorax* followed a biphasic lifecycle consisting of growth and attack phases (Fig. S2.1) visible in SEMs (Fig. 2.1B-D).

We then examined the prevalence of *Halobacteriovorax* across coral microbiomes using a three-year time series of 16S rRNA libraries generated from the surface mucus layer of corals (Supplemental Methods) that were exposed to increased nitrogen and phosphorus to simulate nutrient pollution, environmental factors that indirectly contribute to coral disease (Vega Thurber et al. 2014). *Halobacteriovorax* was prevalent in >79% of all samples (n=198) and not detected in the other samples. Prevalence of *Halobacteriovorax* in nutrient enriched corals (81%; n=115) was not significantly different ($p = 0.28$) from controls (78%; n=83); *Halobacteriovorax* relative abundance was also not significantly different ($p = 0.14$) between the two data sets. The mean relative abundance of *Halobacteriovorax* across all libraries was low (0.40% ± 0.04 SEM), similar to their abundances in estuarine systems (Pineiro et al. 2013) and as characteristic of predators across most ecosystems (Baum & Worm 2009). The high prevalence of this taxon and its predatory lifestyle suggests it is an important low abundance member of the core coral microbiome (core members are detected in ≥75% and are consistent components of the microbial assemblage). Given their low abundance, false negatives cannot be established with certainty, and the reported prevalence values likely represent a lower bound on *Halobacteriovorax* prevalence.

To study the possible interactions between predatory bacteria and other members of the host microbiome, we constructed co-occurrence networks from our coral microbiome dataset using the CoNet plugin (Faust et al. 2012; Faust & Raes 2012) for Cytoscape (Shannon et al. 2003). Networks were generated from the taxonomic order level and provide net effect of all BALO predation. CoNet uses a permutation-renormalization and bootstrap (ReBoot) method to mitigate the effects of spurious

correlations induced by compositionality while assessing the significance of an association. Networks were constructed separately for two known drivers of shifts in the coral microbiome, temperature and nutrient enrichment. Thus, networks were binned by: a) temperature (2ºC intervals), and b) nutrient enrichment, as well as by c) coral host (Table S2.2).

Changing thermal regimes affected bacterial predator-prey interactions (Fig. 2.2, Table S2.3). The largest number of significant taxon co-occurrences (network edges) between BALOs and other bacterial taxa occurred at the highest and lowest extremes of temperature (Fig. 2.2). Networks from the highest temperature datasets formed two distinct clusters. BALOs are a component of the larger cluster in both the control and nutrient networks, where their edges in the network interact with several taxa including Myxococcales, another predatory bacterial taxa, suggesting that bacterial predators in the network respond to common drivers. Bacterial predator-prey interactions in the networks also fundamentally changed under nutrient enrichment (Fig. 2.2). Only three shared co-occurrence taxa (Chromatiales, NB1-j, and unclassfied79) were detected in the networks from the nutrient treatment samples (Table S2.3), and only a single taxa (Cytophagales) was shared between the nutrient and control host network.

Siderastrea and *Agaricia* coral species contributed a majority of the BALO interactions in the host networks while *Porites* accounted for only 8% (Table S2.4). Some the interactions were different among the different coral host. For example, the Bdellovibrionales co-occurred with Vibrionales on *Agaricia* corals but not the two other host taxa (Table S2.4). Interestingly, in all the networks generated, BALOs had exclusively positive co-occurrence interactions (blue) and never exhibited mutual

exclusions (grey) (Fig 2.2). Co-occurrence interactions suggests that: a) Bdellovibrionales populations are increasing due to the increase in their bacterial prey populations, b) conditions are favorable to Bdellovibrionales and the co-occurring taxa, and/or c) the predatory bacteria are removing competitors of co-occurring taxa allowing other members to increase. Given the well-known role of prey abundance in other predator-prey systems, response to prey abundance would parsimoniously explain the co-occurrence data. An alternative hypothesis is that low read depth precludes detection of negative interactions by *Halobacteriovorax*. We examined whether negative interactions were observed for other low-abundance microorganisms. Bacterial relative abundance showed little correlation with the number of negative edges detected (Pearson correlation, r2 = 0.00159). The same relationship held when restricting regression analysis to taxa equally or less abundant than *Halobacteriovorax* (Pearson correlation, r2 = 0.007).

Active predation by host-associated bacteria opens an intriguing area of research into the structure and function of animal microbiomes. Longitudinal analysis of the coral co-occurrence networks simultaneously delivers both a broad census of the microbiome along with a condensed list of potentially interesting biological interactions that can be targeted in future studies using truly quantitative methods (e.g., qPCR of predator-prey cycles). Such truly quantitative approaches would be costly and potentially infeasible without a priori knowledge of which members to target.

Our findings establish that regardless of their low abundance, *Halobacteriovorax* predation may significantly impact microbiome dynamics in a context-dependent manner and, as a corollary, global change could alter predator-prey dynamics on host-associated microbiomes.

Supplementary Materials And Methods

Study Site and Coral Mucus Microbiome Sample Collections

Our study site, Pickles Reef (25° 00' 05"N, 80° 24' 55"W), is located within the Florida Keys Reef Track, approximately 8 km offshore Key Largo, FL, USA. In June 2009 we established a three year experiment where we enriched four replicate, 9 m^2, plots of reef with nitrogen and phosphorus alongside four control plots that experienced ambient nutrient conditions (Vega Thurber et al., 2014). The 9 m^2 plots consisted of 3x3 grids of 1 m^2 plots, and each 1 m^2 plot had nails marking the corners and center. The four plots with the nitrogen and phosphorous enrichment treatment consisted of 175 g of Osmocote® (19-6-12, N-P-K) slow-release garden fertilizer in 15 × 5 cm (length × inner diameter) PVC tubes with 6, 1.2 cm holes drilled into the PVC tube. The PVC tubes or 'nutrient diffusers' were wrapped with mesh window screen to retain the Osmocote® within the diffusers, and a separate diffuser was attached to each of the nails (25 diffusers/ plot) in the enrichment plots (Worm et al. 2000, Burkepile & Hay 2009). This method of enriching benthic water column nutrients increases soluble reactive phosphate (SRP) to a level (ca. 0.25 μm) 9 times that of ambient and dissolved inorganic nitrogen (DIN) to 3 times the level of ambient (ca. 4 μm), and contains no trace metals which might confound the main effects of SRP and DIN (Vega Thurber et al. 2014, Heck et al. 2000).

To examine the microbiomes of corals in the experimental treatments, coral-associated bacteria were isolated, while on SCUBA, using syringe-sampling methods previously described (Sunagawa et al., 2009). Briefly 10 ml of coral mucus samples were

collected from individuals colonies of the following stony coral taxa: *Porites spp., Agaricia spp.,* and *Siderastraea siderea*. Coral taxa included in the genera bins are as follows: Agaricia are likely all *Agaricia agaricites, Porites* consist of *P. asteroides* and *P. porites*, and *Siderastraea* are all *S. siderea.* Samples were brought on board, immediately stored on dry ice for transport to laboratory facilities, and stored at -20°C prior to DNA extraction. Information on frequency of sample collection under nutrient enrichment and control conditions as well as for each of the coral genera sampled has been summarized in Table S1.

Bacteria Culturing and Isolation

From additional *Montastraea cavernosa* corals adjacent to the experimental plots and a subset of the time series corals, approximately 30 ml was used to isolate and culture predatory coral-associated bacteria. Salinity, depth and temperature measurements were recorded during sampling. The viscous mucus samples were size fractionated through glass fiber filter, GF/F 0.7 μm (Whatman, UK), to remove protozoan grazers, and transferred into a 50 ml tube containing a glass microscope slide coated with 2.0% agar and 0.01% yeast extract (modified Chauhan & Williams 2008). The process was repeated with the seawater collected 15 cm above corals as a control. The substrate enrichment tubes then were incubated at room temperature on a gently shaking nutator. A *Vibrio fortis* P1 strain isolated from *Porites* corals in autumn 2010 was selected as a preferred prey to use for *Halobacteriovorax* spp. isolation. *Vibrio fortis* strain P1 was isolated by GFF filtering coral mucus and plating on polypeptone 20 medium, Pp20, consist of 1 g of polypeptone in 1 L of seawater and 1.5% agar (Difco).

Halobacteriovorax spp. were isolated by taking a 2 ml aliquot immediately after GFF purification and at days 3, 5 and 7 from the substrate enrichment tubes. The aliquot was used for a dilution series (100%, 1:100, 1:1000) and plated using a double agar overlay method (Schoeffield & Williams, 1990; Jurkevitch, 2006). Plates were incubated at 25ºC and monitored daily for round plaque forming units (PFU) with sharp boundaries. Plaques were removed from the top layer of agar, re-suspended in sterile seawater, and visualized under phase contrast microscopy for small, highly motile, potential *Halobacteriovorax* cells. Plaques containing highly motile cells were filtered, and a series of dilutions were again plated as described above in order to obtain pure cultures. Cultures were archived in 7% DMSO freezer stocks.

Bacterial prey strain *Vibrio coralliilyticus* ATCC BA450, *Vibrio harveyi* MAC, and *Vibrio fortis* P1 were grown on Pp20 plates. To perform double layer plaque assays, 100 µl of prey were spread evenly over Pp20 plates and incubated at 25°C overnight. Prey plates were flooded with sterile seawater and resuspended to ~10^9 cells/ml (OD_{600} 0.65). Each potential prey was used with serial dilutions of *Halobacteriovorax sp. PA1* to make double layer agar previously mentioned and plaques were confirmed as *Halobacteriovorax* under phase contrast microscopy.

Halobacteriovorax DNA Extraction, Purification, and PCR Amplifications

Environmental DNA from coral mucus samples were obtained using collection and extraction methods previously described (Correa et al., 2009). *Halobacteriovorax* culture isolates were first 0.45 µm filter purified to remove associated prey, and the filtrate was pelleted for 10 minutes at 20,000 X g at room temperature (RT). Pelleted

DNA was extracted using a genomic prep mini spin kit (GE Healthcare, UK). *Bacteriovorax* specific 16S rDNA primers Bac-676F (5'-ATTTCGCATGTAGGGGTA-3') and Bac-1442R (5'-GCCACGGCTTCAGGTAAG-3') (Jurkevitch *et al.*, 2006) were used to confirm the presence of BALOs. 50 µl PCR reactions were performed (10 µl 5x buffer, 2.4 mM of MgCl, 0.2 µM each primer, 2.5U of Taq polymerase, 0.2 nM of each dNTP, and 1 µl extrated DNA) using the following touchdown thermo-cycler program (the conditions used for cloning are in parentheses): 95°C 2 min, 10 cycles of 95°C 1 min, 60°C (63°C) 1 min -0.5°C per cycle, and 72°C 1 min, 20 cycles of 95°C 1 min, 55°C (53°C) 1 min, and 72°C 1 min, and a final extension of 72°C 5 min. Primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3')-1492R (5'- GGCTACCTTGTTACGACTT -3') were used to amplify all material prior to cloning using the Invitrogen TOPO-TA Cloning Kit (pCR4-TOPO vector, Invitrogen).

Phylogenetic Analysis of Halobacteriovorax Isolates.

All sequences were aligned using MUSCLE (MUltiple Sequence Comparison by Log-Expectation; (Edgar, 2004). Gaps and poorly aligned positions were eliminated using Gblocks (Castresana, 2000). The resulting unambiguously aligned 1216 base pair sequences were reconstructed into a maximum likelihood tree using MEGA6 (Tamura et al., 2013) with Deltaproteobacteria NB1-J (AB013831) as the outgroup. The bootstrap consensus tree was inferred from 500 replicates (Felsenstein, 2010). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. Initial trees for the heuristic search was obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the

Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The phylogenetic tree was visualized using the program FIGTREE v1.4.2 (tree.bio.ed.ac.uk/software/figtree/).

Microscopy

Samples were prepared by first preserving 100 µl aliquots of cultures with 900 µl of 0.05 M sodium cacodylate buffer (EMS Sciences Cat #12300) containing 2.5% final concentration fresh glutaraldehyde (EMS Sciences Cat #16320), and stored at 4°C for a minimum of 48 hours before imaging. Fixed samples were transferred to a 0.05 µm Whatman filter using slow even vacuum pressure <5 PSI. Filters were rinsed once with 20% ethanol in order to removing salt build up, affixed to an aluminum stub using carbon adhesive tape, and coated in a thin layer of palladium using a sputter coater (Cressington 108 Sputtercoater, Cressington Scientific). Filters were viewed using a Philips XL30 ESEM-FEG at the University of Miami Center for Advanced Microscopy.

Coral Microbiome DNA Extraction, Sequencing, and Quality Control

Coral microbiome samples were processed as previously described (Soffer, Zaneveld & Vega Thurber, 2014). Briefly, coral mucus samples were concentrated via centrifugation and supernatant decanted. DNA was extracted and purified as described in (Correa et al., 2009), and the microbial amplicon libraries were generated using 515F and 806R primers with 454 sequencing adapters with Golay barcodes added to reverse primers. GoTaq Flexi from Promega (Madison, WI) using the following thermocycling conditions:1 cycle of 94ºC for 3 minutes; 35 cycles of 94ºC for 45 seconds, 50 ºC for 60

seconds, and 72 ºC for 90 seconds; and 1 cycle of 72 ºC for 10 minutes. Triplicate reactions of each sample were pooled and cleaned using AMPure magnetic beads from Agencourt, and these were then quantified using a Qubit dsDNA HS kit from Invitrogen before being pooled into equimolar ratios. Amplicon length and purity was checked on an Agilent Bioanalyzer 2100 prior to sequencing on a 454 Roche pyrosequencer (GSJunior platform) at the Oregon State University's Center for Genome Research and Biocomputing (CGRB) Core Laboratories.

Quality control and selection of operational taxonomic units (OTUs) was performed using the QIIME (v.1.8). Sequences with quality scores less than a mean of 35 were removed. Sequences were clustered into (OTUs) at a 97% 16S rRNA gene identity threshold using USEARCH 6.1.54 (Edgar, 2010), and the subsampled open-reference OTU-picking protocol in QIIME v.1.8 (Rideout et al., 2014), using greengenes 13_8 as the reference (McDonald et al., 2012). Chimeric sequences were removed with UCHIME (Edgar et al., 2011). Singleton OTU sequences found in only one sample were removed. The greengenes taxonomy version 13_8 using the RDP (Ribosomal Database Project) Classifier software v. 2.2 was used to classified taxonomically according (Wang et al., 2007; McDonald et al., 2012). The sequencing stragey of more samples at lower depth was chosen for discovery of new rare diversity within the coral microbiome (Knight et al. 2012). The 198 libraries included in this study consist of only samples with a post quality filtering minimum sequence depth of 500 reads or greater. A detailed description of information on sequencing, i.e. mean number (1602 ± standard deviation 3879) of sequences for all samples and means for each coral genera under the two environmental treatment category, we analyzed in the study are presented in Table S1.

Network construction and analysis

Networks were constructed using CoNet application version 1.0b6 in Cytoscape v 3.2.0, which enable significant correlations between bacterial genera to be identified. The OTU tables used as input matrixes for the networks were from the main microbial analysis summarized into a table of microbial orders (based on RDP classification against the Greengenes v13_8 taxonomic annotations). The main order level OTU table was split by treatment categories and then used to create i) tables separated into 2ºC datasets (four for each treatment) and ii) tables separated by coral host taxa. Temperature bins could not be included for the host network datasets due to samples size limitations after separating by samples by each host taxa. The 'Artic Soils' demonstration was used to set the bootstrap and permutations settings, which is used for tables developed with QIIME inputs. Microbial orders or rows dominated by zero entries for each dataset used to construct networks can be problematic. Thus, microbial orders were discarded from analysis if they were not present in >71% for each network input OTU table. The program, CoNet, does not apply a rarification procedure on the data but rather a ReBoot technique designed specifically to suppress spurious correlations of compositional data such as 16S amplicon datasets. Faust et al. 2012 provides simulation studies detailing how the bootstrap-renormalization methods successfully avoided compositional effects while preserving true correlations. The following co-occurrence measures were employed: Pearson and Spearman correlations; mutual information; and Bray-Curtis and Kullback-Leibler divergences, and their p-values merged. Edges that did not meet the false-discovery rate correction threshold (FDR $q \leq 0.05$) were discarded.

Acknowledgments

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Figures

Figure 2.1Electron Micrographs of Halobacteriovorax Electron micrographs of our coral associated strain, *Halobacteriovorax* PA1, including (A) several attack phase cells and a *Vibrio fortis* prey (the single larger cell), (B) a single attack phase cell, (C) a cell in attachment phase on the surface of a *Vibrio fortis* prey cell, and (D) a *Halobacteriovorax* entering the prey periplasm. (E) Molecular phylogenetic analysis is based on 16S rRNA gene sequences of the order *Bdellovibrionales* by the Maximum Likelihood method. The coral isolated strain (PA1) used in this study is blue, and strains with whole genome sequences are red. The bootstrap consensus tree inferred from 500 replicates and bootstrap values greater than 80% are reported. The analysis involved 23 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1216 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [3].

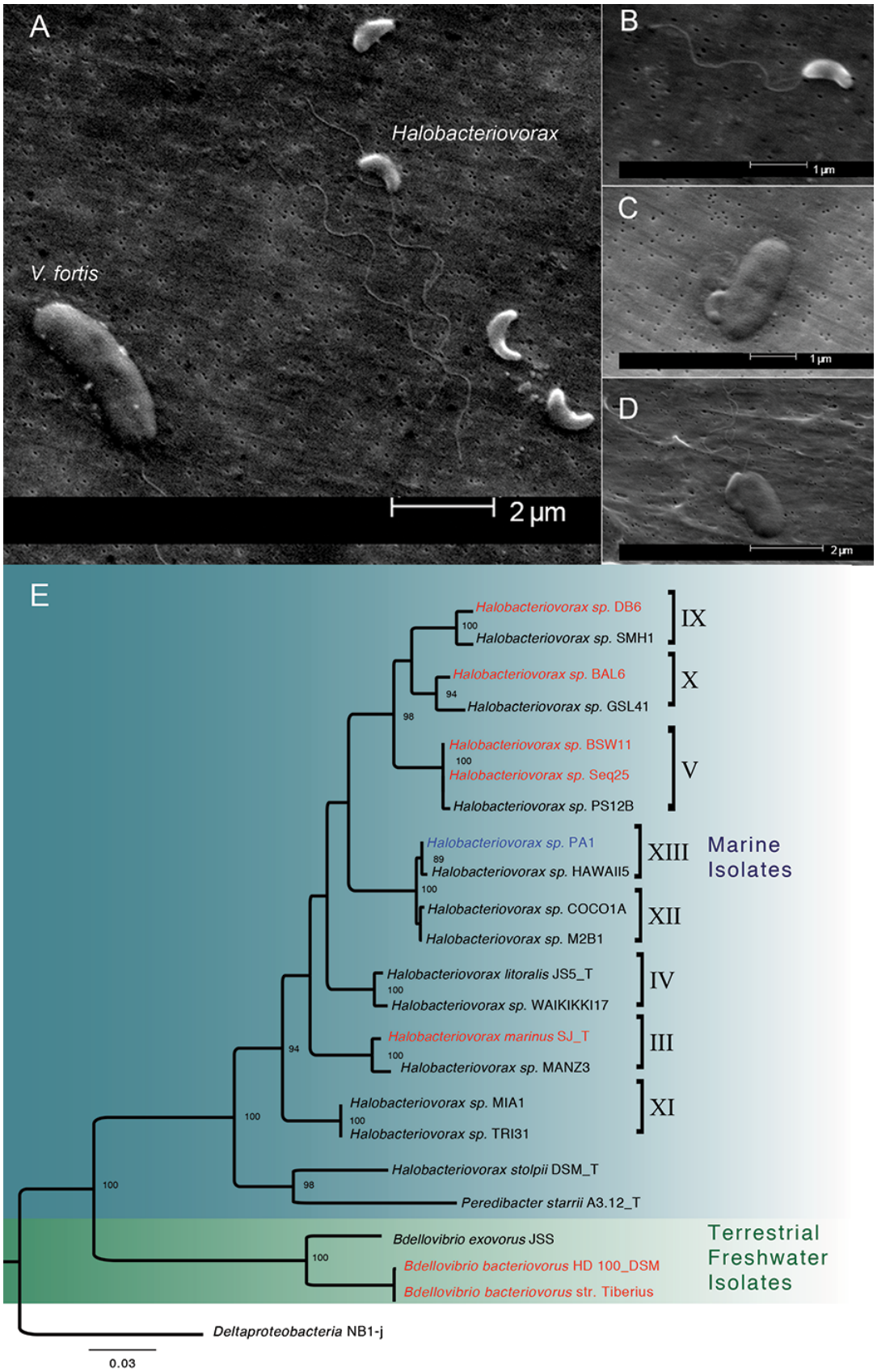

A
Halobacteriovorax
V. fortis
2 µm
B
1 µm
C
1 µm
D
2 µm
E
Halobacteriovorax sp. DB6
Halobacteriovorax sp. SMH1
IX
Halobacteriovorax sp. BAL6
Halobacteriovorax sp. GSL41
X
Halobacteriovorax sp. BSW11
Halobacteriovorax sp. Seq25
Halobacteriovorax sp. PS12B
V
Halobacteriovorax sp. PA1
Halobacteriovorax sp. HAWAII5
XIII
Marine Isolates
Halobacteriovorax sp. COCO1A
Halobacteriovorax sp. M2B1
XII
Halobacteriovorax litoralis JS5_T
Halobacteriovorax sp. WAIKIKKI17
IV
Halobacteriovorax marinus SJ_T
Halobacteriovorax sp. MANZ3
III
Halobacteriovorax sp. MIA1
Halobacteriovorax sp. TRI31
XI
Halobacteriovorax stolpii DSM_T
Peredibacter starrii A3.12_T
Bdellovibrio exovorus JSS
Bdellovibrio bacteriovorus HD 100_DSM
Bdellovibrio bacteriovorus str. Tiberius
Terrestrial Freshwater Isolates
Deltaproteobacteria NB1-j
0.03

Figure 2.2 Host-Associated Microbiome Networks Host-associated microbiome networks focused on members of the *Bdellovibrionales* and like organisms (BALOs), which include the *Halobacteriovorax*. Networks were constructed for control samples falling within the temperature bin (a) 23-24ºC (b) 29-30ºC and nutrient enrichment samples within the temperature bin (c) 23-24°C (d)29-30ºC. Circles in the networks represent bacterial nodes at order taxa level, color-coded by phylum according to the legend in the center. Blue lines connecting nodes pairs indicate significant co-occurrence patterns, while grey lines denote significant mutual exclusion patterns ($P \leq 0.05$) as determined by the ReBoot randomization procedure in the CoNet Cytoscape package (Faust and Raes, 2012; Faust et al., 2012). Network layout was calculated using edge-weighted spring embedded layout in Cytoscape (Shannon et al. 2003).

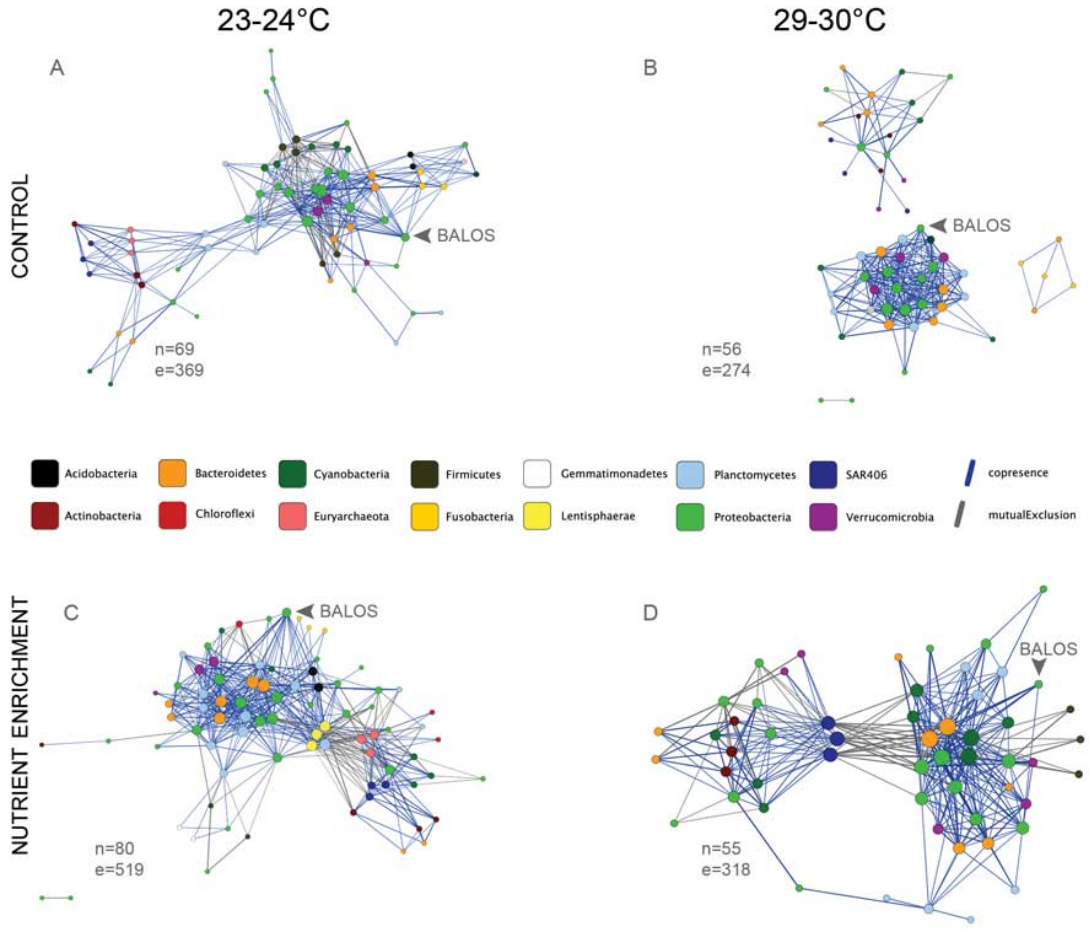
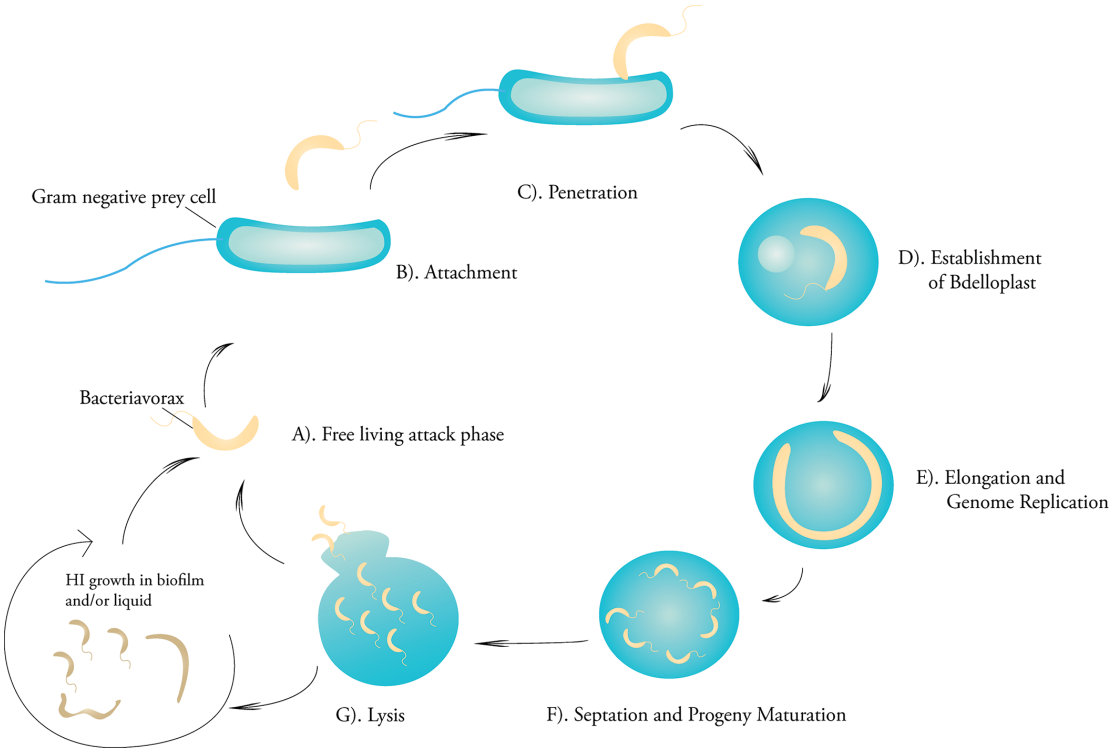


Figure S2.1. Halobacteriovorax host-dependent biphasic life cycle. In predatory attack phase (A. Free Living Attack Phase) *Halobacteriovorax* actively seek out prey. Once contact is made with a potential acceptable host (B. Attachment) *Halobacteriovorax* irreversibly enter the periplasm resealing their point of entry once inside prey (C. Penetration). *Halobacteriovorax* quickly kill the prey and release hydrolytic enzymes (D. Establishment of Bdelloplast) which allows them to take up hydrolyzed nutrients to aid in filamentous growth and genome replication (E. Elongation and Genome Replication). As nutrients become exhausted from the prey, the filament partitions (F. Septation and Progeny Maturation) and the progeny develops into highly motile flagellated predator cells. Lysis of the Bdelloplast cell (G. Lysis) then occurs and *Halobacteriovorax* escape to the environment to repeat the process or enter host-independent (HI) growth.



Supplemental Tables

Table S2.1 Isolates Obtained Number of *Halobacteriovorax* isolates obtained from each host coral taxa in time series study and non-time series *Montasrea cavernosa* corals. Information on sequencing, frequency of sampling, amplicon dataset summary, and *Halobacteriovorax* prevalence (presence/ absence) in 16S rRNA gene amplicon libraries from corals in control or nutrient enriched conditions from time series study.

	Totals	***S. sideraea***	***Agaricia sp.***	***Porites sp.***	***M. cavernosa***
# isolates of Halobacteriovorax	17	5	0	4	8
# Sampling Time Points	124	47	42	35	NA
Control	61	24	14	23	NA
Nutrient	63	23	28	12	NA
# 16S amplicon libraries	198	82	66	50	NA
Control	83	34	16	33	NA
Nutrient	115	48	50	17	NA
Mean (+/- sd) reads per sample	1602 (±3879)	2297 (±5924)	1197 (±907)	1028 (±502)	NA
Control	1269 (±956)	1629 (±1159)	1033 (±825)	1011 (±601)	NA
Nutrient	1842 (±5011)	2781 (±7679)	1249 (±925)	1059 (±437)	NA
range (min - max) of # sequences	511-52316	557 - 52316	511 - 6455	516-3902	NA
Control	516-7635	597 - 7635	537 - 4026	516 - 3902	NA
Nutrient	511-52316	557 - 52316	511 - 6455	547 - 2060	NA
Prevalence of Halobacteriovorax	0.798	0.8415	0.7576	0.78	NA
Control	0.755	0.8824	0.625	0.7576	NA
Nutrient	0.812	0.8125	0.8	0.8235	NA

Table S2.2 Network Interactions Network statistics for interactions between *Bdellovibrionales* and like organisms (BALOs) and other members of the coral microbiome. Node degree distribution (power law fit), average shortest path length (character path), and heterogeneity computed for each network. Total nodes contained in each network and the Between Centrality computed value for the BALOs node in each network.

Data set	R^2	Heterogeneity	AVG. path length	Between Centrality	# Nodes	# of microbial orders significantly correlated with *BALOs*
OTUs binned by temperature						
Control 23-24°C	0.36	0.68	2.7	3.10E-02	69	**14**
Control 25-26°C	0.57	0.66	2.4	8.70E-04	35	**2**
Control 27-28°C	0.75	0.77	2.9	NA	50	**0**
Control 29-30°C	0.29	0.81	1.6	6.90E-04	56	**8**
Nutrient 23-24°C	0.19	0.73	2.4	7.50E-03	80	**16**
Nutrient 23-24°C	0.54	0.59	1.6	NA	29	**0**
Nutrient 23-24°C	0.5	0.66	4.3	5.80E-02	59	**5**
Nutrient 23-24°C	0.04	0.66	2.3	2.50E-05	55	**4**
OTUs binned by coral host species and treatment						
***Agaricia* Control**	0.41	0.83	2.7	7.60E-03	57	**6**
***Agaricia* Nutrient**	0.78	0.86	1.9	4.70E-02	46	**4**
***Porites* Control**	0.37	0.61	2.4	0.00E+00	47	**1**
***Porites* Nutrient**	0.3	0.67	2.4	4.50E-04	48	**1**
***Siderastrea* Control**	0.44	0.74	2	7.90E-03	55	**5**
***Siderastrea* Nutrient**	0.16	0.74	2.8	2.50E-03	66	**7**

Table S2.3 Interactions at Two-Degree Intervals Bacterial taxa that interact with predatory BALOs in networks constructed from all taxa in each sample binned by two-degree intervals (networks from bins without any taxa interacting with BALOs are not shown). Interactions are supported by at least 2 correlation metrics (Pearson, Spearman, Bray-Curtis or Kullback-Liebler) and $P \leq 0.05$.

Table S2.3 Interactions at Two-Degree Intervals

Microbial orders significantly correlated with *BALOs*	Control Treatment			Nutrient Treatment		
	23-24°C	**25-26°C**	**29-30°C**	**23-24°C**	**27-28°C**	**29-30°C**
Chromatiales	+				+	+
Cytophagales		+	+			+
Myxococcales	+		+			+
NB1-j			+	+	+	
Rhodospirillales		+	+	+		
Alteromonadales	+					+
HOC36	+			+		
HTCC2188	+		+			
Saprospirales			+	+		
Thiotrichales	+			+		
unclassified79				+	+	
Verrucomicrobiales	+		+			
Acidobacteria				+		
Bacteroidales	+					
CL500-15					+	
Fusobacteriales	+					
Gloeobacterales	+					
Kiloniellales					+	
Lentisphaerales				+		
Other72	+					
Phycisphaerales				+		
Pirellulales				+		
Planctomycetes				+		
Planctomycetia				+		
Rhizobiales				+		
Sva0725				+		
Thiohalorhabdales				+		
unclassified61				+		
Verrucomicrobia			+			
Verrucomicrobiae	+					
Marinicellales	+					
Saprospirae	+					

Table S2.4 Interactions Under Nutrient Rich Environments Bacteria that interact with predatory BALOs in host binned microbiome networks under either Control or Nutrient Enriched treatments. Interactions are supported by at least 2 correlation metrics (Pearson, Spearman, Bray-Curtis or Kullback-Liebler) and P≤0.05. Temperature bins could not be included due to samples size limitations.

Significant BALOs co-occurring taxa (order level)	***Agaricia***		***Siderastrea***		***Porites***	
	Control	**Nutrient**	**Control**	**Nutrient**	**Control**	**Nutrient**
Cytophagales	+	+	+			
Alteromonadales	+			+		
Thiotrichales		+		+		
Verrucomicrobiales	+		+			
Bacteroidales						+
Chromatiales				+		
Fusobacteriales			+			
HOC36			+			
HTCC2188	+					
Marinicellales			+			
Myxococcales				+		
NB1-j				+		
Pirellulales		+				
Saprospirales		+				
Spirobacillales	+					
Vibrionales	+					
unclassified79					+	
OM190				+		
unclassified16				+		

Chapter 3

Alien vs. Predator: Pathogens open niche space for opportunists, unless controlled by predators

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Abstract

Coral microbiomes are known to play important roles in organismal health, response to environmental stress, and resistance to disease. Pathogens invading the coral microbiome encounter diverse assemblages of resident bacteria, ranging from defensive and metabolic symbionts to opportunistic bacteria that may turn harmful in compromised hosts. However, little is known about how these bacterial interactions influence the overall structure, stability, and function of the microbiome during the course of pathogen challenge. We sought to test how coral microbiome dynamics were affected by interactions between two of its members: *Vibrio coralliilyticus*, a known temperature-dependent coral pathogen, and *Halobacteriovorax*, a unique bacterial predator of *Vibrio* and other gram-negative bacteria. We challenged specimens of the important reef-building coral *Montastraea cavernosa* with *Vibrio coralliilyticus* pathogens in the presence or absence of *Halobacteriovorax* predators, and monitored microbial community dynamics with 16S rRNA gene time-series. In addition to its direct effects on corals, pathogen challenge reshaped coral microbiomes in ways that allowed for secondary blooms of opportunistic bacteria. As expected, *Vibrio coralliilyticus* addition increased the infiltration of *Vibrio* into coral tissues. This increase of *Vibrios* in coral tissue was accompanied by increased richness, and reduced stability (increased beta-diversity) of the rest of the microbiome, suggesting strong secondary effects of pathogen invasion on commensal and mutualistic coral bacteria. Moreover, after an initial increase in *Vibrios,* two opportunistic lineages (*Rhodobacterales* and *Cytophagales*) increased in coral tissues, suggesting that this pathogen opens niche space for opportunists. *Halobacteriovorax* predators are commonly present at low-abundance on coral surfaces.

Based on the keystone role of predators in many ecosystems, we hypothesized that *Halobacteriovorax* predators might help protect corals by consuming gram-negative pathogens. In keeping with a protective role, *Halobacteriovorax* addition alone had only minor effects on the microbiome, and no infiltration of *Halobacteriovorax* into coral tissues was detected in amplicon libraries. Simultaneous challenge with both pathogen and predator eliminated detectable *V. coralliilyticus* infiltration into coral tissue samples, ameliorated changes to the rest of the coral microbiome, and prevented secondary blooms of opportunistic *Rhodobacterales* and *Cytophagales.* Thus, we show that primary infection by a coral pathogen is sufficient to cause increases in opportunists, as seen in correlational studies. These data further provide a proof-of-principle demonstration that, under certain circumstances, host-associated bacterial predators can mitigate the ability of pathogens to infiltrate host tissue, and stabilize the microbiome against complex secondary changes that favor growth of opportunistic lineages.

Introduction

Coral reefs have experienced sharp declines in coral cover from environmental factors (De'ath et al., 2012), temperature induced bleaching (Fitt & Warner, 1995), and disease (Bourne et al., 2009; Burge et al., 2014) with some areas of the Caribbean experiencing as much as 80% coral loss over the past several decades (Gardner et al., 2003). While many studies have identified microbial consortia that increase in diseased corals (e.g. Gignoux-Wolfsohn et al. 2015), etiological agents are unknown for the majority of coral diseases (Mouchka, Hewson & Harvell, 2010). Currently *Vibrio coralliilyticus* is the most well-described model for interactions between corals, the

environment, and pathogenic bacteria (Ben-Haim et al., 2003). Several *V. coralliilyticus* virulence factors are temperature-dependent and upregulated above 27 ºC (Kimes et al., 2012), and it has been suggested that host tissue invasion can only occur above this threshold (Vidal-Dupiol et al., 2011). Given the continuous rise in sea surface temperatures due to global climate change (Hoegh-guldberg et al., 2007), and the projected increased variability of temperature extremes, it is likely that the incidence of infections by *V. coralliilyticus* and other temperature-dependent pathogens will increase (Maynard et al., 2015). Bacterial communities of diseased corals are also known to have large numbers of opportunistic pathogens and secondary colonizers (Gignoux-Wolfsohn & Vollmer, 2015). It has been hypothesized that the majority of coral disease may be the result of normally-benign coral microbionts that become opportunistic pathogens during physiological stress to the host (Lesser et al., 2007a). Thus, the linkages between infection by a primary pathogen and secondary opportunistic infection remain an area of active exploration.

Corals also form mutualistic and commensal partnerships with diverse microorganisms, ranging from endosymbiotic photosynthetic dinoflagellates (*Symbiodinium* spp.), to consortia of archaea, fungi, and bacteria. These multi-domain communities make coral meta-organisms among the most diverse systems on the planet (Bayer et al., 2013). Although the role of *Symbiodinium* in the coral holobiont is well studied, the exact roles (if there are any) of each member of the bacterial portion of the holobiont remains far from clear. Experiments and metagenomic analyses have provided some insights into the roles of individual members of the coral microbiome (e.g., Wegley et al., 2007). It has been suggested that some of these bacteria provide direct benefits to

the coral host, such as nitrogen fixation by symbiotic *Cyanobacteria* in *Montastraea cavernosa* (Lesser et al., 2007b), or ammonia oxidation by archaea (Beman et al., 2007). Other bacteria, particularly those in the coral surface mucus layer, are thought to provide a first line of defense against invading pathogens. Mucosal bacteria are thought to protect the host by several mechanisms, including production of antibiotics (Ritchie, 2006), secretion of chemical compounds that inhibit pathogen metabolism (Rypien, Ward & Azam, 2010), or competition for necessary resources and niche space (Ritchie & Smith, 1997). Increasingly, viruses and phages are recognized as also playing a regulatory role in the holobiont by controlling microbial populations (Barr et al., 2013; Soffer, Zaneveld & Vega Thurber, 2014; Nguyen-Kim et al., 2014).

We have recently described how the predatory bacterium *Halobacteriovorax* also likely influences the diversity and dynamics of the microbial community in the coral surface mucus layer through consumption of a broad range of bacterial prey (Welsh et al., 2015). *Halobacteriovorax* spp. are small, highly motile predatory bacteria that exhibit a biphasic lifestyle and prey exclusively on gram negative bacteria, including known coral pathogens (Williams, Falkler & Shay, 1980, Welsh et al. 2015). *Halobacteriovorax* are the marine component of a group of delta-proteobacteria known as *Bdellovibrio* and like organisms (BALOs). In free-living attack phase, BALOs actively seek out prey in order to attach, burrow inside, and restructure their host cell into a rounded bdelloplast. This kills their prey and provides BALOs with an osmotically stable structure free from competition to utilize prey resources for growth and replication. A new generation of attack-phase predators then bursts forth from the bdelloplast to seek new hosts.

Bacterial predation in the coral microbiome could be a type of top-down control, that directly alters the structure and function of the coral microbiome as demonstrated in other aquatic systems by bacterivorous predators (see reviews by (Jürgens & Matz, 2002; Pernthaler, 2005; Matz & Kjelleberg, 2005). For example, we highlighted potential interactions of *Halobacteriovorax* and other members of the coral holobiont using co-occurrence network analysis of an in-field experimental time series of three coral general, across three years, several treatments, and range of temperature conditions. These networks showed that *Halobacteriovorax* are core members of the coral microbiome, present in >78% of samples from 3 coral genera (Welsh et al. 2015). We also showed that isolated strains of coral-associated *Halobacteriovorax* prey upon known coral pathogens in cultured settings (Welsh et al. 2015). Such antagonisms between predators and prey in the holobiont may have variable effects on the microbiome, such that they could be occlusive to pathogens or disruptive to the coral microbiome itself.

Here we examine how a bacterial predator (*Halobacteriovorax*), a known coral pathogen (*Vibrio coralliilyticus*), and a host (*Montastraea cavernosa*) interact in the complex system of the coral microbiome. We observed that *Halobacteriovorax* prevented the establishment of the pathogen *V. coralliilyticus* on *M. cavernosa* corals, and *V. coralliilyticus* altered microbial population dynamics on the corals. This work has implications for the mitigation of coral disease and increases the scientific understanding of how microbial predator-prey dynamics regulate the microbial community and influence host health.

Methods

Bacterial strains, growth conditions, and prey range assays

Vibrio are a globally distributed marine bacterial genus known to cause disease in several marine organisms (Bally & Garrabou, 2007; Sussman et al., 2009; Richards et al., 2015). For example, both *Vibrio coralliilyticus* and *V. tubiashii* infect oysters and other marine invertebrates (Brown, 1981; Kesarcodi-Watson et al., 2012). Importantly *V. coralliilyticus* are known to be naturally abundant (Wilson et al., 2013) and infect corals (Ushijima et al., 2014; Tout et al., 2015). Thus to better evaluate which *Vibrios* can be preyed upon by coral-associated *Halobacteriovorax*, we conducted a series of predation assays in liquid and solid media.

Bacterial strains *Vibrio fortis* PA1 and *Vibrio coralliilyticus* ATCC BAA450 (Accession # KT626460 and AJ440005, respectively), were grown on Marine 2216 Agar (MA) overnight. A single colony was re-suspended in 50 mL Marine 2216 Broth (MB) in a 250mL flask at 30° C and 250 rpm overnight. Cultures were diluted 1:100 in fresh media and incubated until late exponential growth before use in any experiment.

Our predatory bacterial strain, *Halobacteriovorax* sp. PA1 (Accession # KR493097), was grown as previously reported (Welsh et al., 2015) in Pp20 media. Briefly, a single plaque from a double layer plate was resuspended in 3 mL of 10^9 *V. fortis* cells in filtered seawater in 15 mL test tubes. The culture was incubated at 28° C and shaking at 250 rpm overnight. A 1:100 dilution of the overnight culture was prepared by adding 0.5 mL of 0.45µm filtered culture to 50 mL of 10^9 *V. fortis* PA1 cells in filtered seawater. This new co-culture of predator and prey was grown in a 250 mL

culture flask at 28° C and 250 rpm and monitored until late exponential phase before use in any experiment.

The double layer technique assayed whether *Halobacteriovorax* sp. PA1 was capable of preying on various bacteria (Table 1). One milliliter of the potential prey bacteria suspension (containing 10^9 cells/ml) and one milliliter of the appropriate predator dilution was mixed with 3 ml of molten agar (PP20 medium containing 1.1% Difco agar) held at 42℃, for a final top layer agar concentration of 0.66%. The mixture was immediately spread over the surface of 1.8% agar PP20 Petri dish plates, and three replicates were plated for each predator-prey combination. Plaques were measured after 3–5 days of incubation (Figure 1c).

Triplicate biological replicates for each vibrio prey species were grown overnight in marine broth at 28 ºC and shaking at 250 rpm, transferred using a 1:100 ratio into fresh MB, and monitored until late exponential phase. Prey were washed 3 times in 0.2 μm filtered and autoclaved seawater (FSW) and resuspended to a concentration of 10^9 cells/mL. Three biological replicates of overnight *V. fortis* and *Halobacteriovorax* sp. PA1 in FSW, which had lysed and cleared *V. fortis* prey, were 0.45 μm filtered to isolate predators. Filtered predators were then added to prey species at a 1:100 volume ratio. Predation was measured by OD_{600} values using a microplate reader (Infiniti M200; Tecan Group Ltd, Männedorf, Switzerland). Tecan OD values were reported without conversion to a 1-cm path length (Figure S1). *Halobacteriovorax* in attack phase does not significantly alter the absorbance reading of the prey at 600 nm due to their small cell size. Predation rates in the liquid co-culture assay were measured by the host cell density reduction compared to the predator-free controls. Based on our observed predation rates

and the biological relevance of the strain, we chose to conduct our predator-prey addition experiment using *V. coralliilyticus* BAA 450 (accession # AJ440005).

Collection and Preparation of *Montastraea cavernosa*

Montastraea cavernosa was selected as a model for this work as it is both a common reef-building Caribbean coral and is susceptible to a variety of coral diseases (Sutherland & Ritchie, 2002; Goodbody-Gringley, Woollacott & Giribet, 2012). The *M. cavernosa* colony used in the experiment was obtained from the Florida Keys National Marine Sanctuary (#FKNMS-2010-123) from the Key West (FL, USA), and was maintained for 10 weeks in a shaded flow through raceway tank at the University of Miami Experimental Hatchery. The *M. cavernosa* colony was cored into 3.5 cm diameter cores with skeleton trimmed to ~2 cm. Coral cores were transferred to back into their common garden experimental aquaria and allowed to acclimate for an additional four weeks where they demonstrated signs of growth including lateral tissue extension over exposed skeleton and feeding behavior during recovery. The *M. cavernosa* cores were then transferred to a common garden recirculating seawater tank at FIU prior to being subjected to various experimental treatments in aquaculture. Seawater for the experiment was obtained from the University of Miami Experimental Hatchery (sand and UV-filtered seawater pumped in from Biscayne Bay).

***Montastraea cavernosa* bacterial pathogen and predator additions**

Fragments were held in common garden tank for 10 days while initial bacterial challenges were conducted to establish inoculation conditions. Initial proof-of-principle bacterial challenges were conducted in aerated sterile beakers with water temperatures

held at 31° C to induce pathogen virulence. Pathogen inoculations of coral specimen were conducted at 10^3, 10^6, and 10^9 *Vibrio coralliilyticus* cells. To encourage pathogen invasion all coral cores were taken from common garden tank at time zero, scored with a file to mimic tissue damage, and inoculated in the beakers by transferring the *V. coralliilyticus* cells using a sterile q-tip (Adwin Scientific, Schaumburg, IL). The 10^9 *V. coralliilyticus* was the only inoculation capable of causing visual signs of disease in the *M. cavernosa* fragments and therefore this pathogen abundance was selected for the main experiment (Supplemental Fig. 1).

Following this proof-of-principle test, 48 coral cores were divided into 4 treatments for the main experiment, providing 12 cores per treatment. These treatments were: 1) inoculation of coral with sterile media as a control, 2) inoculation with 10^9 *Vibrio coralliilyticus,* 3) inoculation with 10^6 *Halobacteriovorax,* and 4) inoculation with 10^9 *V. coralliilyticus* and 10^6 *Halobacteriovorax*. 48 coral cores were used in total which allowed 3 replicates cores (one from each of the 3 replicate treatment tanks) at each of 4 time points per treatment (3 replicates x 4 timepoints = 12 per treatment; 12 x 4 treatments = 48 cores total) (Figure 1).

In each experimental inoculation, late exponential bacteria cultures were pelleted and washed 3 times with sterile artificial seawater (ASW) in 2 mL tubes by centrifugation at 10,000 x g for 10 minutes and gently re-suspension with sterile media. The final wash was resuspended in 100 µl of ASW and transferred using a sterile q-tip to apply to freshly abraded corals in sterile beakers. Q-tips were held on corals for 1 hour in the inoculation beakers before transferring cores to recirculating seawater tanks. At each time point (T=0, 4, 8, 24, and 32 hours) one coral fragment from each replicate tanks of each treatment

(n=3 per time per treatment) was removed and placed in a Whirlpak (Nasco, Salida, California) and flash frozen for microbial DNA analysis.

Montastraea cavernosa Microbiome DNA Extraction, Sequencing, and Quality Control

From each core one quadrant of the coral tissue layer was removed using a dental tool (Sup Fig X) and transferred into separate microcentrifuge tubes (4 per core) containing 500 µl of TES Buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, and 100 mM NaCl). A 1.5 mL microtube pestle was used to homogenize the tissue before adding 400 µl of TES buffer with lysozyme (Epicentre; final: 10 U μl^{-1}), followed by incubation at 37 ºC for 30 minutes. A 200 µl aliquot of homogenized sample was used for DNA extraction with the Power Soil DNA extraction kit (MoBio Laboratories, Carlsbad, CA, USA); the remainder was stored at -20ºC. Microbial amplicon libraries were generated using 515F and 806R primers to the V4 region of the 16S with Schloss sequencing adapters (Kozich et al., 2013). AccuStart II PCR ToughMix (Gaithersburg, MD) and the following thermocycling conditions were used for amplification:1 cycle of 94ºC for 3 minutes; 35 cycles of 94 ºC for 30 seconds, 50 ºC for 30 seconds, and 72 ºC for 60 seconds; and 1 cycle of 72 ºC for 10 minutes were used for amplification. Each sample had triplicate reactions that were pooled and cleaned using the Promega Wizard SV Gel and PCR Clean-Up System (Madison, WI). The samples were then quantified using a Qubit dsDNA HS kit (Invitrogen, Oregon) before being pooled in an equimolar ratio. The amplicon purity and length was checked on an Agilent Bioanalyzer 2100 prior to

sequencing on a MiSeq Illumina sequencing platform at the Oregon State University's Center for Genome Research and Biocomputing (CGRB) Core Laboratories.

Quality control and selection of operational taxonomic units (OTUs) was performed using QIIME (v.1.8) (Caporaso et al., 2010). Sequences with quality scores less than a mean of 35 were removed. Sequences were clustered into (OTUs) at a 97% 16S rRNA gene identity threshold using USEARCH 6.1.54 (Edgar, 2010), and the subsampled open-reference OTU-picking protocol in QIIME v.1.8 (Rideout et al., 2014), using greengenes 13_8 as the reference (McDonald et al., 2012). Chimeric sequences were removed with QIIME's wrapper of the UCHIME software (Edgar et al., 2011). Singleton OTUs were removed. The OTUs were assigned using the QIIME wrapper to the UCLUST software package (Edgar, 2010) to classified taxonomically. OTUs that were classified as chloroplast, eukaryotic or mitochondria were filtered out of the dataset.

Statistical analysis

To avoid artifacts due to uneven sampling depth during comparisons of alpha and beta diversity, all samples were rarified (randomly subsampled) to equal sequencing depth. After quality control steps, the least sequenced sample had 11,716 reads, and this was therefore chosen as the rarefaction depth. For alpha diversity (richness), total observed OTUs and Chao1 diversity statistics (Chao, 1984) were calculated in QIIME. The significance of differences in alpha diversity across treatments was determined in QIIME using nonparametric t-tests with 999 Monte Carlo permutations. For beta diversity analysis, weighted UniFrac distances (Lozupone & Knight, 2005) were calculated in QIIME. Distances within samples in each treatment category were

summarized, and tested for significance using Monte Carlo Permutation tests (make_distance boxplots.py, non-parametric p-value, n=999 permutations). To account for multiple comparisons between treatments, Bonferroni-corrected p-values are reported for both alpha- and beta-diversity analyses in the text and figures.

To analyze how order-level taxa responded to bacterial challenge, a generalized linear model (GLM) was fit with the R package DESeq2 (Love, Huber & Anders, 2014). The GLM design specified time point, treatment, and their interaction as factors. For this analysis, the order level OTU table was pre-filtered in QIIME where we excluded all taxa present in fewer than 6 samples. To test for the effect of treatment, a full model was compared to a reduced model fit using only time as a factor, and likelihood ratio tests (LRT) were performed to assess taxon (Table 2). Post hoc Wald tests were performed on the full model object to identify the specific treatments responsible for driving changes in these taxa. To control the rate of false positives due to multiple comparisons, differentially abundant taxa were identified as taxa with Benjamin-Hochberg FDR q-values less than 0.05.

Results

Halobacteriovorax sp. PA1 can prey on multiple *Vibrio* species.

Before conducting a co-infection study, we evaluated the prey range of our previously cultured *Halobacteriovorax sp.* PA1 strain (isolated from a *Porites astreoides* coral (Welsh et al. 2015)) on several cultured *Vibrio*s (Table 3.1). Since differences in pathogenicity occurs among these strains and species (e.g., *Vibrio coralliilyticus* vs *Vibrio tubiashii*; Richards 2014), we also tested whether there were differences in both

the susceptibility to predation and the predation rate among these strains using both liquid co-cultures and the commonly used double layer plaque assay (Table 3.1; Schoeffield & Williams, 1990).

Strain and species level differences in susceptibility to predation were detected among some of the Vibrio species (Table 3.1). For example, among the *V. coralliilyticus* strains, BAA 450 was most susceptible to predation in the liquid assay while strains RE22 and RE98 were less susceptible (Table 3.1). In the double layer plate assay, *Halobacteriovorax sp.* PA1 was capable of killing prey and forming plaques on all *Vibrio* spp. except *V. coralliilyticus* RE22 and *V. tubiashii* ATCC19106 (Table 3.1; Fig 3.1c). *Halobacteriovorax* predation rates (50% killing) ranged from 8.96 hours in *V. cholerae* N1696 to 21.16 hours in *V. coralliilyticus* RE22. The prey rate for *V. coralliilyticus* BAA 450, a model coral pathogen, was 19.00 hours.

Pathogens alter coral microbiome α- and β-diversity unless controlled by predators

Given the observation that *Halobacteriovorax sp.* PA1 was capable of killing *V. coralliilyticus* BAA 450 in co-culture, we conducted an *in situ* challenge experiment that directly inoculated *M. cavernosa* corals with this model pathogen in the presence and absence of the predator (Fig 3.2). An initial pilot bacterial challenge conducted in sterile beakers demonstrated that *V. coralliilyticus* was capable of causing signs of bleaching and tissue disruption only when *M. cavernosa* fragments were inoculated with 10^9 *V. coralliilyticus* and incubated at 31 ºC for 3 days (Fig. S3.1). However, in the larger volume and recirculating tank system used for the main co-inoculation experiment no significant differences were observed in tissue loss or bleaching among the treatments at

any of the time points (data not shown). Despite the lack of any visual signs of pathogenesis, we found significant differences in the tissue associated microbiomes of the inoculated corals using amplicon analysis. We quantified microbial changes using 16S rRNA sequencing of corals in each treatment at 4, 8, 16, and 32 hours post inoculation. After quality filtering of the experimental microbiomes, 4,464,765 reads remained with an average of 85,860 ± 112,003 reads per samples. After rarefaction the mean number of observed OTUs across all samples was 197 (Table S3.1).

Vibrio coralliilyticus addition increased alpha diversity in the tissues of *Montastraea cavernosa* corals. Corals challenged with *V. coralliilyticus* showed significantly increased richness relative to controls (*Vibrio* mean = 259.767 ±14.196; control mean = 178.167 ±47.398) as measured by Chao1 and observed species diversity metrics ($p = 0.048$; Fig. 3.3a). However, when *M. cavernosa* samples were co-inoculated with both 10^9 *V. coralliilyticus* and 10^6 *Halobacteriovorax*, species richness returned to low levels (mean = 163.058 ±36.772) that were indistinguishable from control conditions, but distinct from the *V. coralliilyticus* treatment ($p = 0.018$; Table 3.2). Changes in alpha diversity occurred early in the experiment, but did not change over the remaining time points (Table S3.2). No significant differences in evenness were observed between treatments. Further no significant differences in α-diversity were found between tanks or time points for either the Chao1 or observed species metrics.

Weighted UniFrac distances (β-diversity) were also significantly different between the control treatment and the *V. coralliilyticus* treatment ($p= 0.012$) (Fig 3.3b). In a similar pattern to α-diversity, the *Halobacteriovorax* and *V. coralliilyticus* combination treatment returned β-diversity to control levels and were not significantly

different than the other treatments (Fig 3.3b). The *Halobacteriovorax* sp. PA1 alone did not significantly change β-diversity, and no significant differences were found between tanks or time points for β-diversity metrics.

Pathogens increase growth of opportunists like *Rhodobacterales* and *Cytophagales* unless controlled by predators.

In order to test if inoculation treatments caused any differences among bacterial orders, a generalized linear model was constructed using DESeq2 (Love, Huber & Anders, 2014). Significant differences were detected across treatments in *Vibrionales*, *Rhodobacterales*, *Alteromonadales*, *Cytophagales*, and *Burkholderiales* (Benjamini-Hochberg corrected p = 0.014, 0.045, 0.036, 0.011 and 0.007, respectively). To identify which individual treatments were responsible for driving these changes we used Wald post hoc tests (Table 3.2).

As expected, addition of *V. coralliilyticus* pathogens resulted in increased *Vibrionales* abundances in coral tissues (Figure 3.4 purple lines). In the *V. coralliilyticus* treatment, the mean relative abundance of *Vibrionales* increased over 35% from the 4 to 8 hour time point. Yet corals inoculated with *V. coralliilyticus* in the presence of the predator *Halobacteriovorax* had an 84.74% reduction in *Vibrionales* compared to the *V. coralliilyticus* alone treatment. The combined treatment had a mean *Vibrionales* relative abundance of 1.43%, similar to the controls at 0.98%, while the *V. coralliilyticus* alone treatment had 9.38% mean *Vibrionales* relative abundance across the whole experiment (Table 3.2; Figure 3.4 box plots).

Surprisingly the addition of *V. coralliilyticus* also significantly increased the relative abundance of opportunists *Cytophagales* and *Rhodobacterales* compared to controls. Addition of *V. coralliilyticus* to corals increased the abundance of *Rhodobacterales* in tissues to an even greater extent than the pathogen itself, and this increase persisted after *Vibrio* abundances fell at later time points (Figure 4 red lines). *Rhodobacterales* steadily increased throughout the experiment in the *V. coralliilyticus* addition treatment, nearly doubling at each time point starting from 6.99% (± 0.05 SEM) relative abundance to a maximum mean value of 48.75% (± 0.14 SEM) in the final time point. However, during joint inoculation of *Halobacteriovorax* and *V. coralliilyticus*, *Rhodobacterales* showed no significant differences vs. controls. Similarly, there were no differences in the abundance of *Rhodobacterales* vs. controls in the treatment where *Halobacteriovorax* was added alone (Table 3.2). *Cytophagaes* also was increased by several orders of magnitude, from <0.001% to 3.656%, early in the *V. coralliilyticus* addition experiment (Figure 3.4 brown lines).

The two other taxa that significantly changed, but did so in different patterns, were *Burkholderiales* and *Alteromonadales* (Figure 3.4 blue and green lines respectively). The mean relative abundance for the order *Burkholderiales* was lowest (8.21%) in the *V. coralliilyticus* treatment (Figure 3.4 boxplots) and was significantly lower ($p = 0.015$) in the *V. coralliilyticus* versus the combined *Halobacteriovorax* and *V. coralliilyticus* treatment that had a relative abundance of 21.86% (Table 3.2). *Alteromonadales* were 40.47% more abundant in the controls than the combined *Halobacteriovorax* and *V. coralliilyticus* treatment ($p = 0.014$) (Table 3.2; Figure 3.4 box plots).

Discussion

Manipulating the microbiome: pathogens and predators

High-throughput sequencing and community-based analyses have significantly advanced microbiome research in the past decades. Yet despite these advances we still know little about the interactions among members of many microbiome systems. While we can more easily document membership dynamics and community topology, we often lack the ability to confirm causal relationships among them. Manipulative studies are necessary to link cause and effect. While some host-microbe models can be more readily manipulated (e.g., mouse gut, squid light organ, and rhizosphere), there remain considerable methodological barriers for many systems, especially those for which gnotobiotic (germ-free) host animals are not available.

Vibrio coralliilyticus is a known disease-causing pathogen of corals worldwide (Ben-haim, Zicherman-keren & Rosenberg, 2003; Wilson et al., 2013) and has been documented to induce bleaching (Ben-Haim et al., 1999; Ben-haim, Zicherman-keren & Rosenberg, 2003). Furthermore, experimental evidence has demonstrated that under increased thermal stress *V. coralliilyticus* concentrations rise dramatically (Tout et al., 2015). However, the changes, if any, that *V. coralliilyticus* infection causes to the microbial communities normally present in corals was previously unknown. Determining how pathogens alter the normal flora of a coral may provide insight into whether mutualtists are lost and additional antagonisms arise during an infection cycle and thus contribute to secondary negative effects on animal hosts.

In our study, we used this model coral pathogen and its coral-associated predator *Halobacteriovorax* to manipulate the host microbiome. Here we show that the addition of the pathogen not only changes its own abundance in the system (as would be expected) but also alters the microbiome in various ways, including increases in alpha and beta diversity (Figure 3.3). However, when these corals were challenged with the pathogen in the presence of the predator, these effects were diminished and resulted in almost no changes in the normal coral microbiome.

Addition of the pathogen led to a dramatic increase in a known group of opportunists of corals, the *Rhodobacterales* (Figure 3.4). Remarkably, this increase in *Rhodobacterales* persisted at later time-points, even after the abundance of the pathogen had declined. *Rhodobacterales* have been linked to disease outbreaks as sequence abundances in white plague diseased *Siderastrea siderea* and *Diploria strigosa* corals have been shown to be significantly higher than in healthy controls (Cárdenas et al., 2012). *Rhodobacterales* are fast growing taxa, capable of quickly responding to increasing availability of amino acids (Mayali et al., 2014), and could be responding to resources made available from cells damaged by *V. coralliilyticus.* Such a mechanism would explain associations between *Rhodobacterales* and many stressed or diseased corals. While the present study cannot distinguish whether these secondary, *Vibrio*-induced blooms of *Rhodobacterales* are harmful to corals, the experimental framework used here could test this question in the future.

More broadly, addition of *V. coralliilyticus* allowed a wider variety of bacteria to colonize the tissue (Figure 3.3a). It is likely these invading species infiltrated the tissue shortly after inoculation, as the increase in observed species persisted for the duration of

the experiment (Table S3.2). This suggests that a coral pathogen can alter the microbiome of coral tissue, both by increasing specific opportunists and by increasing richness overall. However, the addition of the predator *Halobacteriovorax* mechanistically dampens the disproportionate impact of the pathogen, and thus increases the resistance of the microbiome to both the invading pathogen itself and other opportunistic taxa that colonize after initial infection (Figure 3.4).

Halobacteriovorax and top down control of pathogens

We have previously cultivated *Halobacteriovorax* from multiple-species of corals, and used long-term microbial time series to show that, despite its low abundance, it is a core member of the microbiome of several coral genera (Welsh et al., 2015). Here we used bacterial challenge experiments to demonstrate that *Halobacteriovorax* can protect its coral host by consuming a temperature-dependent coral pathogen prior to infection of host tissues. We found that the application of *Halobacteriovorax* at the same time as *V. coralliilyticus* can prevent detectable infection of *M. cavernosa* corals. Co-inoculations of this predator with *V. coralliilyticus* showed no significant differences in the abundance of *Vibrionales* in coral tissues versus control inoculations at any time in the course of the experiment (Fig 5 purple lines). Thus it is likely that these predators consumed the *Vibrio* immediately or at the point of infection, and therefore provided a biotic barrier to the host tissues. The ability of *Halobacteriovorax* to mitigate an active infection, if added hours or days after *V. coralliilyticus* infection has begun, remains unknown, but could be tested using similar methods to those we describe here.

Phage have already been shown to be effective against *V. coralliilyticus* (Cohen et al., 2013), and likely play a role in controlling natural populations of *V. coralliilyticus* in the environment--similar to what has been suggested for phage and *V. cholerae* (Faruque et al., 2005). Phages also provide an antimicrobial function in the mucus layer of corals (Barr et al., 2013, 2015) and are often considered the main top-down control mechanism of bacteria in some systems. However in certain circumstances, *Halobacteriovorax* predation has been shown to be a more dominant factor in bacterial mortality than viral lysis (Williams et al., 2015). In addition, predatory bacteria are thought to play a major role in controlling pathogenic *Vibrio* in seawater and shellfish (Richards et al., 2012). In our study we show predatory *Halobacteriovorax sp.* PA1 is effective against *V. coralliilyticus* BAA 450 and other *Vibrio* strains, offering further support to the hypothesis that bacterial predators are likely to play a role in controlling populations in the environment. In a similar fashion to phages, *Halobacteriovorax* thus mediates top-down control of pathogens by preventing initial invasion of the host.

Microbiome manipulation validates previous network analysis predictions

A small but growing body of research suggests *Halobacteriovorax* naturally occur and regularly interact with members of the coral microbiome. For example, a previous metagenomic study of *P. astreoides* from Panama reported that sequences similar to predatory *Halobacteriovorax* were among the most commonly identified bacterial annotations in the coral microbiome (Wegley et al., 2007). Furthermore, we found *Halobacteriovorax* was present in ~80% of samples collected approximately monthly from 3 genera of Caribbean corals across a three-year time span. Network analysis of 198

of these samples detected intriguing co-occurrences between these predators and other taxa (Welsh et al. 2015). Here in the bacterial challenge study we validated several of the co-occurrence patterns detected in our network analysis. For example, in our networks from *Agaricia* corals *Bdellovibrionales* (the order of *Halobacteriovorax*) positively co-occurred with both *Vibrionales* and *Cytophagales*. Here we experimentally demonstrated that *Halobacteriovorax* directly alters the abundance of both of these taxa. *Vibrionales-Cytophagales* also positively co-occurred in our *Agaricia* corals suggesting an interaction that was either dependent or independent of environmental conditions. We show here that *V. coralliilyticus* infection is associated with significant increases in *Cytophagales* abundance *in vivo* as well, suggesting there is a more direct interaction between these two taxa (Table 3.2). This work lends support to the use of networks to provide a predictive understanding of the microbiome's function and dynamics in natural systems.

Conclusions

Pathogens that successfully colonize a coral host are capable of generating a disproportionate impact on community structure. Infection by *V. coralliilyticus* results in a significant shift of the host microbiome to an alternative destabilized state in which opportunists bloom, potentially further exacerbating the negative effects of an infection. Recent evidence supports the hypothesis that host-associated microbes offer protection against invasive pathogens either by depriving these pathogens of essential nutrients or acting as a physical barrier to host attachment (Weyrich et al., 2014). Here we show an additional mechanism by which host-associated predatory bacteria protect the host microbiome: through direct consumption of pathogens prior to the invasion and

colonization of their host. The ability to manipulate the microbiome and therefore test various hypotheses about the principles that govern microbial community assembly, dynamics, and functions, especially in terms of how these relate to host health, remain a challenge for our field (Waldor et al., 2015). As our ability to culture more and more coral microbial taxa improves, so will our methods to manipulate the microbiome. Such efforts will allow us to gain a better understanding of the relationships between members of the microbiota, which ideally will result in better management of *V. coralliilyticus*, and other important coral diseases.

Acknowledgements

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Figures

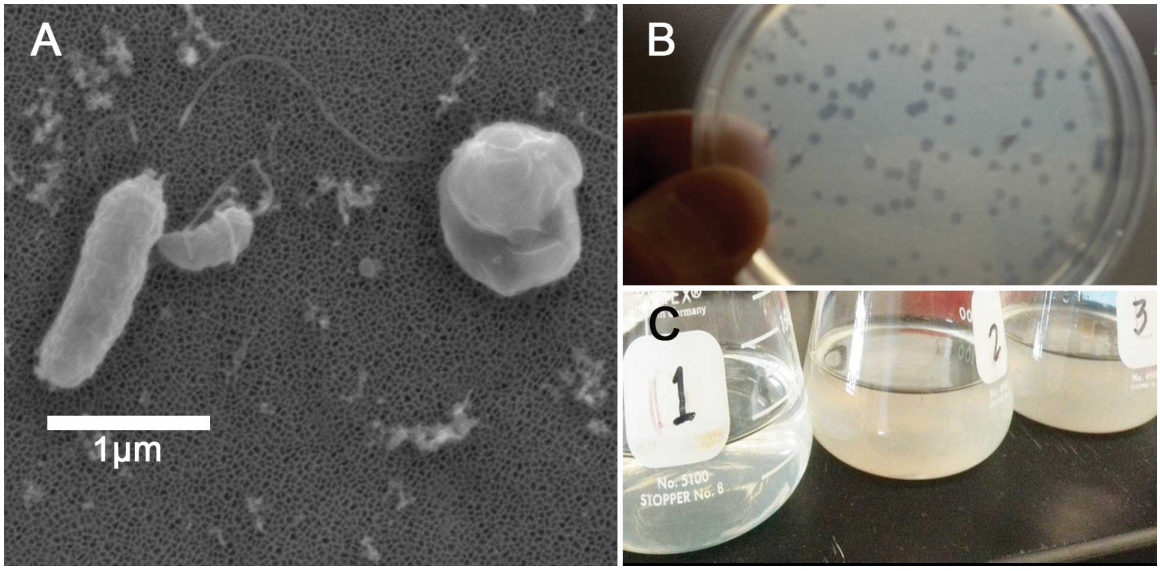

Figure 3.1 Halobacterivorax Predation of *V. coralliilyticus*. a) Micrograph of the pathogen, *Vibrio coralliilyticus* BAA450 being attacked by *Halobacteriovorax* and rounded *V. coralliilyticus* bdelloplast (right) with *Halobacterivorax* inside b) double layer plate showing freshly lysed plaques on a lawn of *V. coralliilyticus* cells c) Overnight liquid cultures of (1) a co-culture of *Halobacteriovorax* and *V. fortis,* (2) *V. fortis* and 0.2µm filtrate from *Halobacteriovorax* culture, and (3) *V. fortis* alone.

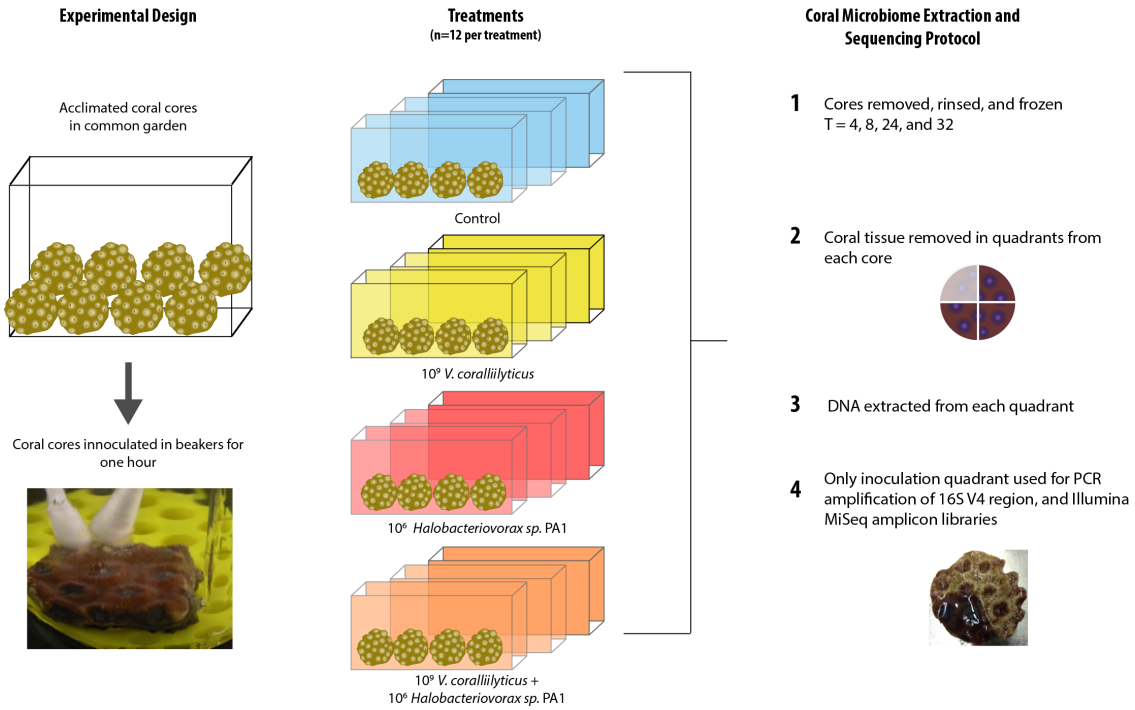

Figure 3.2 M. cavernosa Microbiome Manipulation Experimental Design M. cavernosa microbiome manipulation experimental design (from left) detailing collection and inoculation of coral cores, treatment tanks and replication, sample preservation, tissue removal, DNA extraction, and microbiome sample processing.

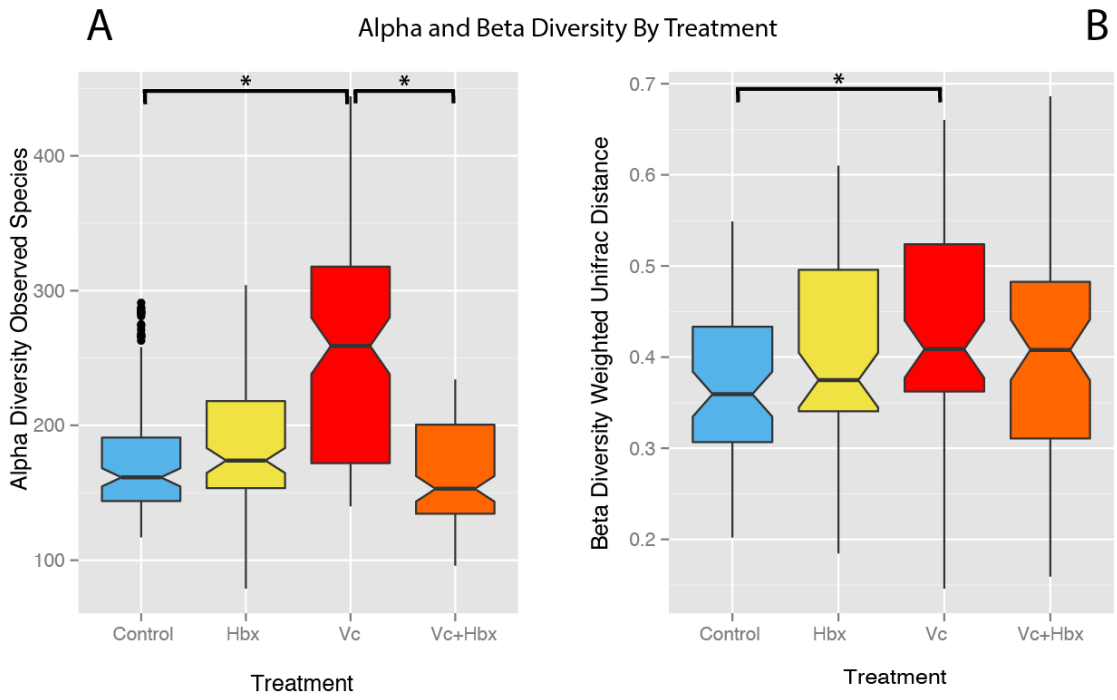

Figure 3.3 Alpha and Beta Diversity by Treatment The bacterial challenge treatment's impacts on microbial diversity. a) Mean alpha diversity (observed species) plotted for each treatment, and b) Mean beta diversity (Weighted UniFrac distance) by treatment. The asterisks indicate Bonferroni-corrected p values < 0.05 for the nonparametric t-test between treatments. In both cases while addition of the pathogen alone increased diversity, predator addition counteracted this effect.

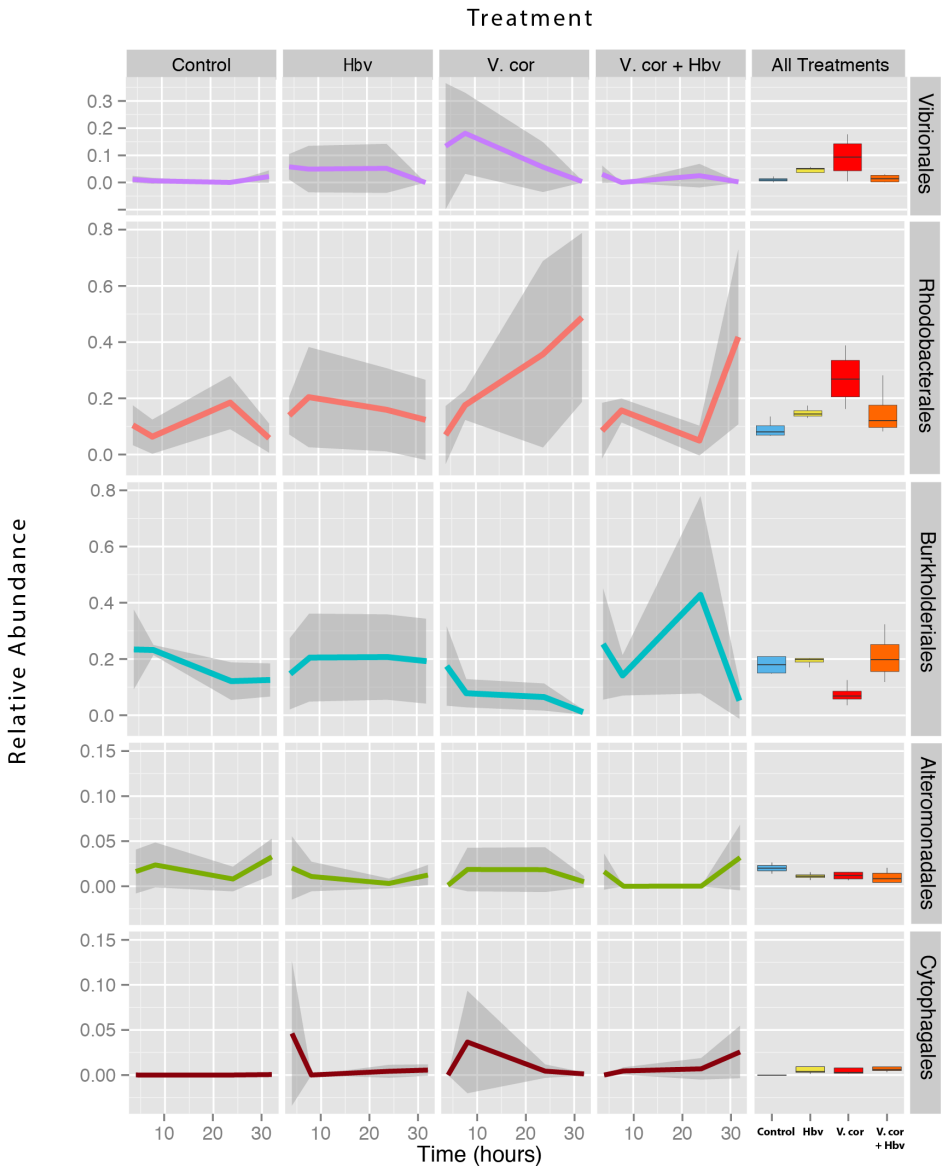

Figure 3.4 Relative Abundance Across Bacterial Challenges Relative abundance of all taxa found to be differentially present under the four bacterial challenge treatments. Colored lines denote mean relative abundance for each time point with grey transparent shading indicating the standard deviation, and boxplots show the mean relative abundance averaged across all time points for each treatment.

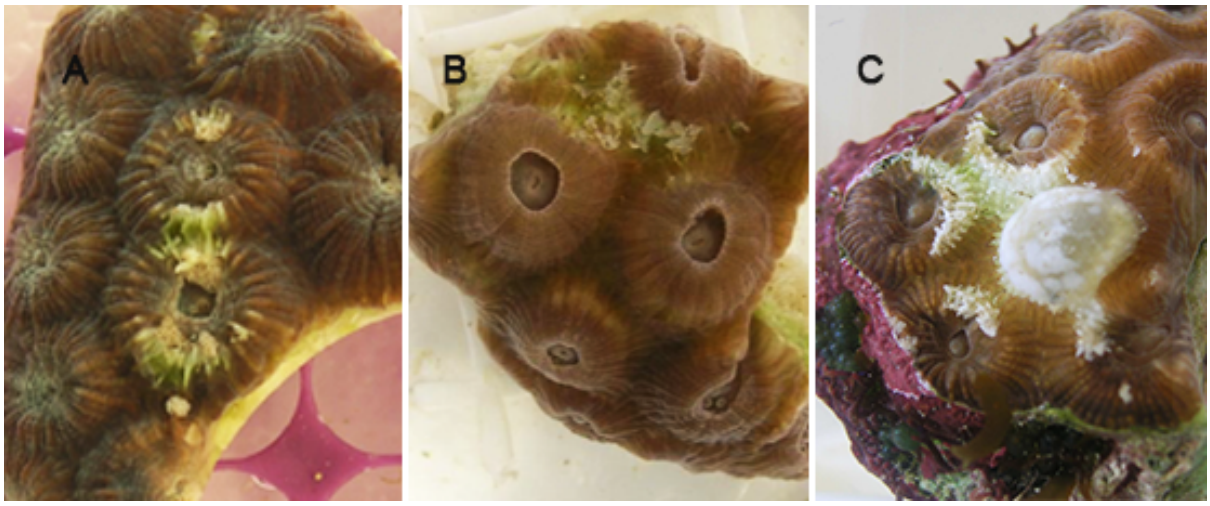

Figure S3.1 Pilot Experiment Inoculations Initial pilot experiment exploring inoculation concentrations needed to produce signs of tissue disruption in *M. cavernosa* corals 3 days post infection by *V. coralliilyticus* BAA 450. A) 10^3 *V. coralliilyticus,* B) 10^6 *V. coralliilyticus,* and C) 10^9 *V. coralliilyticus.*

Table 3.1 Bacterial predation assay results for several pathogenic Vibrio strains

Prey taxon (accession number)	50% killing rate in liquid media (hours)	Predation by Halobacteriovorax? (Double layer assay)
V. coralliilyticus BAA 450 (AJ440005)	19.00	**Yes**
V. coralliilyticus RE 98 (CP009617)	24.16	**Yes**
V. coralliilyticus RE22 (PRJNA168268)	No observable predation	**No**
V. tubiashii ATCC19106 (NZ_AFWI00000000.1)	No observable predation	**No**
V. tubiashii ATCC19109	13.84	**Yes**
V. fortis PA1 (KT626460)	11.67	**Yes**
V. cholerae N1696 (AE003853)	8.96	**Yes**
V. cholerae S10 (accession)	12.76	**Yes**

	Likelihood ratio test for GLMs	Post hoc Wald test on individual treatment comparisons with Benjamini-Hochberg correction					
Taxa Significantly Altered by Treatment (order level)	**P value with Benjamini-Hochberg correction**	**Control vs. Hbv**	**Control vs. Vc**	**Control vs. Vc & Hbv**	**Hbv vs. Vc & Hbv**	**Vc vs. Vc & Hbv**	**Vc vs. Hbv**
Burkholderiales	**0.007**	0.864	0.558	0.210	0.093	**0.015**	0.850
Vibrionales	**0.011**	0.644	**2.08E-04**	0.829	0.429	**0.001**	**0.040**
Cytophagales	**0.014**	**7.21E-05**	**9.23E-05**	**0.002**	0.429	0.424	0.958
Alteromonadales	**0.036**	0.936	0.616	**0.014**	**0.020**	**4.46E-04**	0.835
Rhodobacterales	**0.045**	0.644	**0.001**	0.393	0.987	**0.025**	0.060

Table 3.2 Bacterial taxa significantly altered by bacterial challenge treatment. Likelihood ratio test and post hoc Wald test statistics based on sequences derived from coral samples in microbiome manipulation experiment and Benjamini-Hochberg corrected p-values reported for 5 order level taxa (α below 0.05 reported in bold).

Table S3.1 Alpha Diversity Altered by Bacterial Challenge Alpha diversity altered by bacterial challenge treatment at each time point. Alpha diversity at a rarefaction sequence depth of 11716 reads from sequences derived from coral samples in microbiome manipulation experiment and reported for each time point.

	Bacterial Challenge Treatment			
Time	**Control**	**Hbv**	**V. cor**	**Hbv+V. cor**
4	**188.1 ± 4.13**	**226.2 ±8.0**	**229.6 ±6.6**	**174.6 ±6.7**
8	**187.8 ± 3.92**	**223.7 ±8.0**	**230.2 ±6.4**	**175.3 ±6.6**
24	**187.1 ± 4.27**	**223.6 ±7.9**	**229.3 ±6.4**	**174.5 ±6.8**
32	**187.8 ± 4.26**	**224.9 ±8.0**	**230.0 ±6.6**	**174.8 ±6.9**

Chapter 4

Bacterial predator-prey interactions at the interface between bacteria and their physical and biological environments

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Abstract

In marine ecosystems, the mechanisms that regulate community composition or bacterial population growth rates remain far from clear. However, understanding how bacterial populations respond to predation under different biological and physical environments is critical to modeling predator-prey population dynamics in aquatic and host-associated ecosystems. In addition to providing a boundary zone between host and environment, mucosal surfaces serve multiple roles, including a mechanism to clear bacteria and prevent pathogens from colonizing host epithelial tissue. Here we employed a wide range of methods and culture dependent techniques to study how the physical environment alters cell-cell interactions and impacts microbial predator-prey population dynamics in viscous environments that simulate host-associated mucosal surfaces. In order to study cell-cell interactions in mucosal environments, we employed microfluidic devices and high-resolution video microscopy and image analysis. In simulated mucosal media of various viscosities, real-time imaging with optical microscopy was paired with cell tracking to observe individual predator-prey interactions between our cultured the predatory bacteria, *Halobacteriovorax*, and a model pathogen of a variety of aquatic host organisms, *Vibrio coralliilyticus*. In our contrived two-culture system, we captured striking microscale observations that demonstrate *Halobacteriovorax* are better able to effectively prey on and reduce pathogenic *V. coralliilyticus* populations in viscous environments. Our results suggest predation by *Halobacteriovorax* is more effective against infections of *V. coralliilyticus* in mucosal surfaces and could act as a mechanism to regulate population size of this pathogen in vivo.

Introduction

In marine ecosystems bacterial diseases are an emerging economic and ecological threat (Ward & Lafferty, 2004). Distinct biological and physical environments encountered by the bacteria when migrating from host to host influence transmission dynamics of bacterial pathogens. The seawater to mucosal surface interface is a prime example of the types of highly variable environments bacterial pathogens encounter. Mucosal surfaces not only form the boundary layer between an animal and its environment, but these mucus layers are also often heavily colonized by host microbionts (Sansonetti, 2004). Mucus layers are critical to the resilience of individual host organisms and are often continuously shed to prevent host tissue colonization by pathogens (Neish, 2009). However, some bacteria, like *Vibrio coralliilyticus*, use chemotaxis and chemokinesis to target the mucus of their host prior to infection (Garren et al., 2014). Once in contact with the mucosal surface, pathogens have a limited window for infection due to the constant shedding of the mucus layer. Bacterial motility, which is associated with virulence, would substantially aid pathogens in migrating through the mucosal layer. However, bacterial motility is strongly tied to viscosity and these relationships have been studied for many years (Schneider & Doetsch, 1974; Leshansky, 2009). A critical gap in this area of research is the effect of viscosity on bacteria-bacteria interactions.

In addition the mucosal layer providing a physical barrier to infection, the normal flora that reside within mucosal surfaces often act as effective biological barriers to pathogens via antagonistic interactions or by niche competition (Vine et al., 2004). One particularly interesting interaction from a biological and physical standpoint is the one between an invading pathogen and native bacterial predator in the mucus layer. We

previously found an obligate bacterial predator, *Halobacteriovorax,* to be consistently associated with mucus layer of multiple species of coral and effective against the pathogen *V. coralliilyticus* (Welsh et al. 2015a-b). We also noticed our host associated predatory *Halobacteriovorax* isolates are often more successful at predation in our double layer plate cultures than our liquid cultures (data not shown). Understanding how pathogenic bacteria interact with other bacteria, as well as how changes in their physical environment may mitigate this interaction, could provide insights into transmission dynamics. Bacterial predation and the role of motility in bacterial predator-prey interactions are important to our understanding of both microbial ecosystem dynamics and aquatic bacterial disease, as well as the evolution of pathogen physiology. Here we investigate the effect of viscosity on the interactions between bacterial predators, and an important motile pathogen. The bacterial population dynamics were studied in co-cultures that were continuously monitored, yielding accurate, precise data with instantaneous real-time results using a high throughput 96-well plate reader. These data were paired with real-time imaging of bacterial predator and prey velocities using optical microscopy; this dual approach allows us to test theoretical predictions about the role of the environment in predator-prey interactions. We found that fundamental ecological principles in this area of biology vastly underestimates the impact of the physical environment on prey defenses.

Methods

Bacterial strains and growth conditions

Bacterial strains *Vibrio fortis* PA1, *V. coralliilyticus* strain BAA-450 from the American Type Culture Collection (www.atcc.org, Manassas, VA, USA), and the nonmotile *Vibrio coralliilyticus* strain *flhA* mutant (Meron et al., 2009) were grown in autoclaved 0.2 μm filtered artificial seawater (FASW) (Instant Ocean, Spectrum Brands Company, Cincinnati, OH, USA) with 10% 2216 media (BD Difco) in a shaking incubator at 30°C. *Vibrio* sp. were grown overnight from a single CFU, diluted 1:100 in fresh media, and grown to late exponential phase ($OD_{600\,nm} = 0.5$) before use in any experiment. The bacterial predator strain *Halobacteriovorax* sp. PA1 was grown in FASW in co-culture with *Vibrio* sp. prey (for details see Chapter 3) that were washed 3 times in FASW before being resuspended to a concentration of 10^9 cells/ml.

Microfluidic experiments

A variety of cell dynamics (cell-cell interactions, velocity, cell division) were visualized in a straight polydimethylsiloxane (PDMS) microchannel with a 400 μm wide injector and a 120 μm deep microchamber. Visualization was conducted using phase-contrast video microscopy on a Nikon Ti microscope (Tokyo, Japan) equipped with an Andor Neo CCD camera (6.5 um/ pixel; Belfast, UK), at 400 frame per second for 20 seconds.

The viscosity of FASW was adjusted using methylcellulose (Sigma Chemical Co., St. Louis, MO) to produce mediums with three different viscosities: 1 centipoise units (cP) (FASW), 2 cP (0.5 % methylcellulose), and 25 cP (2% methylcellulose). Cells were pelleted and resuspended in FASW, 0.5% methylcellulose in FASW, or 2% methylcellulose in FASW and then injected into the channel at a moderate flow rates (2

µl per minute). Flow was ceased stopped to image the cells velocity under zero flow in the middle of the chamber. Predation events were captured by allowing non-motile cells to naturally adhere to the glass substrate via non-specific binding and recording predator-prey interaction.

Cell tracking analysis

All analysis for cell tracking and trajectories were performed in Matlab (MathWorks, Natick, MA, USA) using automated in-house segmentation software to measure cell positions, size, shape, and track cell movements. Analysis was limited to cells tracked for a minimum of 10 consecutive frames. Detection and removal of non-motile cells and other particles were accomplished using background subtraction and cross-correlation functions to exclude these non-active particles (Garren et al. 2014).

Viscosity predation assay

The viscosity of FASW was adjusted using methylcellulose as described above. In this study the predator-to-prey ratio was identical in all three mediums, and thus allowed for direct comparison of population dynamics between media viscosity and prey phenotype. Triplicate biological replicates were grown for each predator-prey combination and suspended in each viscosity. Each predator-prey-viscosity combination was split into triplicate technical replicates in flat bottom 96-well plates (Greiner-Bio One, Germany). The bacterial population dynamics of the co-cultures in the three separate viscosities were continuously monitored yielding accurate, precise data with instantaneous real-time results using a high throughput 96-well plate reader with 200 µl

per well. Predation was measured by $OD_{600\,nm}$ values using a microplate reader (Infiniti M200; Tecan Group Ltd, Männedorf, Switzerland). Optical density values were reported without conversion to a 1 cm path length. *Halobacteriovorax* in attack phase does not significantly alter the absorbance reading of the prey at 600 nm due to their small cell size. Predation rates in the liquid co-culture assay were measured as the host cell density reduction in the predator-prey cultures compared to the predator-free controls.

Results

Microfluidic predator-prey interaction experiments

In this study, the life cycle and physical cell-cell dynamics of predation by *Halobacteriovorax* were recorded using microfluidics and phase contrast microscopy. Capturing predator-prey interactions was made possible by first allowing non-motile mutant cells of *V. coralliilyticus* to naturally adhere to the glass substrate via non-specific binding and then documentation of the position of attachment and time required for the predators to successfully attack the immobilized cells. Upon contact, the *Halobacteriovorax* point of entry into their prey cell (Figure 4.1) occurred at the polar region of the *V. coralliilyticus* cells ~33.3% of the time and at the midpoint region ~66.6% (Table 4.1). The time from initial predator-prey contact to predatory entry and structural modification of the host cell into a bdelloplast took on average 7.31 minutes (±28.25 sec) (Table 4.1). The entire maturation of *Halobacteriovorax* in the bdelloplast, as measured by the length of time between attachment to progeny release and lysis of bdelloplast, took on average 3.51 hours (±38.10 mins) (Table 4.1).

The responses of *V. coralliilyticus* to predation were captured using microfluidics and 8 hour time lapse videography, the approximate length of two complete *Halobacteriovorax* lifecycles. After the initial pulse of *Halobacteriovorax*, the flow of liquid media into the chamber was stopped in the microfluidic system, to prevent additional cells from entering the system. A ratio of 1:3 (predator to prey) was established at time zero, which allowed for subsequent infection to be observed after the first round of bdelloplast lysis. The remaining uninfected *V. coralliilyticus* cells did not grow or divide until the first round of *Halobacteriovorax* progeny were released via bdelloplast lysis around 3 hours and 23 minutes, with the average time to the first cell division at 4.32 hours (±1.06 hours) (Figure 4.2a bars). Exponentially more predators were observed in the microfluidic chamber (a result progeny lysis of the bdelloplast), and 89% of the vibrios were observed to be lysed by the time final image (Figure 4.3). Video analysis of these uninfected *V. coralliilyticus* showed these cells continuing to divide over the remaining length of the time lapse if predators in the chamber did not consume them (Figure of pictures), suggesting that lysate from bdelloplasts provided necessary resources for those few cells to divide. In contrast, only 5.56% of the cells from control channels with *V. coralliilyticus* alone divided over the entire 8-hour time lapse, and these control cells only divided once and then stopped unlike the cells in the ~4 rounds of division seen in the predator treatment (Figure 4.2).

The effect of viscosity on predation rates of wild type and non-motile mutants of *V. coralliilyticus*

The effect of motility and viscosity on bacterial predation was measured by studying predator–prey interactions in three separate viscous media: 1, 2, and 25 centipoise units (cP). To test differences in predation across these viscosities we used the wild type strain and a mutant, *V. coralliilyticus flhA* strain, that is incapable of forming the flagella and thus is non-motile. As measured by differences in the optical density of the prey alone control subtracted from the predator-prey co-culture, predators were found to effectively prey on both strains in all three conditions, but striking differences in predation efficiencies of the motile versus non-motile strains were observed across the viscosities. In the 1 cP lowest viscosity, which was ambient seawater conditions, the non-motile mutants experienced significantly higher predation than the wild type at the 15-hour time point ($p = 0.0004$) (Figure 4.4). Although the non-motile mutant also experienced significantly higher predation than the wild type in 2 cP ($p= 0.0441$), the wild types were now predated upon more efficiently (see below). In the 25 cP media, the non-motile *V. coralliilyticus* flhA cells experienced a 44% higher predation rate than the wild type at the 10 hour time point, the wild type ended up with a 27% higher predation rate at time final, but no significant differences were observed in this highest viscosity ($p = 0.209$) (Table 4.2).

The effect of viscosity on bacterial predation was determined by comparisons within a strain (i.e., wildtype or mutant) in the separate viscosities. Comparing predation of wild type *V. coralliilyticus* in all three media, we observed the pathogen to be most susceptible to predation in 2 cP media followed by 25 cP media. Significantly higher predation in 2 cP ($p= 0.002$) and 25 cP media ($p= 0.002$) was observed compared to 1 cP FASW (Table 4.2). The same trend was not observed in non-motile mutant. Here the

non-motile mutants in the mid and lowest viscosity media were quickly reduced to the lower boundary of the detection as the predation rate reached an asymptote after the initial 15 hours of the co-culture assay in both the 1 cP and 2 cP media (Figure S4.1). The non-motile mutants had the highest predation rate in FASW media, followed by 2 cP, and then 25 cP media (Figure 4.4).

Increases in viscosity reduce prey velocities and increase predator velocities

To test if reduced prey velocity is the mechanism behind this enhanced predation rate at higher viscosities, we tested the effect of viscosity on bacterial motility by quantifying individual cell (both prey and predator) mean velocities in three separate mediums (Figure 4.5). In ambient conditions in FASW, *V. coralliilyticus* cell mean velocities (55.37 μm * sec^{-1}) were significantly higher ($p = < 0.0001$) than *Halobacteriovorax* (45.37 μm * sec^{-1}). Conversely, in 2 cP media the mean velocity of *V. coralliilyticus* cells (56.30 μm * sec^{-1}) was significantly lower than ($p = 0.0001$) than *Halobacteriovorax* (65.46 μm * sec^{-1}). In the highest viscosity media test (25 cP) both species reported the slowest speeds, but no significant difference was detected between *V. coralliilyticus'* mean velocity (32.12 μm * sec^{-1}) and the mean velocity of *Halobacteriovorax* (32.16 μm * sec^{-1}).

Both bacterial types followed a similar trend in that their individual mean velocity were highest in a medium with twice the viscosity of seawater, or 2 cP viscosity, and were slowest in 25 cP media. However, the predatory bacteria *Halobacteriovorax* were significantly faster in 2 cP media ($p = < 0.00001$) than in FASW, while *V. coralliilyticus* were not significantly faster in the 2 cP media than they were in FASW (Figure 4.5)

suggesting that higher viscosities actually enhance the predator's speed advantage. We then plotted the predator's speed advantage (mean predator velocity – mean prey velocity) verses the predation rate at each viscosity to further demonstrate *Halobacteriovorax* predation is strongly correlated with predator speed advantage (Figure 4.6).

Discussion

BALOs are highly motile single flagellated bacteria, with observed swimming speeds from 35 μm * sec^{-1} for strain *Bdellovibrio bacteriovorus* 109J up to 160 μm * sec^{-1} for strain *B. bacteriovorus* HD100 (Lambert et al., 2006). Our study is the first report of swimming speeds for the marine genus *Halobacteriovorax,* and describes the mean velocities in three different methylcellulose amended medias (Figure 4.5). Methylcellulose has been used to study the effects of viscosity on bacterial motility (Pijper, 1947) and to successfully mimic the physical environments of host mucosal surfaces (Worku et al., 1999). Recent studies have shown that the coral pathogens *Vibrio shiloi* and *V. coralliilyticus* use chemotaxis to migrate towards coral mucus, enhancing the ability of these pathogens to locate and colonize their host (Banin et al., 2001; Garren et al., 2014). Yet, the mucus layer is heavily colonized by bacteria, including *Halobacteriovorax,* which these pathogens are likely to encounter and interact with before adhering to the coral surface. Our findings demonstrate how changing the viscosity of the bacteria's physical environment alters fundamental biological cell-cell interactions such as predation.

Encounter rates in bacterial predator-prey interactions are affected by cell size and motility (Blackburn & Fenchel, 1999). In our co-culture system, cell size is fixed, leading us to hypothesize that the combination of the highest observed swimming speeds detected in 2 cP media for the wild type *V. coralliilyticus* (56.30 μm * sec^{-1}) and the predator (65.46 μm * sec^{-1}) would lead to the highest encounter rate and thus the highest predation rate in our macroscale predation studies. However, the highest predation rates were detected for the non-motile mutant and predator in FASW (Figure 4.4) indicating the motility of the prey is a key defense mechanism against bacterial predation. Previous studies have shown that highly motile bacteria experience significantly lower ingestion rates for eukaryotic bacterivorous nanoflagellate (Matz & Jurgens, 2005). Here we provide the first quantification of how prey motility decreases bacterial predation rates of an important marine pathogen by bacterial predators.

The predator's velocity relative to their prey was also correlated with predation rates and further demonstrates the role of motility in bacterial predation. The average velocity of wild type *V. coralliilyticus* was not significantly different in 1 vs. 2 cP media, but the predator's mean velocity went from being lower than the prey in 1 cP to significantly faster than the prey in 2 cP (Figure 4.5). Furthermore, the predation rate was significantly higher in the 2 cP media compared to 1 cP.

The non-motile mutant *V. coralliilyticus flhA* was effectively cleared by *Halobacteriovorax* in the first 15 hours of the experiment in FASW and 2 cP media, and experienced significantly higher predation than the wild type in of the two viscosities (Figure 2). *Halobacteriovorax* did graze upon wild type at a slightly higher rate than the

non-motile *flhA* mutant in 25 cP media, although the increase in predation rate was not significant ($p = 0.209$).

The viscosity of the physical environment also affected predation rates on the wild type *V. coralliilyticus,* which were significantly higher in the viscous environments than in FASW ($p= 0.002$ and 0.020 in 2 and 25 cP media, respectively). Conversely, an opposite trend was reported for non-motile strain in that although no significant difference was detected between FASW and 2 cP, bacterial predation rates were significantly higher in the less viscous 2 cP ($p = 0.029$) than 25 cP media and in FASW compared to the 25 cP ($p= <0.001$) viscous environment. Studying the differences in the predator's and prey's velocities at these different viscosities, we see that in 2 cP media the predators are significantly faster and that correlates with their higher predation rates in 2 cP compared to FASW where the prey are faster.

In our study we explored the microscale cell-cell interactions and describe the temporal details of the major life cycle events for *Halobacteriovorax* as it attacks, enters, grows, and lysis an important marine pathogen, *V. coralliilyticus* (Figure S4.1). These results greatly expand upon previous studies of Bdellovibrio and like organisms (BALO), which were only available for the freshwater predatory bacteria *Bdellovibrio* (Kessel & Shilo, 1976; Fenton et al., 2010; Lerner et al., 2012). The marine predator *Halobacteriovorax* exhibits a much faster attachment to cell invasion time, 7.31 minutes (28.25 sec), when preying on *V. coralliilyticus* compared to the ~20 minutes for *Bdellovibrio* preying on *Escherichia coli*. Whether the faster invasion times are due to differences in the prey, the predators, or a synergistic combination of the two is unclear, but represents an area for future studies as the mechanisms behind these differences may

provide insights to possible prey recognition by BALOs. The nature of prey recognition by BALOs and whether specific receptor sites of host cells exist is not yet clear. Another interesting observation is that the freshwater *Bdellovibrio* are reported to preferentially attack and invade the polar region of their prey cell (Tudor, Mccann & Acricht, 1990), yet in our study *Halobacteriovorax* seems to be equally capable of invading vibrios from either the middle region (33.33%) or the polar ends (66.66%) (Table 1).

Halobacteriovorax completes an entire lifecycle in 3.51 hours (38.10 min), which is similar to previous observations using *Bdellovibrio* (Rittenberg & Shilo, 1970; Ruby & Rittenberg, 1983). *Bdellovibrio* are remarkably efficient at the utilization of prey components and exhibit carbon assimilation rates of up to 55%, respiration of another 15%, and the remainder is discarded (Ruby & Rittenberg, 1983). Yet in our time-lapse studies using *Halobacteriovorax*, we observed significantly more cell divisions of *V. coralliilyticus* post-bdelloplast lysis than pre-lysis or in the control with *V. coralliilyticus* alone (Figure 4.2). These data indicate predation by *Halobacteriovorax* is responsible for nutrient cycling, which once liberated can be taken up by their prey or other bacteria. Furthermore, these results indicate we are likely under-reporting the total active predation when using the optical density of the co-cultures to measure predation rates.

Our findings demonstrate *Halobacteriovorax* as an effective predator against *V. coralliilyticus* in the marine environment. Interestingly, in the viscous environments used in this study to mimic animal host mucosal surfaces, the scales are tipped in the predator's favor, and the pathogens are more efficiently preyed upon in these environments. In the water column *V. coralliilyticus* is likely more able to use its higher swimming speed to escape from predation than in host mucosal surfaces.

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Figures

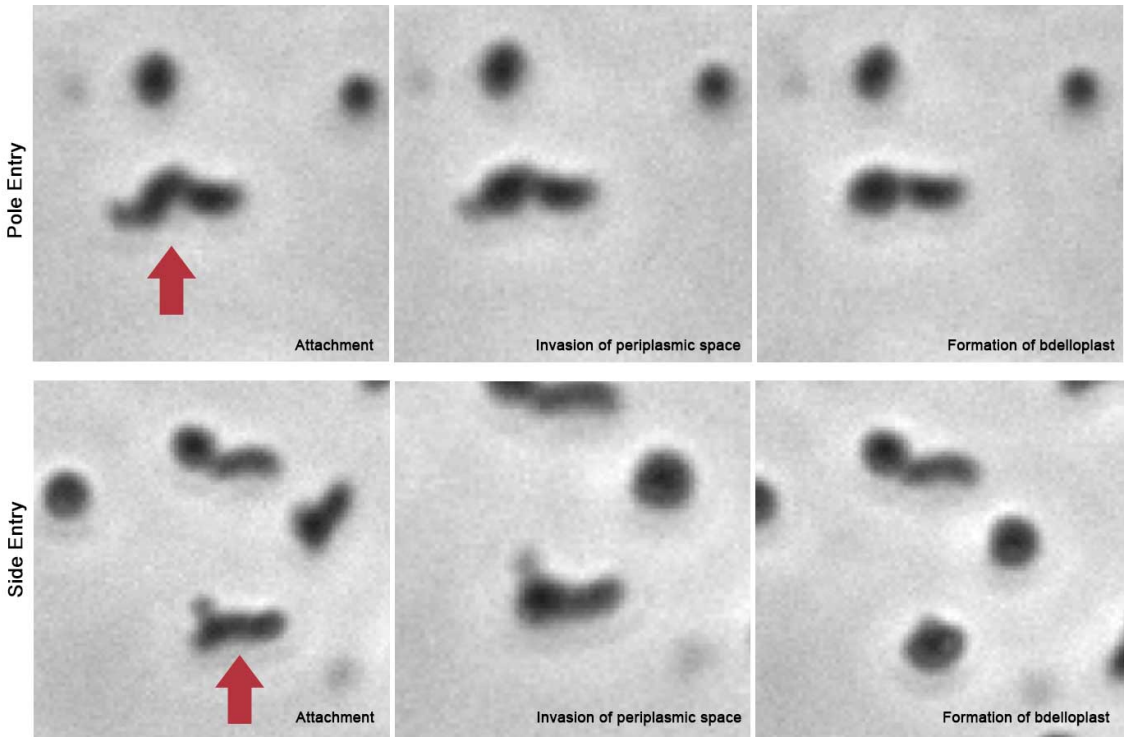

Figure 4.1 Point of Entry Point of entry into the periplasmic space of *V. coralliilyticus.* The top is an example of predatory attack and entry from the pole of the cell and the bottom panels show non-polar attack and entry on the side of *V. coralliilyticus.*

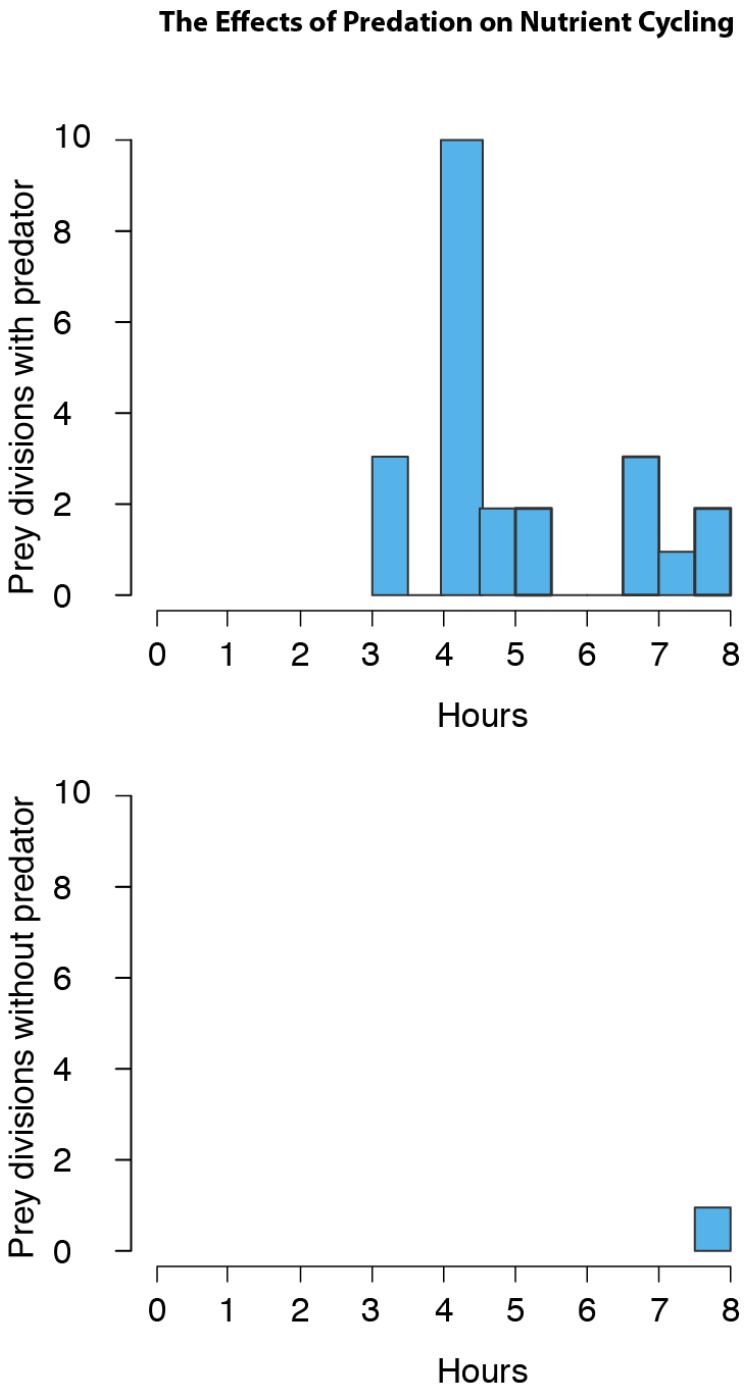

Figure 4.2 The Effects of Predation on Nutrient Cycling Prey divisions post bdelloplast lysis and nutrient release. Histogram showing total number of *V. coralliilyticus* cell divisions binned by time for the microfluidic system with the predators (top) and without (bottom). No *V. coralliilyticus* cell division is observed prior to one complete life cycle of the predators (~3.5 hrs), and the highest number of uninfected cell division occurs shortly after bdelloplast lysis.

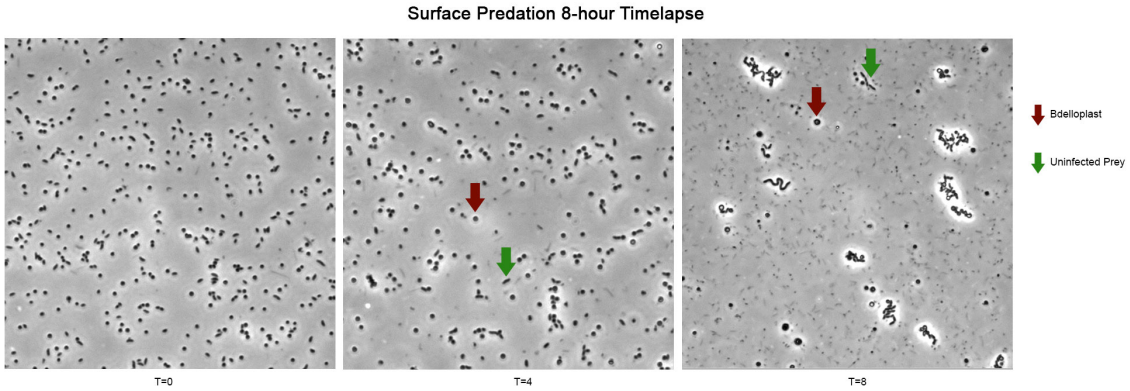

Figure 4.3 Surface Predation 8-hour Timelapse Time lapse of *Halobacteriovorax* clearing a lawn of non motile *Vibrio coralliilyticus flhA* cells. Red arrows indicate examples of rounded bdelloplast of infected *V. coralliilyticus* and green arrows indicate examples of uninfected *V. coralliilyticus* cells.

Figure 4.4 Effect Of Viscosity On Predation Rates. The effect of viscosity on predation rates of the wild type *V. coralliilyticus* strain and a non motile mutant, *V. coralliilyticus flhA*. The higher the change in optical density the higher the observed predation rate in the three different viscosities for both prey types (1, 2, and 25 cP).

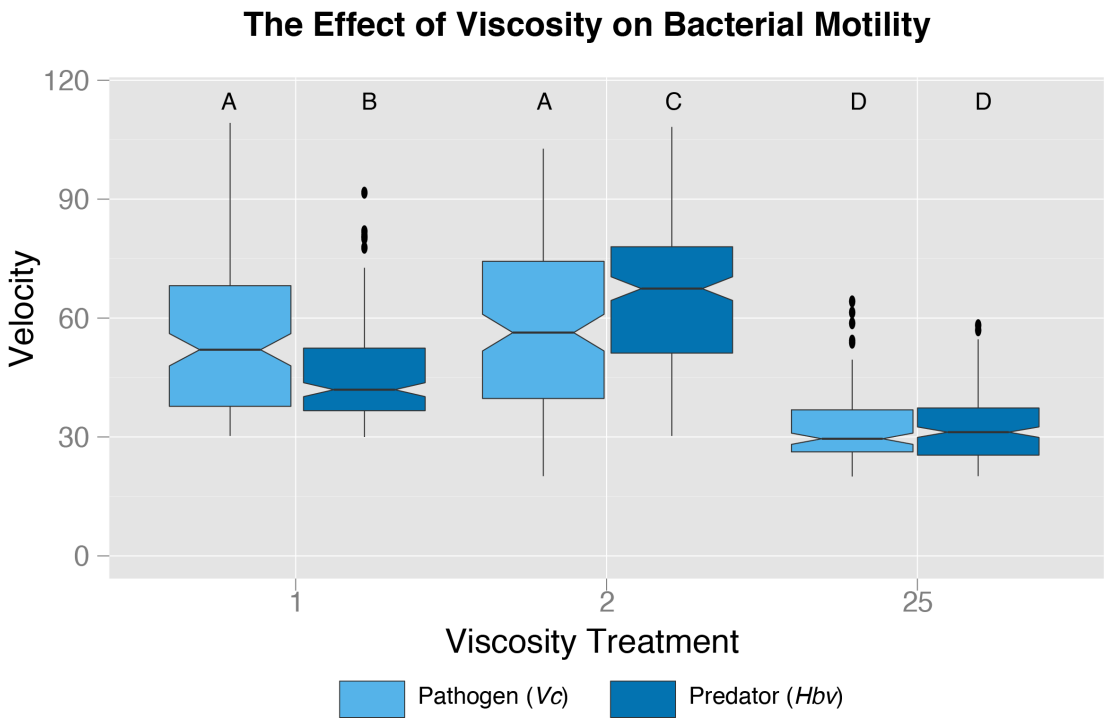

Figure 4.5 The Effect Of Viscosity On Bacterial Motility. Boxplots representing average velocities of each predator and prey bacteria (n=150 tracks) at three different viscosities. *V. coralliilyticus* are represented as the light blue boxes on the left side at each viscosity and *Halobacteriovorax* are dark blue boxes.

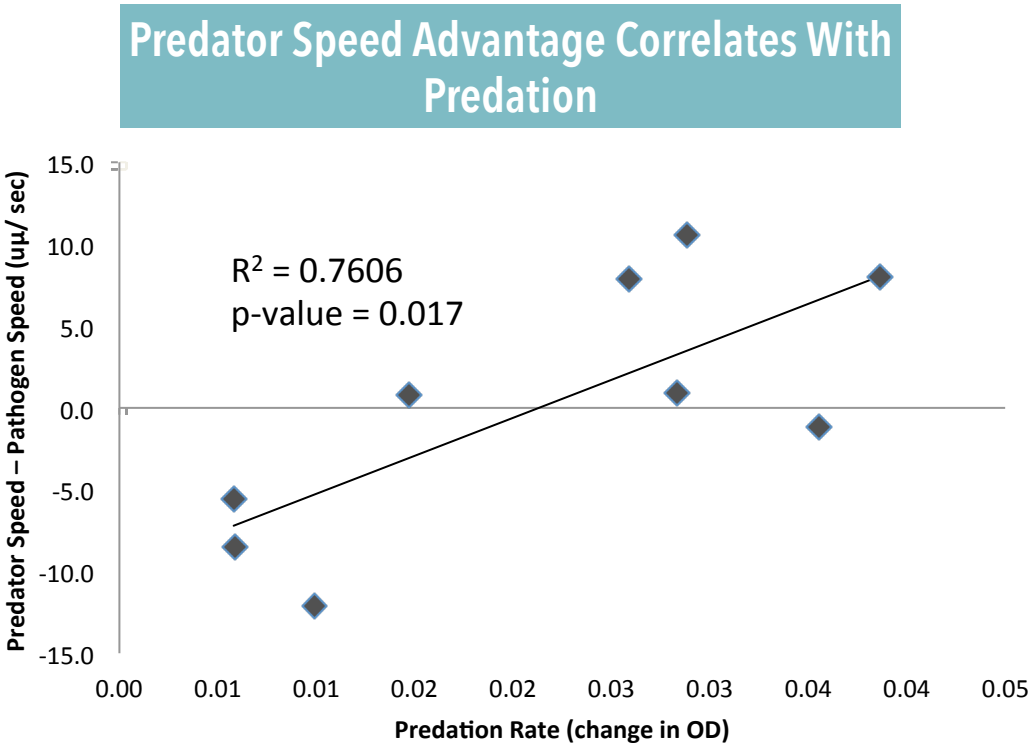

Figure 4.6 Predator Speed Advantage Correlates with Predation Rate. The speed advantage of *Halobacteriovorax* (mean predator velocity – mean prey velocity) verses the predation rate of *V. coralliilyticus* is plotted to measure the strength of a linear association between the two variables for all three viscosities. The Pearson correlation was calculated and the value of the R^2 and p-value are shown in the plot.

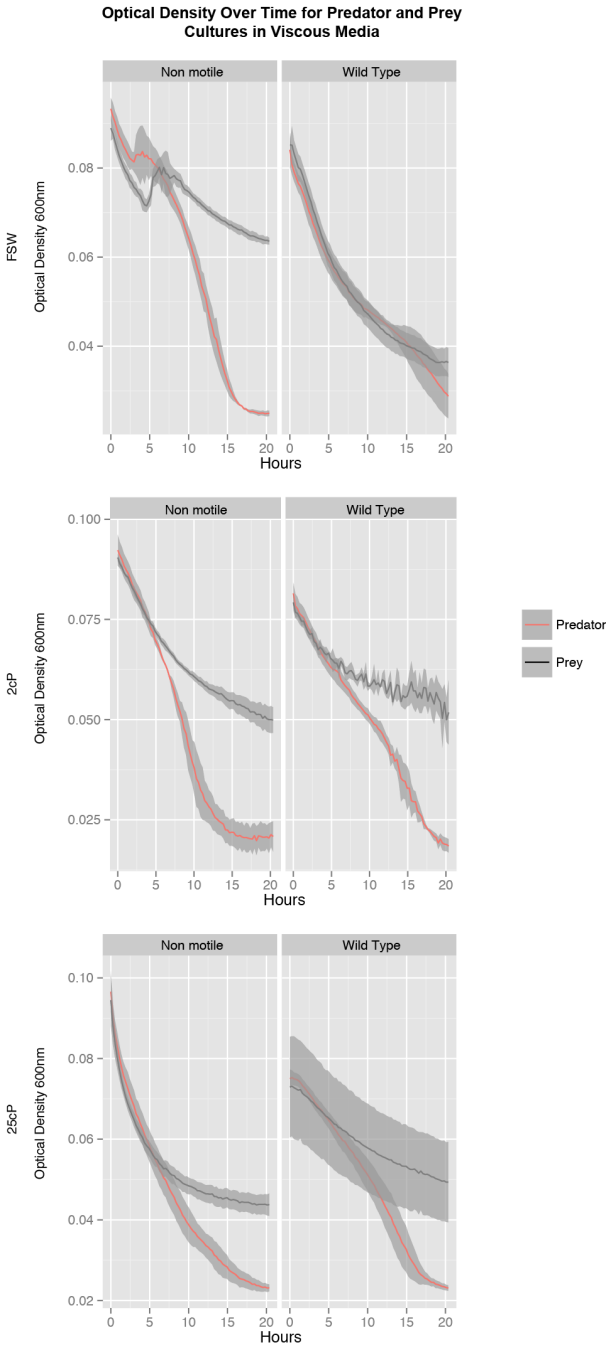

Figure S4.1 Optical Density Over Time for Predator and Prey Cultures in Viscous Media Mean optical density of Halobacteriovorax, wild type V. coralliilyticus strain and a non motile mutant, V. coralliilyticus flhA. Observed OD over time at three different viscosities (1, 2, and 25 cP).

Tables

Table 4.1 Microscale Observations Of Predator-Prey Interactions Microscale observations of predator-prey interactions in the lifecycle of the predatory bacteria *Halobacteriovorax* preying on non-motile *V. coralliilyticus flhA.* Averages reported are of a subpopulation cells (n=30) in filtered seawater from image analysis of predator-prey interactions in a straight channel microfluidic device.

Observations of Predator-Prey Interactions	Summary of Predator-Prey Interactions
Percent pole end entry to prey periplasms	***66.66%***
Percent mid-point entry to prey periplasms	***33.33%***
Average predator-prey contact to bdelloplast formation (minutes)	***7.31 (±0.47)***
Average contact to bdelloplast lysis (hours)	***3.51 (±0.64)***

Table 4.2 Macroscale Observations Of The Effect Of Viscosity And Motility On Predation Rates Student's T-test compared to wild type.

Media Viscosity	wild type vs. non motile (20 hour)	wild type vs. non motile (15 hour)	wild type vs. non motile (10 hour)	wild type	non motile
1	**0.010**	**1.00E-05**	**2.00E-05**	**-**	**-**
2	**0.014**	**0.044**	0.338	**-**	**-**
25	0.376	0.344	0.209	**-**	**-**
1 vs. 2	**-**	**-**	**-**	**0.002**	**0.029**
1 vs. 25	**-**	**-**	**-**	**0.020**	**7.40E-05**
2 vs. 25	**-**	**-**	**-**	0.265	0.052

Chapter 5

Discussion and Conclusions

Microbiome research has surged over the past decade due to advances in high-throughput sequencing technologies and the increased availability of powerful computing software to analyze such data. Explorations into the composition and dynamics of microbial communities have transformed our understanding of the diversity of microbial ecosystems (Waldor et al., 2015). For example, coral microbiome research has greatly expanded over the past decade largely due to the desire to understand interactions between the host and their symbiotic microorganisms and to use this knowledge to prevent the spread of coral disease (Rosenberg, Kellogg & Rohwer, 2007). Although there have been advances in the understanding of the microbiomes on and within healthy, stressed and diseased corals (Frias-Lopez et al., 2002; Pantos and Bythell, 2006 Sunagawa et al. 2009, Vega Thurber *et al.*, 2009), the mechanisms and interactions regulating the structure and dynamics of the coral microbiome is still far from clear. In this dissertation I combined traditional microbiology and emerging high-throughput technologies to go beyond cataloging the composition of microbial communities and attempt to elucidate the principles that govern microbial dynamics, structure and functions. Specifically, I used a known coral pathogen, *Vibrio coralliilyticus,* and its natural predator, a coral-associated predatory bacteria, *Halobacteriovorax,* to study how individual members of the microbiome contribute to the structure of the community.

Predation is pervasive in nearly every ecosystem on the planet and is a significant cause of mortality. Predation is a strong evolutionary force on both predator and prey. For prey, there is a strong selection for predator avoidance. For predators, there is a strong selection for predatory efficiency (Day, Abrams & Chase, 2002). In many ecological communities, predation plays a key role in regulating community structure or function

(Stanley, 1973). While predation has been extensively explored in animals and microbial eukaryotes, predation by bacteria is less well understood. Predatory bacteria are ubiquitously distributed in aquatic and terrestrial environments, and ecologically relevant members of microbial communities (Pineiro et al. 2007; Snyder et al. 2002; Davidov and Jurkevitch 2004, and this work). *Halobacteriovorax* are a halophilic genus of the obligate predatory bacteria, Bdellovibrio and like organisms (BALOs). As obligate predators they contribute to the highly diverse Deltaproteobacteria class. Although BALOs are tiny, about 20 % of the size of a typical bacterium, their known genomes are large (~3.73 MB), which is a contrast to typical obligate parasite/ predators which have much smaller genomes than free-living species. Among the predatory bacteria, BALOs are the best studied for their predatory behavior, but a majority of this research focuses on the freshwater and terrestrial genus *Bdellovibrionales.*

Predation by the bacteria *Halobacteriovorax* represents a virtually untapped resource for coral microbial ecologists, as well as for microbiologists in general. Previous studies reported *Halobacteriovorax* to be associated with corals (Sutton & Besant, 1994; Wegley et al., 2007; Vega Thurber et al., 2009). In chapter 2, I discussed our study that confirmed *Halobacteriovorax's* presence on three genera of reef building corals through cultures. I also presented full-length 16S rRNA sequencing of cultures, phylogenetic analysis of the full length reads, and microscopic evidence to describe this unique biphasic predatory lifecycle and its stages (Welsh et al. 2015). Isolating *Halobacteriovorax* from natural samples and then working with them in laboratory presented unique challenges. The modified isolation method for coral mucus samples I developed in Chapter 2 helped address these issues, and I was then able to detail culture-

independent approaches that other researches can use in future investigations. I also conducted network analysis using 16S amplicon libraries of 198 samples from a larger a three-year time study on a Caribbean reef to detail the temporal and spatial dynamics of BALOs and their potential prey across the reef (Welsh et al. 2015) (Chapter 2). Our networks indicated that BALOs promote maintenance of a community with even distribution of a consistent set of coral-associated microbes. Furthermore, despite their low abundance, and predatory lifestyle, BALO were positively associated with the members of the coral microbiome community structure (Welsh et al. 2015) (Chapter 2).

In Chapter 3 I tested how coral microbiome dynamics were affected by interactions between our coral isolate *Halobacteriovorax* sp. PA1, which is effective against known vibrio coral pathogens (Chapter 2), and the model coral pathogen *Vibrio coralliilyticus,* the bacteria most-studied for coral disease and microbial-associated bleaching (Ben-Haim et al., 1999; Ben-haim, Zicherman-keren & Rosenberg, 2003; Wilson et al., 2013). *Montastraea cavernosa* coral specimens were treated *V. coralliilyticus* in the presence or absence of *Halobacteriovorax,* and microbial community dynamics were monitored with 16S rRNA gene time-series. Specimens treated with *V. coralliilyticus* alone experienced increases in Vibrios and microbiome richness, with reduced community stability (increased beta-diversity). In specimens treated with both *V. coralliilyticus* and *Halobacteriovorax*, the predators ameliorated those effects. Importantly, *Halobacteriovorax* was not detected in coral tissue specimens treated with *Halobacteriovorax* alone or the combination of *Halobacteriovorax* and *V. coralliilyticus.* Thus, I demonstrate that these coral associated predatory bacteria

primarily colonize the surface mucus layer of their host (Welsh et al. 2015b in review) and provide a top down control of pathogens in that habitat (Chapter 3).

In Chapter 4 I tested questions about the role of the physical environment on predator-prey dynamics by mimicking the viscosity of the coral mucosal surface layer. This approach assisted in my studies on the potential cell-cell interactions occurring between *Halobacteriovorax* and *V. coralliilyticus* in the coral mucus layer. I combined traditional methods and emerging technologies to study the effects of viscosity on motility and predator-prey interactions. I used methylcellulose to alter the viscosity of bacterial media to study the effect of viscosity on motility and employed high throughput 96-well plate reader assays to test the effect of multiple viscosities on predator-prey population dynamics. My results indicated that predators are most effective against *V. coralliilyticus* in viscous environments such as those found in mucosal surfaces (Chapter 4). Using real-time imaging with optical microscopy and particle tracking, I established the differential swimming speeds found between predator and prey in three separate viscosities. Thus I also established that viscosity induced changes in swimming speed was the underlying mechanism responsible for changes in bacterial predation rates (Chapter 4).

Future studies should explore the impact of *Halobacteriovorax* on bacterial mortality and how bacterial predation effects environmental processes. Such studies could have transformative effects on microbial ecology and lead to potential breakthrough therapies for bacterial infections. Given the rise of antibiotic resistance, alternative therapies are urgently needed across many sectors. One such example is the aquaculture industry, the fastest growing food-producing sector worldwide, which loses

billions of dollars per year due to disease (Lafferty et al. 2015). Future studies using predatory bacteria as an alternative to antibiotics should explore predator-prey cycles using truly quantitative methods such as qPCR, and test whether the addition of predatory bacteria promote nutrient availability. Furthermore, studies should focus on the health of the host and how that correlates with changes in the microbiome. The studies to date that have explored the use of predatory bacteria as a viable alternative antibiotics, primarily focusing solely on the health of the host aquaculture species (Cao et al. 2015). We now have the ability to test fundamental ecological theories on predation's cascading impacts in real-time on living hosts. My work represents an exciting first step in this emerging field of discovery.

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