Microbial Ecology and Genetic Mechanisms of Virulence and Pathogenicity in White Band Disease-Infected Staghorn Corals

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Dedication

To my grandfather, Seymour Amkraut. I see this achievement as yours as much as mine.
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Abstract of Dissertation

Coral disease represents a massive threat to coral reefs around the globe. This dissertation focuses on white band disease (WBD), an epizootic responsible for the near eradication of two important reef-building Caribbean corals, *Acropora cervicornis* and *Acropora palmata*. The worsening effect of WBD – and coral disease in general – necessitates an increased understanding of the ecology and genomics of coral-associated microbes. The following studies explore the microbial community structure of diseased corals and the mechanisms of pathogenesis.

Chapter 2 introduces the idea that WBD initiation and progression may rely on quorum sensing (QS) in bacterial residents. Quorum sensing as a mechanism for disease transmission will be a theme of this dissertation. Healthy *A. cervicornis* fragments were exposed to autoinducer-supplemented microbial assemblages. Incubation with the AI-1 autoinducer N-Hexanoyl-DL-homoserine lactone led to WBD transmission in all test corals. This finding indicates that activation of QS-controlled pathways in coral-associated bacteria may lead to opportunistic pathogenesis. Chapter 2 also employed culture-based methods to determine the relationship between certain bacterial species and *A. cervicornis* disease state. *Vibrio* and Flavobacteria were grown on media infused with healthy and WBD 0.2 µm-filtered coral homogenate. Diseased filtrate promoted growth of Flavobacteria while healthy filtrate inhibited this group. The results of this study solidified the theme of this dissertation; each subsequent chapter will focus on the influence of QS as well as the taxa *Vibrio* and Flavobacteria on WBD infection and transmission in *A. cervicornis*.
Chapter 3 utilizes next-generation high-throughput sequencing of the hypervariable region of the 16S rRNA gene. This chapter explores the opposite effect of autoinduction by employing an autoinducer antagonist – a QS inhibitor (QSI) – to stop WBD in healthy *A. cervicornis*. WBD-associated microbiomes exposed to QSI were unable to transmit disease to healthy corals while control WBD microbiomes spread disease. QSI-supplemented communities were found to contain a lower abundance of Vibrionaceae and Flavobacteriaceae OTUs suggesting that QSI constrains the population of disease-causing bacteria. These microbiomes also contain a higher abundance of the putative coral symbiont *Endozoicomonas* and the related bacterial family Halomonadaceae. The findings of this chapter imply that Vibrionaceae and Flavobacteriaceae are involved in WBD infection, *Endozoicomonas* and Halomonadaceae are potential *A. cervicornis* symbionts, and that WBD transmission can be halted via the application of QSI.

Chapter 4 employs next-generation sequencing methods in the context of metatranscriptomics as well as 16S rRNA. Microbial communities from paired healthy and WBD-infected *A. cervicornis* fragments were isolated and RNA was sequenced in order to determine differential bacterial gene expression between healthy and diseased coral-associated microbiomes. The 16S rRNA gene was also sequenced for these same samples in order to compare the overall community to the metabolically active species. A relatively small number of genes contribute to WBD infection and thus the 16S results were used to determine the important bacterial taxa on which to focus differential gene expression: Vibrio and Flavobacteriaceae. WBD-associated Vibrios employ the SOS response while WBD-associated Flavobacteriaceae utilize lipid metabolism and antibiotic resistance.
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Coral-bacterial interactions from mutualism to parasitism through the lens of white band disease in *Acropora cervicornis*

**Overview**

Coral reefs are among the most important and valuable ecosystems on earth (De Groot et al. 2012). However, due to climate change and other anthropogenic impacts, reefs have entered a state of decline globally with regards to coral cover and health. In addition to coral bleaching, one of the main drivers behind this unprecedented deterioration is coral disease. Yet, despite the importance of coral disease impacts globally, the high diversity and variability of coral microbiomes has complicated the identification of specific coral disease agents. Coral disease is often correlated with disruptions to the coral’s resident microbial assemblage, but the mechanisms that drive infection and transmission of specific pathogens are not well characterized. Conventional wisdom identifies approximately 20 coral diseases, only a handful of which have been linked to a pathogen via fulfillment of Koch’s postulates (Sutherland et al. 2004). However, in this dissertation we suggest that coral disease is caused by a consortium of opportunistic bacterial species rather than a single pathogen. Furthermore, we propose that quorum sensing allows these pathogens to establish infection on vulnerable corals.

**The coral holobiont**

Microbial communities play a vital role in a vast array of marine ecosystems, including coral reefs (Harvell et al. 2007, Knowlton and Rohwer 2003). In fact, corals are now viewed as a
holobiont – comprising the coral animal, its algal symbionts, and associated microbes – rather than a single organism. Symbiosis between the coral animal and its endosymbiotic dinoflagellates is an established and well-studied phenomenon. Corals derive the majority of their chemical energy from their mutualism with *Symbiodinium* – symbiotic dinoflagellates colloquially known as zooxanthellae – which photosynthesize and translocate sugars in the form of glucose and glycerol to the coral and receive a safe and stable habitat in return (Hoegh-Guldberg and Smith 1989). Coral bleaching occurs under increased temperatures and UV stress causing the host to digest or expel its *Symbiodinium* thereby exposing the animal skeleton underneath (Hoegh-Guldberg and Smith 1989). In some cases, corals may reacquire their zooxanthellae after a bleaching incident, but in general bleached corals only survive for a couple weeks (Grottoli et al. 2006).

Compared to the well-documented coral-algal symbiosis, much less is known about the relationship between the coral animal and its diverse bacterial microbiome (Knowlton and Rohwer 2003). Corals provide a number of discrete locations for microorganisms to colonize including the surface mucus layer, the coral tissue itself, and the calcium carbonate skeleton (Rosenberg et al. 2007). Each of these habitats harbors a distinct bacterial population that also differs from the surrounding environment (Bourne and Munn 2005, Koren and Rosenberg 2006).

Given that coral-associated microbes differ from bacterial populations found in reef seawater, it can be assumed that the relationship between a coral and its microbiota is specific in that corals of the same species harbor similar microbiomes despite their geographic location. (Morrow et al. 2012, Rohwer et al. 2001, Rosenberg et al. 2007, Thurber et al. 2009). Other microbes – like
Endozoicomonas – appear to be generalists and associate with many species of healthy coral in large numbers (Bayer et al. 2013b). Regardless, breakdowns in the coral-bacterial community structure during times of environmental stress can lead to coral disease (Bourne et al. 2009, Harvell et al. 2007). Indeed, the hallmark of coral disease is a shift from a healthy coral microbiome towards one that is characterized by disease-associated microbial species (Ritchie 2006, Thurber et al. 2009).

**Opportunistic bacteria as drivers of coral disease**

Coral diseases are often necessarily characterized by the visual appearance of phenotypic disease signs on the coral host. Unfortunately, disease signs are often inappropriate markers for distinguishing diseases because many coral diseases share similar physical characteristics (e.g. bleaching) as well as putative bacterial pathogens. In addition, it is notoriously difficult to fulfill Koch’s postulates for coral diseases. Koch’s postulates are four criteria historically used to establish a causative relationship between a pathogen and a disease: (1) the bacteria must be found on all diseased organisms but not healthy organisms (2) the bacteria must be isolated from a diseased organism (3) the isolated bacteria should cause disease when introduced to a healthy organism and (4) the bacteria must be re-isolated from the inoculated organism. Although these criteria have major limitations, they represent a good guide for establishing pathogenicity. Very few coral diseases sufficiently fulfill these rules, the first difficulty being that many bacteria are unculturable via traditional methods. Moreover the absence of a dominant pathogen indicates that a causative agent does not exist for most coral diseases. Rather, the same coral disease can be associated with a variety of microbes (Gignoux-Wolfsohn and Vollmer 2015, Gil-Agudelo et al. 2006) suggesting that a consortium of species is responsible. In addition, environmental
pressures – such as increasing sea surface temperatures – exacerbate coral disease prevalence, implying that opportunist microbes in the coral host native microbiome may contribute to disease pathologies.

In recent decades, the relationship between corals and their microbial residents has been destabilized by a variety of anthropogenic impacts, most notably the rising seawater temperatures and ocean acidity associated with global climate change (Aronson and Precht 2001, Harvell et al. 1999). Environmental changes, especially in temperature, increase stress on the coral animal. These abiotic stressors compromise the coral’s immune system leading to shifts in the affected coral’s bacterial communities away from symbiotic and commensal microbes towards general and opportunistic pathogens (Aronson and Precht 2001, Muller and van Woesik 2012, Ritchie 2006, Rosenberg and Ben-Haim 2002). This phenomenon, known as the compromised host hypothesis, states that commensal species may become pathogenic as certain bacterial residents take advantage of their host’s decreased resistance to infection (Lesser et al. 2007). Essentially, the increasing incidence of coral disease is not due to the introduction of novel pathogens or increased pathogen virulence but rather results from the inability of susceptible corals to fight off infection (Lesser et al. 2007, Muller and van Woesik 2012). As a result, worldwide coral epizootics have escalated in incidence and severity over the last 30 years and have contributed to the unprecedented decline in global coral reef health (Aronson and Precht 2001, Harvell et al. 2007). This hypothesis may partly explain the difficulty in isolating coral pathogens as coral disease is thought to be caused by resident microbes run amok.

Mutualistic bacteria as coral symbionts
Although less of a focus, coral-associated bacteria can also act as symbionts. Several nitrogen-fixing bacterial species provide their hosts with invaluable chemical compounds (Chimetto et al. 2008, Lema et al. 2012). Similar to zooxanthellae, endolithic cyanobacteria transfer organic products of photosynthesis to the coral tissue and as a result, may act as a defense against starvation in the face of bleaching events (Schlichter et al. 1995). Recent research has also identified bacteria from the genus *Endozoicomonas* (family Hahellaceae) as widespread symbionts (Bayer et al. 2013a, Neave et al. 2016). *Endozoicomonas* frequently associates with marine invertebrates and dominates the microbiomes of certain corals indicating a conserved symbiotic relationship (Bayer et al. 2013a).

Despite accumulating evidence for coral-bacteria mutualisms, efforts to identify essential microbial species to the coral microbiome have been unreliable. In fact, only a very small fraction of bacteria are found in the majority of individuals from the same coral species (Ainsworth et al. 2015). This indicates that a core microbiome may not exist and that a species’ symbionts are not tightly conserved. Bacterial symbionts are more likely transient, influenced by environmental changes including temperature, availability of nutrients, dissolved oxygen, location on the reef, and anthropogenic impacts such as pollution (Kimes et al. 2013, Littman et al. 2009, Roder et al. 2015).

Examples of coral-bacteria mutualisms support the coral probiotic hypothesis, which states that stressful environmental conditions select for beneficial coral-microbe relationships (Reshef et al. 2006). Beneficial coral bacteria may create a barrier to infection by producing antibiotics and/or by occupying space on the host surface, thus preventing potential pathogens from colonizing and
overgrowing (Reshef et al. 2006). Many healthy coral-associated bacteria produce antibiotic compounds as part of their basal metabolism (Castillo et al. 2000, Kelman et al. 2006, Ritchie 2006). These beneficial compounds reside in the mucus layer, protecting the coral from pathogen invasion and subsequent infection. Interestingly, these antimicrobial properties are lost during bleaching events, suggesting that abiotic stress changes the coral habitat from a barrier to an environment more favorable to opportunists (Ritchie 2006).

The probiotic hypothesis (Reshef et al. 2006) goes hand in hand with the emerging hologenome theory of evolution. Since all animals and plants associate with microorganisms, it is logical to consider the microbiome as an extension of the host. Since the diversity and fast growth rates of bacteria allow for rapid changes to their genomes – far quicker than eukaryotic genomes – hosts may utilize their microbiome like an adaptive immune system, employing their symbiotic microbiota to ward off infection. This is especially valuable to corals and other species that possess only innate immune systems (Libro et al. 2013). Multiple studies have shown that corals from the same species host similar bacteria compared to sympatric corals from different species (Morrow et al. 2012, Roder et al. 2014). This suggests that a coral’s microbiome evolves in tandem with the host, further evidence supporting the hologenome theory.

**Acropora cervicornis and white band disease**

White band disease (WBD) is an epizootic which has had a particularly devastating impact on the Caribbean acroporids *Acropora cervicornis* and *Acropora palmata* (Aronson and Precht 2001). Since its first described outbreak in 1979, WBD has decimated populations of these important reef-building corals, landing both species on the IUCN List of Threatened Species
WBD belongs to a group of related coral diseases known as white syndromes (Bythell et al. 2004), which are classified through a shared phenotype of creeping tissue loss as the disease progresses along the coral. The WBD phenotype is well documented across the Caribbean and is characterized by the appearance of a white band of dying and necrotic tissue that starts at the base of an acroporid branch and travels upwards towards the tip (Aronson and Precht 2001). This upwards movement is likely due to the circulation of gastrovascular fluid towards the axial tip of the coral (Gladfelter 1983). WBD, like many coral diseases, is temperature-dependent and increased sea surface temperatures have been correlated to WBD outbreaks (Randall and Van Woesik 2015). WBD is highly transmissible both via the water column onto injured individuals as well as through coral-coral contact and animal reservoirs, including the carnivorous snail *Coralliophila abbreviata* (Gignoux-Wolfsohn et al. 2012). Although an etiological agents(s) has not been identified, the disease is most likely bacterial in nature as transmission can be halted by filtration and by the addition of antibiotics (Kline and Vollmer 2011) and transmission can be intensified by the addition of bacterial autoinducers (Certner and Vollmer 2015). Several bacterial taxa have been associated with WBD-infected *A. cervicornis* including Vibrionaceae (Gignoux-Wolfsohn and Vollmer 2015, Gil-Agudelo et al. 2006), Rickettsiales (Casas et al. 2004), and Flavobacteriales (Gignoux-Wolfsohn and Vollmer 2015).

**White band disease as an opportunistic infection**

Many studies have attempted to isolate the WBD pathogen(s). However, Koch’s postulates have never been satisfactorily fulfilled. A 2006 study by Gil-Agudelo et al. managed to fulfill three of the four assumptions although the candidate pathogen, *Vibrio charshariae*, has not been
specifically identified in other WBD studies (Gil-Agudelo et al. 2006). Furthermore, WBD-infected corals have variable microbiomes although a few key bacterial taxa appear across studies. Interestingly, these species generally also appear in healthy corals but in smaller numbers indicating opportunistic infection.

As a result, it is less important to identify the putative pathogen(s) than it is to establish types of likely infectious species. Time and again, bacteria belonging to the Gammaproteobacteria class – particular from the genus *Vibrio* – surface as candidate pathogens during coral disease studies, including WBD (Gignoux-Wolfsohn and Vollmer 2015), black band disease (Sekar et al. 2008), white plague (Sunagawa et al. 2009), white pox (Patterson et al. 2002), and yellow band disease (Cervino et al. 2008) to name a few. Gammaproteobacteria are a large and diverse class of species, including most of the known human and animal pathogens. Bacteria from this taxon are also united by quorum sensing, an instrumental strategy of opportunistic pathogens.

**Quorum sensing**

Quorum sensing (QS) refers to the mechanism by which populations of bacteria regulate gene expression through the production and accumulation of signaling molecules called autoinducers (Miller and Bassler 2001). It is a finely-tuned cell-cell communication system designed to coordinate the behavior of a group and stimulated by a growing bacterial population (Miller and Bassler 2001). Bacteria synthesize autoinducers which quickly diffuse out of the cell and accumulate in the extracellular environment until their concentration crosses a detection threshold (Miller and Bassler 2001). Bacterial recognition of these extracellular autoinducers initiates a signaling cascade resulting in the regulation of certain population-based actions.
controlled by gene expression. This allows bacterial populations to synchronize their behaviors and act collectively. QS has been shown to regulate a variety of important metabolic functions including biofilm formation, virulence, antibiotic production, motility, and sporulation (Hammer and Bassler 2003, Miller and Bassler 2001).

In general, autoinducers can be classified as either species-specific (AI-1) or universal (AI-2) (Miller and Bassler 2001). AI-1s, generally act in a species-specific manner, whereas AI-2s are general inducers that influence signaling in a variety of gram-negative and gram-positive species of bacteria (Fuqua et al. 2001, Miller and Bassler 2001). One of the best-characterized species-specific AI-1 QS systems derives from gram-negative Proteobacteria and relies on acylated homoserine lactones (AHLs) as autoinducers (Fuqua et al. 2001). AHLs belong to a class of neutral lipid molecules composed of an acyl chain of varying lengths and a homoserine lactone ring (Fuqua et al. 2001). The universal autoinducer, AI-2, is an unusual furanosyl borate diester molecule that is produced and detected by most gram-negative bacterial species (Hammer and Bassler 2003). These two QS systems act in tandem to regulate transcription of several density-dependent metabolic processes such as biofilm formation and virulence (Miller and Bassler 2001). Gram-positive bacteria rely on a similar system but communicate via short peptides as their autoinducer (Miller and Bassler 2001).

The role of quorum sensing in coral disease

Recent research suggests that QS may contribute to the ability of certain bacterial species – particularly Vibrios – to outcompete other microbes, especially in a compromised host like a heat-stressed coral (Certner and Vollmer 2015, Ritchie 2006, Tait et al. 2010). It has been
established that resident coral-associated bacterial populations include QS species. In fact, specific coral-associated bacteria in culture have been shown to produce autoinducers. A study by Tait et al. 2010 screened several Vibrio species isolated from healthy, diseased, and bleached individuals from a variety of corals species (Tait et al. 2010). All of the bacteria screened produced AI-2 molecules and the majority also produced at least one type of AHL (Tait et al. 2010). This result was corroborated by Golberg et al. 2011, which found that Vibrios are the overwhelming source of autoinducer production in coral-associated bacteria.

However, coral-associated bacteria do not only react to QS molecules in isolation. In the first chapter of this thesis, I show that adding exogenous AHL to a healthy coral microbiome has the ability to convert that bacterial population into a disease-causing agent, inducing tissue loss in corals exposed to the treated microbiome. Conversely, we show in Chapter 3 that an AHL antagonist – a quorum sensing inhibitor – in combination with a WBD-associated microbiomes has the ability to cease disease transmission in healthy test corals. These two studies indicate that QS molecules, even in small concentrations, have a significant effect on certain opportunistic bacteria within coral microbiomes. In turn, these relatively few QS-utilizing bacteria ultimately drive the behavior of the population as a whole. A small change in the availability of certain signaling molecules can stimulate certain bacteria to become opportunistic pathogens.

Interestingly, other coral microbial symbionts and even the coral animal itself may be actively combatting the opportunistic pathogenesis facilitated by QS-utilizing bacteria. Microbial species isolated from healthy corals have the ability to inhibit biofilm formation by known pathogens (Golberg et al. 2013). This finding corroborates the coral probiotic hypothesis, clearly indicating
the existence of coral-associated bacteria in a beneficial partnership with their host.

Unsurprisingly, QS inhibition (QSI) has been established in a number of other marine organisms including sponges and algae (Manefield et al. 1999, Skindersoe et al. 2008). The highly connected nature and nutrient-rich conditions of marine environments enable bacterial growth. In order to prevent colonization, marine organisms must launch a defense against opportunistic species. The third chapter of this thesis further addresses the role of QS in WBD-infected *A. cervicornis* and we show that adding exogenous QSI to disease-associated microbiomes prevents the transmission of WBD to healthy corals.

**Dissertation objectives**

The goal of this dissertation is to explore the etiology of coral disease and the role of bacterial QS, specifically in WBD on *A. cervicornis*. Chapter 2 focuses on culturing potential WBD pathogens to estimate the extent of their presence (or absence) on healthy versus diseased corals. *Vibrios* and Flavobacteria were chosen as the candidate WBD pathogens since they have often been associated with WBD in previous studies. Each chapter of this dissertation will incorporate these two groups of bacteria and explore their role in coral disease. Chapter 2 also introduces the hypothesis that QS may be involved in the onset and progression of WBD in *A. cervicornis*. It demonstrates that that a healthy-associated coral microbiome can be converted into a disease vector via the addition of autoinducer.

Chapter 3 moves beyond culture-based methods to next-generation 16S sequencing in order to gain a better understanding of the entire coral-associated microbial community. This chapter addresses the opposite effect of autoinduction and examines the influence of QSI on WBD
transmission as well as disease-associated microbiomes. Chapter 3 characterizes the specific bacterial species – including Vibrionaceae and Flavobacteriaceae – influenced by QS.

Finally, Chapter 4 investigates the theory that coral disease can be overwhelmingly attributed to opportunistic pathogenesis by identifying the gene expression patterns of disease-associated microbiomes. While chapter 3 looks at the population structure of healthy versus diseased coral microbiomes, this chapter emphasizes the behavior of these microbes. Chapter 4 addresses this question through a bacterial metatranscriptome in order to establish and put into context differentially expressed bacterial gene between healthy and WBD-infected corals. Again, *Vibrio* and Flavobacteriaceae are specifically addressed with regards to WBD-associated genes belonging to these two taxa.
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Evidence for autoinduction and quorum sensing in white band disease-causing microbes on *Acropora cervicornis*


**Abstract**

Coral reefs have entered a state of global decline partly due to an increasing incidence of coral disease. However, the diversity and complexity of coral-associated bacterial communities has made identifying the mechanisms underlying disease transmission and progression extremely difficult. This study explores the effects of coral cell-free culture fluid (CFCF) and autoinducer (a quorum sensing signaling molecule) on coral-associated bacterial growth and on coral tissue loss respectively. All experiments were conducted using the endangered Caribbean coral *Acropora cervicornis*. Coral-associated microbes were grown on selective media infused with CFCF derived from healthy and white band disease-infected *A. cervicornis*. Exposure to diseased CFCF increased proliferation of *Cytophaga-Flavobacterium* spp. while exposure to healthy CFCF inhibited growth of this group. Exposure to either CFCF did not significantly affect *Vibrio* spp. growth. In order to test whether disease symptoms can be induced in healthy corals, *A. cervicornis* was exposed to bacterial assemblages supplemented with exogenous, purified autoinducer. Incubation with autoinducer resulted in complete tissue loss in all corals tested in less than one week. These findings indicate that white band disease in *A. cervicornis* may be caused by opportunistic pathogenesis of resident microbes.
Introduction

Worldwide coral epizootics have escalated in incidence and severity over the last 30 years and have contributed to the unprecedented decline in global coral reef health (Aronson and Precht 2001, Harvell et al. 2007). Despite the devastating effects of coral disease, key details regarding their etiology and ecology remain unknown. A prime example of one such disease is white band disease (WBD), an epidemic which has nearly eradicated two of the Caribbean’s most important reef-building corals (Aronson and Precht 2001). Since 1979, WBD has decimated 95% of regional *Acropora* populations, resulting in the listing of both *Acropora cervicornis* (Lamarck, 1816), staghorn coral and *Acropora palmata* (Lamarck, 1816), elkhorn coral as critically endangered on the IUCN Red List of Threatened Species (Gignoux-Wolfsohn et al. 2012). Although its etiological agent has not been identified, the WBD phenotype is well documented across the Caribbean (Libro et al. 2013, Ritchie 2006). Like most coral diseases, it is characterized by rapid tissue loss as the disease progresses along a coral branch (Aronson and Precht 2001).

Growing evidence indicates that WBD – and many other coral diseases – may be the result of opportunistic pathogenesis of resident coral bacteria (Ritchie 2006). Corals are host to dynamic and complex microbial communities that vary greatly within and between species (Knowlton and Rohwer 2003, Rohwer et al. 2002). These microbes, along with symbiotic dinoflagellates and a variety of other metazoans, constitute the coral holobiont (Bourne et al. 2009). Abiotic stressors, such as increased water temperatures, can heavily impact the coral holobiont, disrupting the balance of various host-symbiont relationships (Thurber et al. 2009). One of the hallmarks of coral disease is a shift in the composition of coral-associated microbial communities from
symbionts to opportunistic species (Ritchie 2006, Thurber et al. 2009). It has been postulated that during times of sustained environmental stress, certain bacteria take advantage of their vulnerable coral host and outcompete commensal microbes (Ritchie 2006). This phenomenon, known as the compromised-host hypothesis, suggests that the increasing incidence of coral disease is not due to the introduction of novel pathogens or increased pathogen virulence but rather results from the inability of susceptible corals to fight off infection (Lesser et al. 2007, Muller et al. 2008, Muller and van Woesik 2009). Recent research has implicated Vibrios as likely opportunistic pathogens, as diseased corals often host bacterial populations dominated by Vibrios (Roder et al. 2014, Tait et al. 2010). In addition, many Vibrio species engage in a phenomenon called quorum sensing (QS) as a regulator of virulence in a variety of hosts, potentially including corals (Henke and Bassler 2004, Kushmaro et al. 1997, Tait et al. 2010, Yildiz and Visick 2009).

Quorum sensing refers to the mechanism by which populations of bacteria regulate gene expression through the concentration-dependent detection of signaling molecules called autoinducers (Miller and Bassler 2001). It is a finely-tuned cell-cell communication system designed to coordinate the behavior of a group of conspecifics and is stimulated by a growing bacterial population (Miller and Bassler 2001). Bacteria synthesize autoinducers, which diffuse out of the cell and accumulate in the extracellular environment until their concentration crosses a detection threshold (Fuqua et al. 2001). Bacterial recognition of these extracellular autoinducers initiates a signaling cascade resulting in the regulation of certain population-based actions controlled by gene expression. This allows bacterial populations to synchronize their behavior and act collectively. QS has been shown to regulate a variety of important metabolic functions
including biofilm formation, virulence, antibiotic production, motility, and sporulation (Hammer and Bassler 2003, Miller and Bassler 2001).

In general, autoinducers are classified as either species-specific (AI-1) or universal (AI-2), the former used by a narrow, closely-related group of bacteria while the latter is used by many gram-negative and gram-positive species (Miller and Bassler 2001). One of the best-characterized species-specific AI-1 QS systems was first studied in gram-negative gammaproteobacteria and relies on acylated homoserine lactones (AHLs) as autoinducers (Fuqua et al. 2001). AHLs belong to a class of neutral lipid molecules composed of an acyl chain of varying lengths and a homoserine lactone ring (Churchill and Chen 2010). N-Hexanoyl-DL-homoserine lactone, the autoinducer used in this study, is a mid-sized molecule containing an acyl chain of six carbon atoms (Zhu et al. 1998). It is a common, virulence-associated AHL produced by a number of gram-negative bacteria including species of the genera Pseudomonas, Aeromonas, and Serratia (Delalande et al. 2005, Winson et al. 1995). N-Hexanoyl-DL-homoserine lactone is also employed by a variety of Vibrio species (Garcia-Aljaro et al. 2012, Huang et al. 2007).

Numerous coral-associated bacteria, including many Vibrios, have been shown to produce AHLs as part of their metabolism, although the connection between QS and coral disease has yet to be characterized (Golberg et al. 2011, Ransome et al. 2014, Tait et al. 2010).

Here we explore the effects of (1) cell-free culture fluids (i.e. filtered coral homogenates) from diseased and healthy corals on coral-associated bacteria and (2) an established QS autoinducer on the transmission and progression of WBD-associated disease characteristics in a healthy A. cervicornis microbiomes. We extracted cell-free culture fluid (CFCF) from diseased and healthy
coral homogenates in order to determine whether CFCF induces or inhibits the growth of *Cytophaga-Flavobacterium* and *Vibrio* spp., two groups associated with WBD and known to employ QS systems (Dobretsov et al. 2007, Garcia-Aljarro et al. 2012, Henke and Bassler 2004, Natrah et al. 2011, Tait et al. 2009). Next, we exposed *A. cervicornis* to N-Hexanoyl-DL-homoserine lactone (AHL) in controlled tank environments to determine if exogenous AHLs can induce WBD-like disease signs (i.e. tissue loss) in otherwise healthy corals.

**Methods**

*Coral Collection for Selective Culturing of Coral-Associated Bacteria Experiment*

All corals were sampled in February 2014 from Crawl Cay (9° 14’ 00” N, 82° 08’ 00” W) in Bocas del Toro, Panama. Six healthy and six diseased (active WBD) *A. cervicornis* fragments (5 cm in length) were collected from 12 coral colonies (CFCF Group). In addition, three paired samples of healthy and diseased *A. cervicornis* fragments (also 5 cm in length) were collected from coral colonies displaying both the healthy and the WBD phenotype (Bacterial Homogenate Group). Coral fragments were transported to the Smithsonian Tropical Research Institute in separate containers and corals from the Bacterial Homogenate Group were acclimated in flow-through aquaria (separating diseased and healthy corals).

*Preparation of Healthy and Diseased Cell-Free Culture Fluid (CFCF)*

Within two hours of collection, each healthy *A. cervicornis* fragment from the CFCF Group was separately homogenized in a sterile tube containing 10 mL of 0.2 µm-filtered seawater by vortexing with sterile 3 mm glass beads. The coral skeleton was then removed with sterile tweezers and the resulting coral tissue homogenates were pooled and filtered at 0.2 µm to obtain
the healthy CFCF. Homogenates were pooled in order to ensure sufficient production of CFCF for 250 mL media. Differences among coral colonies were accounted for during the plating step when individual bacterial homogenates were plated onto media containing pooled CFCF. These steps were repeated for the diseased *A. cervicornis* fragments from the CFCF Group, resulting in diseased CFCF.

**Preparation of Media Containing CFCF**

Selective media were created in order to cultivate certain bacterial genera. TCBS Agar (Thiosulfate Citrate Bile Salts Sucrose, from VWR International) and Cytophaga Media (from VWR International) were chosen to select for *Vibrio* spp. and *Cytophaga-Flavobacterium* spp. given their prevalence on diseased corals and strong association with WBD (Garcia et al. 2013, Gignoux-Wolfsohn and Vollmer 2015). 500 mL of each type of media was prepared according to manufacturer instructions and split into two equal volumes of 250 mL. After media had cooled to 50°C, 25 mL of each CFCF (healthy and diseased) was added to 250 mL of each media type. This resulted in the following four combinations of media and CFCF: (1) TCBS medium & healthy CFCF (2) TCBS medium & diseased CFCF (3) Cytophaga medium & healthy CFCF and (4) Cytophaga medium & diseased CFCF. All media/CFCF combinations were mixed before plates were poured.

**Plating of Healthy and Diseased *A. cervicornis* Bacterial Homogenates onto Media/CFCF**

The paired healthy and disease *A. cervicornis* fragments from the Bacterial Homogenate Group were homogenized using the aforementioned vortexing technique. Start and end weights (before coral was added and after coral skeleton was removed) were taken of the tubes to normalize for
grams of tissue plated in subsequent calculations. Each coral tissue homogenate was filtered at 5 µm to remove coral cells and symbiotic dinoflagellates. Based on initial dilution series, we decided to dilute the bacterial homogenates ten-fold in 0.2 µm-filtered seawater. 100 µL of each 1:10 dilution was added to each of the four media/CFCF combination plates and was spread using sterile 3 mm glass beads. Plates were incubated for 24 hours at 27°C, after which photos were taken of each plate to record colony densities.

Coral Collection for AHL Addition Experiment

Ten healthy and ten diseased (active WBD) A. cervicornis fragments (5 cm in length) were collected from 20 coral colonies to prepare bacterial homogenates (Homogenate Group). In addition, 12 fragments each (6 cm in length) were collected from three healthy A. cervicornis colonies to serve as the test coral fragments. Organisms were transported to the Smithsonian Tropical Research Institute in separate containers. For the test corals, three fragments from each of the three colonies (genotypes) were acclimated in four separate flow-through aquaria for several hours prior to the start of the experiment.

Preparation of Healthy and Diseased Coral Homogenate and Addition of AHL

Within two hours of collection, each healthy A. cervicornis fragment from the Homogenate Group was separately homogenized in a sterile tube containing 10 mL of 0.2 µm-filtered seawater by vortexing as described above. The coral skeleton was then removed with sterile tweezers and the resulting ten healthy coral tissue homogenates were pooled. These steps were repeated for the ten diseased A. cervicornis fragments from the homogenate group. Both of the homogenate pools were split into equal halves of 50 mL and labeled as follows: “Healthy
Control,” “Healthy + AHL,” “Disease Control,” and “Disease + AHL.” A 25 mM stock solution of the Proteobacteria autoinducer N-Hexanoyl-DL-homoserine lactone (Sigma Aldrich) was created using deionized water. This concentration was chosen to ensure a final 1.3 µM concentration of AHL in the aquaria. In other studies, AHLs were found to interact with cell membranes in the micromolar concentration range (Davis et al. 2010, Mathesius et al. 2003, Von Bodman et al. 1998). This particular AHL was chosen based on the length of its acyl chain as six carbon AHLs are produced by multiple gammaproteobacteria, including a number of known marine Vibrio pathogens (Tait et al. 2010, Zhu et al. 1998). The AHL solution was mildly heated and mixed until all AHL was fully dissolved. One mL of the AHL stock solution was then added to the “Healthy + AHL” and the “Disease + AHL” homogenates. One mL deionized water was added to the “Healthy Control” and the “Disease Control” homogenates. The four homogenates were incubated for five hours at room temperature with intermittent swirling.

Lesioning and Dosing of Test Corals
Within the last hour of the AHL incubation period, all test coral fragments were lesioned by removing ~7.5 mm² of tissue with an airbrush and 0.2 µm-filtered seawater to ensure waterborne disease transmission (Gignoux-Wolfsohn et al. 2012). Immediately prior to dosing, the flow-through aquaria were converted to closed 20 L systems containing a water circulation pump. Each aquarium was then dosed with one of the four homogenates. After dosage, the experiment was checked every 12 hours and the health status of each coral fragment was recorded. We equated total tissue loss with coral mortality.

Statistical Analyses
All statistical analyses were performed using R statistical software. For the Selective Culturing experiment, bacterial growth was measured by determining the percent bacterial coverage of each plate per mg of coral tissue using the point intercept method of counting in ImageJ (Abràmoff et al. 2004). Point intercept was used instead of traditional CFU counts in order to account for the fact that treatments containing diseased CFCF or diseased bacterial homogenates displayed a swarming phenotype. We believe this methodology does not alter the results as we found very significant percent coverage differences between diseased CFCF plates compared to healthy CFCF plates. Percent bacterial coverage per mg coral tissue plated was analyzed with a two-way analysis of variance (ANOVA) that considered the CFCF in the media (healthy or diseased) and the plated bacterial homogenate (healthy or diseased) as fixed effects. The *Cytophaga-Flavobacterium* (Supplementary Table S1) and *Vibrio* (Supplementary Table S2) datasets were analyzed separately. All ANOVA conditions were satisfied for the *Cytophaga-Flavobacterium* dataset. Levene’s test was used to test for homogeneity of variance (p = 0.5600) and the Shaprio-Wilk test was used to test for normality (p = 0.1991). In order to satisfy the ANOVA condition of homoscedasticity for the *Vibrio* dataset, percent bacterial coverage values were converted to a proportion. A small delta of 0.001 was added to each proportion to account for the zeros within the datasets. Values were then logit transformed (Warton and Hui 2011). Homogeneity of variance was satisfied (Levene’s test, p = 0.4320). Although the data were not normally distributed (Shapiro-Wilk, p = 0.1991), our solidly significant results are likely to be robust to this departure from the assumptions of ANOVA (Supplementary Table S2). All means were analyzed using Tukey HSD post-hoc tests with an α value of 0.05.
For the AHL Addition experiment, time to total tissue loss in healthy test *A. cervicornis* fragments was analyzed with a two-way ANOVA that considered the bacterial homogenate (healthy or diseased) and the addition or lack of AHL as fixed effects (Supplementary Table S3). Homogeneity of variance was satisfied (Levene’s test, *p* = 0.2327). The data were not normally distributed. All means were analyzed using Tukey HSD post-hoc tests with an *α* value of 0.05.

**Results**

*Selective Culturing of Coral-Associated Bacteria*

Coral-associated bacterial assemblages from WBD-infected and healthy *A. cervicornis* were plated onto selective media infused with cell-free culture fluid (Figure 1). CFCF was obtained by filtering diseased and healthy coral tissue homogenates at 0.2 μm. Plated bacterial homogenates were obtained by filtering coral tissue homogenates at 5 μm to remove *A. cervicornis* cells and symbiotic dinoflagellates.

Origin of the CFCF (diseased versus healthy) had a significant effect on *Cytophaga-Flavobacterium* growth, which was defined as percent bacterial coverage per mg coral tissue plated (ANOVA, *p* = 0.0004) (Figure 2a) (Supplementary Table S1). Disease state (diseased versus healthy) of the plated bacterial homogenate did not have a significant effect on *Cytophaga-Flavobacterium* growth (ANOVA, *p* = 0.6770) (Figure 2a) (Supplementary Table S1). The effects of CFCF and plated bacterial homogenate were additive (*p* = 0.1890). Across healthy plated bacterial homogenates, the addition of CFCF from diseased corals to Cytophaga medium yielded significantly greater *Cytophaga-Flavobacterium* growth than did the addition of healthy CFCF (Tukey HSD, *p* = 0.0042) (Figure 2a).
608% more *Cytophaga-Flavobacterium* growth was observed per mg healthy coral tissue when plated onto diseased CFCF as opposed to healthy CFCF (Figure 2a). We found almost no culturable *Cytophaga-Flavobacterium* within healthy bacterial homogenates when grown in the presence of healthy CFCF (Figure 2a). Although the effect was not significant, diseased bacterial homogenates grown in the presence of healthy CFCF yielded more *Cytophaga-Flavobacterium* growth – an 85% increase in percent coverage – than did healthy bacterial homogenates grown in the presence of healthy CFCF (Figure 2a). Similarly, diseased bacterial homogenates grown in the presence of healthy CFCF yielded less *Cytophaga-Flavobacterium* growth – an 81% decrease – than did diseased bacterial homogenates grown in the presence of diseased CFCF (Tukey HSD, p = 0.0625).

CFCF origin (diseased versus healthy) did not have an effect (ANOVA, p = 0.8697) on *Vibrio* growth (Figure 2b) (Supplementary Table S2). Only the disease state of the plated bacterial homogenate had a significant effect on percent bacterial coverage per mg coral tissue (ANOVA, p = 0.0232) (Figure 2b) (Supplementary Table S2). Regardless of CFCF, diseased plated homogenates produced significantly more bacterial growth than did healthy plated homogenates on TCBS media. Healthy plated homogenates produced no culturable *Vibrio* spp. (Figure 2b).

*AHL Addition Experiment*

36 healthy *A. cervicornis* fragments were exposed to four combinations of WBD- and healthy-associated coral homogenates incubated with the either the AHL N-Hexanoyl-DL-homoserine lactone (Sigma Aldrich) or DI. N-Hexanoyl-DL-homoserine lactone was chosen based on the
length of its acyl chain as six carbon AHLs are produced by multiple bacterial species, including a number of known marine Vibrio pathogens (Tait et al. 2010, Zhu et al. 1998). All coral fragments within the Healthy Control treatment remained healthy (no tissue loss) for the entire duration of the experiment (14 days) (Figure 3). All corals in both the Disease Control and the Disease + AHL treatments experienced tissue loss consistent with WBD signs (tissue sloughing beginning around the lesioned area and traveling up the fragment) and total mortality within three to four days (Figure 3). Similarly, all A. cervicornis fragments in the Healthy + AHL treatment experienced tissue loss and experienced total mortality within 4.5 to 5.5 days.

Disease state of the tissue homogenate had a significant effect on the average number of days to total tissue loss in healthy A. cervicornis (ANOVA, p < 0.0001) (Figure 3) (Supplementary Table S3). Addition of AHL to tissue homogenates versus lack of AHL also produced a significant effect on days to tissue loss (ANOVA, p < 0.0001) (Figure 3) (Supplementary Table S3). The two disease treatments (Disease Control versus Disease + AHL) yielded statistically equivalent effects on tissue loss (Tukey HSD, p = 0.5434) (Figure 3). However, the two healthy treatments (Healthy Control versus Healthy + AHL) produced significant differences in time to tissue loss in the test corals (Tukey HSD, p < 0.0001) as fragments in the Healthy Control treatment displayed no disease symptoms whatsoever (Figure 3). The AHL addition treatments (Healthy + AHL versus Disease + AHL) also yielded a significant effect on coral tissue loss (Tukey HSD, p < 0.0001) (Figure 3). On average, the Healthy + AHL treatment took 26% longer to kill the healthy coral fragments compared to the Disease + AHL treatment.
Discussion

Cytophaga-Flavobacterium spp. within bacterial homogenates derived from healthy A. cervicornis grew significantly more in the presence of cell-free culture fluid obtained from diseased corals than in the presence of CFCF obtained from healthy corals (Figure 2a). This result indicates that diseased CFCF may enhance the proliferation of coral-associated Cytophaga-Flavobacterium either by containing molecules that induce growth and/or by lacking molecules that suppress growth. This finding is consistent with recent research suggesting that opportunistic pathogenesis of resident microbes can develop in vulnerable corals (Ritchie 2006, Thurber et al. 2009). Diseased corals exist in a state of severe physiological stress, which may destabilize the relationship between the coral host and its microbial symbionts. As a result, commensal bacteria may turn pathogenic as they take advantage of their host’s decreased resistance to infection (Lesser et al. 2007, Muller et al. 2008, Muller and van Woesik 2012). Our results support the compromised-host hypothesis in that Cytophaga-Flavobacterium spp. are present in small numbers on healthy corals but are able to grow unchecked when exposed to diseased coral CFCF (Muller et al. 2008, Muller and van Woesik 2012) (Figure 2a). To our knowledge, this phenomenon has never before been demonstrated with coral-associated Cytophaga-Flavobacterium as it is usually linked with Vibrio species (Ritchie 2006, Thurber et al. 2009).

Not only do our results demonstrate that diseased CFCF induces Cytophaga-Flavobacterium growth, they also imply that healthy CFCF may inhibit the growth of bacteria in this group. The growth of Cytophaga-Flavobacterium within diseased A. cervicornis bacterial homogenates appears to be hindered by CFCF obtained from healthy corals (Figure 2a). Compared to diseased
bacterial homogenates grown on diseased CFCF, diseased bacterial homogenates grown on healthy CFCF show a trend toward a significant (81%) decrease in *Cytophaga-Flavobacterium* growth (Figure 2a). Inhibition of disease-associated microbes by healthy coral CFCF is consistent with recent findings that healthy corals are able to suppress the growth of potential pathogens via their symbiotic microbes (Reshef et al. 2006, Rosenberg et al. 2007). This phenomenon, more commonly known as the coral-probiotic hypothesis, states that stressful environmental conditions select for beneficial coral-microbe relationships (Lesser et al. 2007, Muller et al. 2008). Resident coral bacteria may prevent infection by producing antibiotics and/or by occupying space on the coral surface, thus preventing potential pathogens from colonizing (Kelman et al. 2006). Our study suggests that healthy CFCF may contain antimicrobial molecules that significantly inhibit the growth of the *Cytophaga-Flavobacterium* group (Figure 2a). Diseased corals lose this ability, demonstrated by the proliferation of *Cytophaga-Flavobacterium* from healthy bacterial homogenates on plates infused with diseased CFCF (Figure 2a). It is not yet clear whether those antimicrobials are derived from the coral host and/or its resident microbes (Libro et al. 2013).

Unexpectedly, CFCF did not have a significant impact on *Vibrio* spp. growth. This may be attributed to a low abundance of culturable *Vibrios* living within healthy *A. cervicornis* tissue (Figure 2b). A high abundance of *Vibrio* spp. was observed within diseased coral homogenates, indicating that they are associated with the WBD state (Figure 2b). Increases in *Vibrio* species living within diseased or bleached coral tissue is a relatively common observation (Ritchie 2006, Thurber et al. 2009).
The AHL Addition Experiment may provide greater insight into *Vibrio* activity on *A. cervicornis*. Selective culturing of bacterial homogenates showed an apparent difference in chemical composition between healthy and diseased coral CFCF. Interestingly, the addition of QS chemical signaling molecules to healthy coral homogenates has the ability to convert a healthy-associated microbiome into a pathogenic vector. Specifically, incubation with exogenous N-Hexanoyl-DL-homoserine lactone can shift a healthy-associated coral homogenate into a disease-inducing agent. This is demonstrated by the significant difference in time to tissue loss between the corals in the Healthy Control versus the Healthy + AHL treatments (Figure 3). The Healthy + AHL treatment showed a greater similarity to both the Disease Control and the Disease + AHL treatments as far as inducing WBD-like tissue loss in healthy *A. cervicornis* fragments (Figure 3). This result suggests that the addition of a common AHL can transform a population of commensal bacteria into pathogens.

The acyl-homoserine lactone QS system is well characterized in many *Vibrio* species, including *Vibrio cholerae*, *Vibrio fischeri*, and *Vibrio harveyi*. *V. harveyi*, in particular, is closely related to a number of suspected and established marine pathogens from the *Vibrio* genus (Golberg et al. 2011, Miller and Bassler 2001, Rosenberg and Falkovitz 2004). In addition, *V. harveyi* (synonym *V. charchariae*) has recently been implicated as a potential primary pathogen for WBD (Gil-Agudelo et al. 2006, Sweet et al. 2014). Three parallel QS systems regulate gene expression in *V. harveyi*: an AI-1 mechanism controlled by an AHL, the AI-2 mechanism controlled by the universal gram-negative autoinducer, and a Cqs system similar to the AI-1 mechanism which functions at lower cell densities (Henke and Bassler 2004). Together these three systems control
many functions including bioluminescence, type III secretion, and polysaccharide, siderophore, and metalloprotease production (Henke and Bassler 2004).

In many marine Vibrios, virulence is positively controlled by QS in similar pathways to those found in V. harveyi (Henke and Bassler 2004, Natrah et al. 2011, Yildiz and Visick 2009). In other cases, QS negatively controls virulence as part of a late-stage infection strategy (Gode-Potratz and McCarter 2011, Hammer and Bassler 2003). Many Vibrio species, including known marine pathogens Vibrio parahaemolyticus and Vibrio vulnificus, employ a combination of positive and negative QS regulation systems to control virulence-associated genes (Gode-Potratz and McCarter 2011, Henke and Bassler 2004, Kim et al. 2003). It is likely that QS in marine Vibrios is also involved in repression of host immunity. Pseudomonas aeruginosa, a related gammaproteobacteria, is a well-studied opportunistic pathogen that has demonstrated an ability to disrupt host cells through extensive QS-controlled protease production (Caballero et al. 2001, Engel et al. 1998, Lyczak et al. 2000, Malloy et al. 2005). This has profound implications for organisms – such as corals – that rely on protective mucosal barriers as part of their innate immunity. Although the connection between protease activity and coral disease has not been established, it is interesting to note that protease production occurs in V. harveyi and has been tied to successful V. harveyi infections of marine shrimp (Austin and Zhang 2006, Lee et al. 1999).

Although lesser known than the gammaproteobacteria systems, species belonging to the Bacteroidetes phylum – which includes the Cytophaga-Flavobacterium group – also employ QS and are associated with marine diseases (Dobretsov et al. 2007, Romero et al. 2010). In many
aquatic ecosystems, *Cytophaga-Flavobacterium* are chemoorganotrophs responsible for significant decomposition of dissolved organic matter (DOM) (Kirchman 2002). In addition, a biofilm-forming member of the *Cytophaga-Flavobacterium* group, *Tenacibaculum maritimum*, has recently been shown to produce both short- and long-chain AHLs similar to the molecules produced by *Vibrios* (Romero et al. 2010). The *Cytophaga-Flavobacterium* group also contains a number of known marine pathogens, including the aforementioned *T. maritimum*, which is responsible for the fish disease tenacibaculosis (Alvarez et al. 2004, Bernardet 1998, Mudarris et al. 1994, Romero et al. 2010). The preferred DOM energy source and newfound QS potential of *Cytophaga-Flavobacterium* gives credence to a number of possible outcomes regarding this group’s role in coral disease. *Cytophaga-Flavobacterium* growth within diseased CFCF could indicate that this group contributes to secondary infection by simply proliferating on decaying coral tissue (in line with the compromised-host hypothesis). However, the *Cytophaga-Flavobacterium* group also has the potential to emerge as a primary pathogen responding to AHLs of its own. Further studies are needed in order to determine the QS capabilities of the *Cytophaga-Flavobacterium* group on corals.

Overall, our results confirm that diseased CFCF has the ability to stimulate the growth of potential primary or secondary coral pathogens including *Cytophaga-Flavobacterium*. We also demonstrate that the addition of the autoinducer N-Hexanoyl-DL-homoserine lactone can convert a healthy coral homogenate into a disease-causing agent. In this study, exogenous AHL was able to convert a healthy microbial population into a disease vector that produced WBD-like symptoms in healthy *A. cervicornis* (Figure 3). This result strongly suggests that autoinduction, via the addition of AHL, can manipulate QS pathways and their downstream genetic targets in
coral-associated bacteria. Although we cannot be sure of the molecular mechanisms involved, it is clear that the addition of N-Hexanoyl-DL-homoserine lactone somehow influences the growth of coral-associated bacteria. Molecular characterization of CFCF and bacterial metatranscriptomic studies will reveal details regarding presence/absence of AHLs and coral-associated microbial gene expression. Furthermore, as bacterial communities vary greatly within and between ecosystems, these results should be corroborated by similar experiments throughout the Caribbean.

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Literature Cited


Figure 2.1. Experimental design for selective culturing of coral-associated bacteria. Diseased and healthy CFCF were added to both Cytophaga (*Cytophaga-Flavobacterium*-selective) and TCBS (*Vibrio*-selective) media resulting in four combinations of media/CFCF: Cytophaga medium/healthy CFCF, Cytophaga medium/diseased CFCF, TCBS medium/healthy CFCF, and TCBS medium/diseased CFCF. Diseased and healthy *A. cervicornis* bacterial homogenates were then diluted 1:10 with filtered seawater and plated onto all four media/CFCF combinations.
Figure 2.2. Bacterial growth for each CFCF/bacterial homogenate combination for *Cytophaga-Flavobacterium* (a) and *Vibrio* (b) species. Growth was measured by determining the percent bacterial coverage of each plate per mg of coral tissue plated using the point intercept method of counting in ImageJ (Abràmoff et al. 2004). Growth was analyzed with a two-way ANOVA that considered disease state of the CFCF and disease state of the bacterial homogenate as fixed effects. Standard error bars are shown.
Figure 2.3. Time to mortality in healthy *A. cervicornis* exposed to coral tissue homogenates supplemented with AHL. Tissue homogenates of WBD-infected and healthy *A. cervicornis* were incubated with N-Hexanoyl-DL-homoserine lactone. Aquaria were then converted to closed systems and healthy test *A. cervicornis* were dosed with one of the four tissue homogenate/AHL combinations: Healthy + AHL, Disease + AHL, Healthy Control, and Disease Control. After dosage, the experiment was checked every 12 hours and the health status of each coral fragment was recorded. We equated total tissue loss with coral mortality. Time to coral mortality was analyzed with a two-way ANOVA that considered disease state of the dosed tissue homogenate and addition or lack of AHL as fixed effects. Standard error bars are shown.
## Supplementary Information

**Table 2.S1.** ANOVA table for *Cytophaga-Flavobacterium* dataset. Percent bacterial coverage per mg coral tissue plated was analyzed with a two-way ANOVA that considered the CFCF in the media (healthy or diseased) and the plated bacterial homogenate (healthy or diseased) as fixed effects.

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<td>3,738</td>
<td>3,738</td>
<td>2.062</td>
<td>0.188952</td>
</tr>
<tr>
<td>Residuals</td>
<td>8</td>
<td>14,502</td>
<td>14,502</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**Table 2.S2.** ANOVA table for *Vibrio* dataset. Percent bacterial coverage per mg coral tissue plated was analyzed with a two-way ANOVA that considered the CFCF in the media (healthy or diseased) and the plated bacterial homogenate (healthy or diseased) as fixed effects.

<table>
<thead>
<tr>
<th>Source</th>
<th>Df</th>
<th>Sum Sq.</th>
<th>Mean Sq.</th>
<th>F-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFCF (cell-free culture fluid)</td>
<td>1</td>
<td>0.52</td>
<td>0.52</td>
<td>0.029</td>
<td>0.8697</td>
</tr>
<tr>
<td>Bacterial Homogenate</td>
<td>1</td>
<td>141.69</td>
<td>141.69</td>
<td>7.835</td>
<td>0.0232</td>
</tr>
<tr>
<td>CFCF:Bacterial Homogenate</td>
<td>1</td>
<td>0.52</td>
<td>0.52</td>
<td>0.029</td>
<td>0.8697</td>
</tr>
<tr>
<td>Residuals</td>
<td>8</td>
<td>144.67</td>
<td>18.08</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**Table 2.S3.** ANOVA table for AHL Addition Experiment. Time to total tissue loss in healthy test *A. cervicornis* fragments was analyzed with a two-way ANOVA that considered the bacterial homogenate (healthy or diseased) and the addition or lack of AHL as fixed effects.

<table>
<thead>
<tr>
<th>Source</th>
<th>Df</th>
<th>Sum Sq.</th>
<th>Mean Sq.</th>
<th>F-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>1</td>
<td>309.17</td>
<td>309.17</td>
<td>2508</td>
<td>&lt;2e-16</td>
</tr>
<tr>
<td>AHL</td>
<td>1</td>
<td>162.56</td>
<td>162.56</td>
<td>1319</td>
<td>&lt;2e-16</td>
</tr>
<tr>
<td>Homogenate:AHL</td>
<td>1</td>
<td>180.01</td>
<td>180.01</td>
<td>1460</td>
<td>&lt;2e-16</td>
</tr>
<tr>
<td>Residuals</td>
<td>32</td>
<td>3.94</td>
<td>0.12</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Inhibiting bacterial quorum sensing arrests white band disease transmission and disease-associated microbes in *Acropora cervicornis*

Abstract

Among the greatest threats to coral reefs are coral epizootics, which are increasing in frequency and severity. In particular, white band disease (WBD) has devastated Caribbean acroporid populations since its initial outbreak in 1979. However, despite its widespread and damaging effects, the etiology of WBD remains largely uncharacterized. Here we examine the role of quorum sensing within bacterial communities associated with WBD-infected *Acropora cervicornis*. Microbial communities isolated from WBD-infected corals were exposed to quorum sensing inhibitor (QSI) – a N-acyl homoserine lactone autoinducer antagonist – and then dosed onto healthy test corals. WBD-associated bacteria supplemented with QSI lost the ability to transmit disease, whereas healthy corals exposed to uninhibited WBD bacterial communities became infected within two days. Microbial 16S rRNA metagenomic sequencing analyses were then used to identify shifts in bacterial communities due to QSI on WBD-associated bacterial communities. Our results demonstrated that Vibrionaceae and Flavobacteriaceae abundances were strongly inhibited by the addition of QSI to WBD microbiomes, whereas putative coral symbiont *Endozoicomonas* and Halomonadaceae abundances decrease dramatically in immune-compromised corals.
**Introduction**

Marine epizootics are on the rise globally, across a diverse range of ecosystems and species (Harvell et al. 2004). Increases in marine epizootics are often attributed to changes in host-bacterial relationships that are exacerbated by global warming and other anthropogenic activities (Harvell et al. 1999, Harvell et al. 2004, Knowlton and Rohwer 2003). Coral reefs in particular have undergone an unprecedented decline in recent decades, partially due to coral diseases caused by disruption to coral-microbe mutualisms (Bourne et al. 2009, Harvell et al. 2007, Rosenberg et al. 2007). As the incidence and severity of coral disease continues to grow, key details regarding the coral microbiome as well as the etiology for many coral diseases remain elusive.

White band disease (WBD) is an especially devastating epizootic that affects the Caribbean acroporids: the staghorn coral *Acropora cervicornis* and the elkhorn coral *Acropora palmata* (Aronson and Precht 2001). Since the initial outbreak in 1979, WBD has severely reduced staghorn and elkhorn populations landing both species on the IUCN Red List (Aronson et al. 2010) and US Endangered Species Act (2006). WBD is classified by a phenotype of advancing necrotic tissue that moves up the coral branch as the infection progresses, sometimes at rates in excess of one cm per day (Vollmer and Kline 2008). Research has established that WBD is bacterial, as transmission can be stopped with filtration and through the application of antibiotics (Kline and Vollmer 2011, Sweet and Bythell 2015). Koch’s postulates have not been fulfilled for WBD, however, several bacterial taxa have been associated with WBD-infected corals including Vibrionaceae (Gignoux-Wolfsohn et al. 2012, Gil-Agudelo et al. 2006, Ritchie 2006), Rickettsiales (Casas et al. 2004), and Flavobacteriales (Gignoux-Wolfsohn and Vollmer 2015).
Quorum sensing (QS) is a mechanism by which bacteria regulate gene expression in response to changes in cell density (Hammer and Bassler 2003, Miller and Bassler 2001). This cell-cell communication system is designed to synchronize group behavior and is stimulated by a growing bacterial population through the concentration-dependent detection of signaling molecules known as autoinducers (Miller and Bassler 2001). Bacteria produce and secrete autoinducers, which accumulate in the extracellular environment until their concentration reaches a threshold. Cellular binding of these autoinducers initiates a signaling cascade in individual bacteria, resulting in collective gene expression of a bacterial population. Quorum sensing can control a number of important biological functions including virulence, biofilm formation, antibiotic production, motility, and sporulation (Miller and Bassler 2001).

Coral-associated bacteria – both innocuous and WBD-causing – likely employ quorum sensing as part of their regular metabolism and infection processes. Our recent research has shown that exposing bacteria collected from healthy A. cervicornis colonies to the exogenous autoinducer N-hexanoyl-DL-homoserine lactone converts healthy coral microbiomes into a WBD-causing agent when dosed onto healthy corals (Certner and Vollmer 2015). As a result, we infer that not only do coral-associated bacteria use quorum sensing-controlled pathways, they rely on quorum sensing to establish virulence. Many genera of coral-associated bacteria originating from healthy, diseased, and bleached hosts have been shown to produce autoinducers similar to N-hexanoyl-DL-homoserine lactone (Golberg et al. 2011, Tait et al. 2010). Autoinducers have also been isolated from black band disease-infected Diploria strigosa tissue, indicating that quorum sensing may be involved in black band disease progression (Zimmer et al. 2014).
Autoinducers can be classified as either specific (AI-1) or universal (AI-2); AI-1s typically act upon a few species whereas AI-2s are general inducers that influence signaling in a variety of gram-negative and gram-positive bacteria (Miller and Bassler 2001). One of the best-characterized species-specific AI-1 quorum sensing systems comes from gram-negative Proteobacteria and relies on acylated homoserine lactones (AHLs) as autoinducers (Fuqua et al. 2001). While autoinducers initiate quorum sensing-controlled gene expression, quorum sensing inhibitors (QSIs), obstruct quorum sensing pathways through a variety of means including autoinducer antagonism (Hentzer et al. 2002, Hentzer et al. 2003). One of the first naturally-occurring QSIs – a halogenated furanone – was discovered in the red alga Delisea pulchra and is thought to inhibit bacterial colonization of the seaweed (Manefield et al. 1999). Due to their structural similarity to AHLs, furanones successfully mimic autoinducers and prevent quorum sensing controlled biofilm formation and virulence in certain bacteria including Pseudomonas aeruginosa (Hentzer et al. 2002, Hentzer et al. 2003, Manefield et al. 1999).

Given that AHL autoinducers can convert a healthy staghorn coral microbiome into a WBD-causing agent (Certner and Vollmer 2015), we explore the possibility that a quorum sensing inhibitor (QSI) can inhibit transmission of a WBD-associated microbiome to healthy corals. Since the addition of an autoinducer has the ability to kill coral, then perhaps the addition of an autoinducer antagonist has the ability to halt infection. In this study, we examine the role of quorum sensing in WBD transmission and progression in A. cervicornis through a phenotypic (transmission rate) and microbial lens (microbial 16S rDNA sequencing). Microbiomes from WBD-infected staghorn corals were incubated with the QSI (Z-)-4-bromo-5-(bromomethylene)-
2(5H)-furanone or seawater and then dosed onto healthy corals, which were monitored for physical WBD signs (tissue loss). The V4 region of the 16S gene was sequenced for samples taken before dosage and 12 and 24 hours post-dosage in order to identify community-level and OTU level bacterial differences between healthy and WBD-infected A. cervicornis microbiomes and bacterial species influenced by the QSI.

Methods

Coral collection and creation of WBD pools

Sixty asymptomatic (healthy) A. cervicornis fragments were collected from Coral Cay in Bocas del Toro, Panamá in February 2015. Corals fragments were acclimated in 12 aquaria (five fragments per aquaria) for 24 h in a flow-through system. The following day, 30 fragments of A. cervicornis exhibiting active WBD signs were collected from the same reef. The diseased coral fragments were homogenized in 15 ml 0.2 µm-filtered seawater (FSW) with sterile glass beads. Six WBD pools were created from the disease homogenates by combining five ml from five homogenates. The six pools were standardized to 37 mg ml⁻¹ of tissue using FSW. The pooled homogenates were then spun at 500 rpm for five min to remove large cellular debris and 20 ml supernatant from each spun pool was transferred to a new tube. A sample of each WBD pool was also saved in Chaos DNA buffer (Fukami et al. 2004) for subsequent 16S analysis.

Creation of dosage treatments and incubation with QSI

Two levels of disease exposure (−WBD or +WBD) and two levels of QSI addition (−QSI or +QSI) were fully crossed to create four dosage treatments: (1) −WBD/−QSI, no WBD and no QSI (2) +WBD/−QSI, WBD but no QSI (3) −WBD/+QSI, QSI but no WBD and (4)
+WBD/+QSI, both WBD and QSI. Treatments were created in triplicate. 20 ml FSW was used to create the −WBD treatments and the six 20 ml WBD pools were used to create the +WBD treatments. The +QSI treatments were supplemented with 50 µM QSI ((Z)-4-bromo-5-(bromomethylene)-2(5H)-furanone, Sigma-Aldrich) (Hentzer et al. 2003, Janssens et al. 2008). Treatments were incubated for five hours with shaking at room temperature in the dark.

Lesioning, 16S sampling, and dosage of healthy A. cervicornis

During the treatment incubation, acclimated coral fragments were given a ~7.5 mm² lesion using an airbrush with FSW to mimic naturally occurring injury (Certner and Vollmer 2015, Gignoux-Wolfsohn et al. 2012). Immediately prior to lesioning, three polyps were aseptically removed from each coral fragment using flame-sterilized tweeters and placed into DNA buffer for subsequent 16S analysis (Fukami et al. 2004). This sampling represents Time 0, the pre-dosage, baseline community. Corals were allowed to recover for one hour before aquaria were converted into 10 l closed systems supplemented with a circulating water pump. Aquaria were then dosed with the normalized tissue treatments (three aquaria per treatment, five corals per aquarium).

Subsequent 16S sampling of treated A. cervicornis

Corals were closely monitored for WBD signs, characterized by a growing band of necrotic tissue on the coral fragment. All corals were sampled as described above at 12 hours post-dosage (Time 1), and again at 24 hours post-dosage (Time 2). Upon detection of WBD signs, the diseased coral was removed from its aquarium to prevent infection of other fragments within the same aquarium.
16S library preparation and bioinformatics

Total DNA was extracted from all 144 samples using the BioSprint 96 DNA Blood Kit (Qiagen). The V4 hypervariable region of the 16S rRNA gene was amplified and sequenced according to Fadrosh et al. 2014, without using the spacers (Fadrosh et al. 2014). A separate 25 µl PCR was performed for each sample with a unique combination of barcoded primers that did not contain spacers and Phusion High-Fidelity DNA Polymerase (New England Biolabs). PCR products were normalized using the SequalPrep Normalization Plate Kit (Thermo Fisher Scientific), pooled, and concentrated with Agencourt AMPure XP magnetic beads (Beckman Coulter). The concentrated pools were quantified with qPCR using the KK4835 KAPA Library Quantification Kit (KAPA Biosystems). Resulting multiplexed libraries were sequenced on the Illumina 2500 HiSeq platform with 250 base pair sequencing. Single-end sequences comprising the V4 region were demultiplexed using a custom Python script. OTUs were clustered at 97% similarity and singletons were removed using the open reference picking script from QIIME (Caporaso et al. 2010) and UCLUST against the SILVA database (Caporaso et al. 2010, Quast et al. 2013). OTU taxonomy was assigned using BLAST against the SILVA database.

Statistical analyses

Aquaria experiment analysis

For the disease phenotype portion of the experiment, the percentage of WBD-infected corals was calculated for each of the 12 aquaria across the four treatments (three replicate aquaria per treatment). A generalized linear model (GLM) with a quasibinomial error distribution that accounts for overdispersion was used to determine whether the proportion of infected A. cervicornis varied across disease exposure and QSI addition using the R package MASS (Ripley
et al. 2013). Significance of effects was determined via Likelihood Ratio Tests (LTR) using the R package car (Fox et al. 2016).

**Community and OTU-level analysis**

For the 16S data, OTU counts were normalized using the metagenomeSeq method which calculates scaling factors equal to the sum of counts up to a particular quantile; this accounts for sparsity due to undersampling (Paulson et al. 2013a, Paulson et al. 2013b). Dissimilarity between samples at each time point was visualized using principal coordinate analysis (PCoA) applying the Hellinger transformation – which gives low weights to low abundance species – using the function decostand in the R package Vegan (Oksanen et al. 2007). Community level differences between the two factors (disease exposure and QSI addition) were determined by PERMANOVA of Bray-Curtis dissimilarities using the adonis function in the R package Vegan. Alpha diversity of each treatment was determined using the Shannon index. At each time point, coral-associated bacterial diversity was analyzed with a two-way ANOVA that considered disease exposure and QSI addition as fixed effects. All means were analyzed using Tukey HSD post-hoc tests with a $\alpha$ value of 0.05.

At each time point, significantly differentially abundant OTUs for the main effects – WBD exposure and QSI addition – as well as the interaction were determined using the fitZig function from the R package metagenomeSeq, which computes the weighted fold-change estimates and t-statistics (Paulson et al. 2013a, Paulson et al. 2013b). In this two factor comparison, OTUs with significant interaction terms are most interesting because they shows the differential effects of QSI addition by disease exposure. The fitZig function is based on a zero-inflated Gaussian
mixture model. The metagenomeSeq package relies heavily on the topTable function from the R package limma (Ritchie et al. 2015). Log2-fold change values were also determined with these packages and functions.

Results

Effects of QSI on Disease Transmission

Both disease exposure and QSI addition had a significant effect on coral transmission ($\chi^2(1) = 5.546, p = 0.0185, \chi^2(1) = 13.258, p = 0.000271$) (Figure 1, Supplementary Table S1). After six days, the proportion of corals in each treatment that displayed WBD signs was as follows: 33% of corals in the seawater (–WBD/–QSI) treatment, 6.7% of corals in the QSI (–WBD/+QSI) treatment, 100% of corals in the WBD (+WBD/–QSI) treatment, and 6.7% of corals in the WBD QSI combination (+WBD/+QSI) treatment (Figure 1). The interaction was not significant ($\chi^2(1) = 2.376, p = 0.123$) (Figure 1, Supplementary Table 1).

Effects on the Microbial Community Composition and Diversity

One hundred forty-four coral-associated bacterial samples were sequenced for 16S (48 samples from each of the three time points). 97% clustering yielded 4,283 OTUs with an average number of 89,327 reads per sample. Six additional samples were sequenced to represent the WBD pools used to create the WBD doses and these yielded 2,191 OTUs with an average number of 99,135 reads per sample. The WBD pools were dominated by Proteobacteria, particularly Gammaproteobacteria and Alphaproteobacteria, and Bacteroidetes (Supplementary Figure 1). Principal coordinate analysis plots show little difference between coral-associated bacterial communities at Time 0 (pre-dosage) (Supplementary Figure 2). However, at Time 1 (12 hours
post-dosage) (Figure 2) and Time 2 (24 hours post-dosage) (Figure 3), bacterial communities from corals dosed with WBD minus QSI (+WBD/−QSI) cluster away from the other three treatments (Figure 4A, 4B).

Relative species abundance plots also show a clear difference between microbiomes from +WBD/−QSI corals compared to microbiomes from corals dosed with one of the other three treatments at Times 1 and 2 (Figure 2, 3). At Time 1, +WBD/−QSI microbiomes have a higher abundance of Colwelliaceae and Vibrionaceae OTUs compared to the other treatments, which have a higher abundance of Hahellaceae (Endozoicomonas) and Halomonadaceae OTUs (Figure 2). At Time 2, Hahellaceae and Halomonadaceae are still more abundant in these three treatments while +WBD/−QSI microbiomes have a high number of Chalmydiae OTUs (Figure 3).

PERMANOVA analyses of the microbial communities corroborate the PCoA results. At Time 1, disease exposure (PERMANOVA: F = 5.668, R² = 0.0899, p = 0.002) and QSI addition (PERMANOVA: F = 7.973, R² = 0.126, p = 0.001) as well as the interaction (PERMANOVA: F = 5.394, R² = 0.0856, p = 0.003) are significant (Table 1). The same holds true at Time 2 where both of the main effects of disease exposure (PERMANOVA: F = 5.298, R² = 0.0836, p = 0.002) and QSI addition (PERMANOVA: F = 8.736, R² = 0.138, p = 0.001) and the interaction (PERMANOVA: F = 5.331, R² = 0.0841, p = 0.002) are significant (Table 1).

Alpha diversity of the coral-associated bacterial community increases when the coral host is exposed to WBD-associated microbes. Corals in the +WBD/−QSI treatment contain a more
diverse bacterial community than corals in the other three treatments. At Time 0, the Shannon diversity indices of the four treatments are statistically equivalent (Supplementary Table 2). However, at Time 1, both disease exposure (ANOVA: $F = 24.08, p < 0.001$) and QSI addition (ANOVA: $F = 19.20, p < 0.001$) are significant as well as the interaction (ANOVA: $F = 16.40, p < 0.001$) (Figure 5A, Supplementary Table 2). Similarly, at Time 2 the main effects (ANOVA: $F = 26.01, p < 0.001$; $F = 24.82, p < 0.001$) and the interaction (ANOVA: $F = 24.76, p < 0.001$) are significant (Figure 5B, Supplementary Table 2). At Times 1 and 2, corals in the +WBD/–QSI treatment had a significantly higher diversity than corals in the other three treatments (Tukey HSD, $p < 0.0001$) (Figure 5).

OTU Differential Abundance

At Time 0 (pre-dosage), no OTUs were significantly differentially abundant for disease exposure, QSI addition, and the interaction between these two main effects (Supplementary Table 3). At Time 1, 634 OTUs were significant for disease exposure, 3 OTUs were significant for QSI addition and 387 OTUs were significant for the interaction (Supplementary Table 3). At Time 2, 831 OTUs were significant for disease exposure, zero OTUs were significant for QSI addition, and 615 were significant for the interaction (Supplementary Table 3).

At Time 1 (12 h post-dosage), 106 of the 387 differentially abundant interaction OTUs had an average abundance of greater than 100 across all samples (Figure 6A). 91 OTUs were more abundant in the corals from the +WBD/–QSI treatment including Flavobacteriaceae (15), Vibrionaceae (15), Alteromonadaceae (10), Colwelliaceae (13), and Rhodobacteraceae (8).
(Figure 6A). 15 OTUs were more abundant in the +WBD/+QSI treatment, 14 of which are Hahellaceae (*Endozoicomonas*) (Figure 6A).

At Time 2 (24 h post-dosage), 111 of the 615 differentially abundant OTUs had an average abundance of greater than 100 across all samples (Figure 6B). 104 OTUs were more abundant in +WBD/–QSI corals and these OTUs mirror the taxonomy of Time 1 and include Flavobacteriaceae (26), Vibrionaceae (8), Rhodobacteraceae (12), Colwelliaceae (14), and Alteromonadaceae (8) (Figure 6B). 27 OTUs were more abundant in +WBD/+QSI corals and, similar to Time 1, this group was dominated by *Endozoicomonas* (19) (Figure 6B).

Based on the univariate analysis, we chose to focus on four taxa – Flavobacteriaceae, Vibrionaceae, *Endozoicomonas*, and Halomonadaceae – that showed the strongest signatures in WBD-infected (+WBD/–QSI) corals versus healthy (other three treatments) corals. Two of these families, Flavobacteriaceae and Vibrionaceae, are significantly more abundant in +WBD/–QSI microbiomes post-dosage (Figure 7A, 7B). Specifically, Vibrionaceae is virtually absent from the Time 0 +WBD/–QSI microbiome and the other three treatments. In contrast, *Endozoicomonas*, and Halomonadaceae are relatively less abundant in +WBD/–QSI microbiomes (Figure 7C, 7D). Their numbers drop significantly post-dosage at Times 1 and 2.

**Discussion**

*The addition of QSI to WBD homogenates prevents disease transmission*

We previously demonstrated that adding autoinducer to a healthy *A. cervicornis* microbiome has the ability to convert a healthy-associated coral bacterial community into a WBD vector (Certner...
and Vollmer 2015). In this study, we show that adding a QSI (an autoinducer antagonist) to WBD microbiomes inhibits disease transmission when dosed onto healthy *A. cervicornis*. All corals dosed with WBD microbiomes lacking QSI (+WBD/–QSI) developed WBD signs within 24 hours, whereas only one of the 15 corals dosed with WBD microbiomes treated with QSI (+WBD/+QSI) contracted disease (Figure 1). The stark contrast in survivorship between corals exposed to WBD with and without QSI demonstrates the significant influence of QSI on WBD-associated bacterial communities. This result suggests that WBD transmission is controlled, at least in part, by quorum sensing. The QSI used in this study ((Z)-4-bromo-5-(bromomethylene)-2(5H)-furanone) acts as an antagonist to AI-1 AHL-dependent quorum sensing pathways (Wu et al. 2004), many of which have been implicated in pathogenicity (Hammer and Bassler 2003).

Consequently, our findings demonstrate that quorum sensing is necessary for the WBD pathogen(s) to elicit disease signs in healthy corals. More specifically, the addition of QSI likely prevents WBD-causing bacteria from activating virulence-related genes, thus hindering infection by pathogenic microbes on the dosed corals and preventing their overgrowth.

*QSI prevents the establishment of a WBD-causing microbiome*

16S rDNA data demonstrate that the bacterial communities of *A. cervicornis* dosed with WBD change before visual WBD signs appear. At Time 1 (12 h post-dosage), the microbiomes of +WBD/–QSI corals undergo a dramatic shift compared to the microbiomes associated with the other three treatments (Figure 2, 4A). In contrast, bacterial populations on +WBD/+QSI corals remain statistically indistinguishable from the –WBD/–QSI and –WBD/+QSI treated corals (Figure 2, 4A). These strong shifts in the +WBD/–QSI treated corals continued into Time 2 (24 h post-dosage) (Figure 3, 4B). These results demonstrate that significant changes occur in the
microbiomes of corals exposed to WBD prior to the development of disease signs (Table 1). The absence of this shift in corals exposed to QSI-treated WBD microbiomes indicates that QSI hinders the development of a WBD-associated microbiome seen in +WBD/–QSI corals (Figure 2-4). Again, this suggests that QSI neutralizes the disease-causing bacteria in WBD homogenates.

Interestingly, QSI does not appear to act upon healthy-associated coral microbiomes as indicated by the clustering of +WBD/+QSI with –WBD/–QSI, –WBD/+QSI microbiomes. As a result, we can conclude that the primary effect of QSI on the WBD microbial community is to inhibit disease-associated bacteria. These results are supported by the alpha diversity values from the four treatments. The Shannon indices of +WBD/+QSI microbiomes are statistically equivalent to those from –WBD/+QSI and –WBD/–QSI microbiomes (Figure 5, Supplementary Table 2). Similar to the PCoA results, the three asymptomatic treatments group together while microbiomes from the +WBD/–QSI treatment yield significantly higher Shannon values (Figure 5, Supplementary Table 2). Since increased alpha diversity is commonly linked to coral disease and bleaching (Bourne et al. 2008, Pantos et al. 2003), this phenomenon indicates that the addition of QSI not only changes WBD-associated microbiomes, but rescues these communities. The low Shannon values of +WBD/+QSI microbiomes imply that QSI converts WBD-associated bacteria into innocuous or even beneficial assemblages with a lower alpha diversity (Figure 5).

*QSI specifically inhibits WBD-associated bacteria*

Based on the OTUs significantly differentially abundant for the interaction between disease exposure and QSI addition, we can conclude that QSI specifically inhibits putative coral
pathogens. At Time 1 (12 h post-dosage), of the 387 significant interaction OTUs, 175 were found to be more abundant in WBD-infected corals from the +WBD/~QSI treatment compared to healthy corals from the +WBD/+QSI treatment (Supplementary Table 3). The majority (133, 76%) of the WBD-associated OTUs belong to the phylum Proteobacteria, including the families Vibrionaceae, Alteromonadaceae, Colwelliaceae, and Rhodobacteraceae (Figure 6). Proteobacteria and especially the class Gammaproteobacteria (including Vibrionaceae, Alteromonadaceae, and Colwelliaceae), relies heavily on quorum sensing and contains a large number of putative coral pathogens (Gignoux-Wolfsohn and Vollmer 2015, Sunagawa et al. 2009, Thompson et al. 2006). These bacteria are largely missing from +WBD/+QSI microbiomes at both time points (Figure 6). As a result, we can conclude that QSI effectively inhibits the growth of bacterial species associated with WBD. The addition of QSI to WBD homogenates rich in quorum sensing Proteobacteria and Gammaproteobacteria allows for the targeted elimination of disease-causing species while preserving beneficial coral mutualists.

Our results demonstrate that the WBD-associated microbiome is dominated by a few key families including Vibrionaceae, Alteromonadaceae, Colwelliaceae, Rhodobacteraceae, and Flavobacteriaceae (Figure 6). For the WBD-associated microbiome, Vibrionaceae and Flavobacteriaceae make up the majority of differentially abundant interaction OTUs with an average expression of greater than 100 per coral (Figure 6). All of the families in the WBD-associated profile have previously been implicated as primary pathogens of WBD on A. cervicornis (Gignoux-Wolfsohn and Vollmer 2015, Gil-Agudelo et al. 2006, Roder et al. 2014). In particular, the family Vibrionaceae contains a number of marine pathogens (Austin and Zhang 2006, Colwell and Grimes 1984) and known coral pathogen like Vibrio coralliilyticus (de O
Santos et al. 2011). In addition, Gil-Agudelo has been able to cause WBD-like disease signs on *Acropora cervicornis* exposed to *Vibrio charachariae* (Gil-Agudelo et al. 2006).

*Vibrios* are well known for quorum sensing and many *Vibrio* virulence genes are controlled by quorum sensing pathways (Hammer and Bassler 2003). Flavobacteriaceae are less studied, but this family has been associated with diseases in marine fish (Bernardet 1998, Starliiper 2011). Although, quorum sensing is not as well established in Flavobacteriaceae, there exists proof of quorum sensing-controlled behaviors. A biofilm-forming member of the Cytophaga-Flavobacteria group, *Tenacibaculum maritimum*, has been shown to produce AHLs similar to those produced by *Vibrios* (Romero et al. 2010). In addition, QSIs have the ability to decrease bacterial densities and alter community structure in Cytophaga-Flavobacteria (Dobretsov et al. 2007).

The two families – Vibrionaceae and Flavobacteriaceae – that emerge as dominant components of WBD-associated microbiomes follow a similar pattern across the four treatments (Figure 7A, 7B). Both Vibrionaceae and Flavobacteriaceae OTUs are highly abundant in +WBD/~QSI (diseased) microbiomes but relatively uncommon in ~WBD/~QSI, ~WBD/+QSI, and +WBD/+QSI (healthy) microbiomes. These two groups are also highly abundant in the WBD pools (family Blattabacteriaceae belongs to the order Flavobacteriales) indicating that these populations continue to increase and persist into late-stage infection (Supplementary Figure 1). This suggests that Vibrionaceae and Flavobacteriaceae possess virulence genes controlled by quorum sensing, since the addition of QSI inhibits these bacteria in +WBD/+QSI microbiomes.
The Vibrionaceae family is known to rely on quorum sensing and contains a large number of human and animal pathogens. Many disease-causing *Vibrios* are characterized by toxin production and protease activity (Shinoda 1999) and high toxin concentrations have been isolated from corals undergoing bacterial-induced bleaching (Banin et al. 2000). Like Vibrionaceae, the families Alteromonadaceae and Colwelliaceae also belong to class Gammaproteobacteria and are therefore likely to have similar mechanisms of pathogenicity.

Alteromonadaceae and Colwelliaceae are often found along with Vibrionaceae as coral pathogens (Sunagawa et al. 2009, Thompson et al. 2006). Interestingly, Flavobacteriaceae display the same pattern (i.e. they are abundant in diseased corals and relatively rare in healthy corals) as Vibrionaceae where they are abundant in diseased corals and despite the fact that these families are not closely related (Figure 7A, 7B) (Wu et al. 2009). Flavobacteriaceae belong to the phylum Bacteroidetes and though they are known fish pathogens (Bernardet 1998, Starliper 2011) their relationship to coral disease is not well-established. The similarities between Vibrionaceae and Flavobacteriaceae abundance on WBD-infected corals implies that WBD may not have one primary pathogen but may result from multiple pathogens possibly acting as a consortium of opportunistic species.

Another interesting family associated with WBD in this study is Kordiimonadaceae (class Alphaproteobacteria). Only Six OTUs at Time 1 and 11 OTUs at Time 2 belong to Kordiimonadaceae but this rare family may wield great influence. At Time 1, a single Kordiimonadaceae OTU appears nearly 1000 times in +WBD/–QSI microbiomes and has the highest B-statistic (log-odds that the OTU is differentially abundant) of any OTU (Figure 6A). Furthermore, Kordiimonadaceae was found to be a notable component of the WBD pools,
indicating that this family thrives in mature WBD infections (Supplementary Figure 1). Kordiimonadaceae has been previously associated with WBD (Gignoux-Wolfsohn and Vollmer 2015) as well as implicated as a potential white syndrome pathogen of Acropora muricata (Gignoux-Wolfsohn and Vollmer 2015, Sweet and Bythell 2015). As such, Kordiimonadaceae may represent a rare but powerful opportunistic coral pathogen helping to initiate WBD infection.

**QSI maintains putative coral symbionts**

Recently, the genus Endozoicomonas (family Hahellaceae) has received attention as a putative coral endosymbiont associated with coral health (Bayer et al. 2013, Yang et al. 2010). We found that Endozoicomonas is indeed associated with healthy A. cervicornis microbiomes from the −WBD/−QSI, −WBD/+QSI, and +WBD/+QSI treatments (Figure 6, 7C), whereas their abundance is relatively low on +WBD/−QSI exposed corals (Figure 6, 7C), even before WBD disease signs develop. This suggests that QSI helps to maintain Endozoicomonas populations when corals are exposed to WBD pathogens. Without QSI, these symbionts are quickly lost as the WBD-associated microbiome overwhelms the coral. Their rapid disappearance is a hallmark of diseased microbiomes in the same way that Vibrionaceae and Flavobacteriaceae overgrowth characterizes the WBD profile. Halomonadaceae follows a similar pattern to Endozoicomonas in that they are also more abundant on healthy corals compared to diseased corals (Figure 7D). Both Endozoicomonas and Halomonadaceae belong to the same halophilic order of bacteria Oceanospirillales indicating a related group of potential coral symbionts, and thus Halomonadaceae should also been investigated as putative symbionts of A. cervicornis.
*Endozoicomonas* appear to reside primarily within the coral endoderm where they form dense aggregates (Bayer et al. 2013). This intracellular clumping behavior may be common in Oceanospirillales symbionts and is found in other marine animals (Jensen et al. 2010). Interestingly, *Endozoicomonas* are also Gammaproteobacteria and aggregation is typically controlled by quorum sensing in other bacterial species (Waters and Bassler 2005). Therefore, it is possible that while our QSI (an AHL antagonist) has a negative effect on WBD-associated bacteria, another quorum sensing system may help to sustain *A. cervicornis* symbionts within healthy corals.

**Conclusion**

This study highlights the important of quorum sensing to disease-causing coral bacteria. Our results show that QSI prevents WBD transmission by inhibiting quorum sensing bacteria as WBD homogenates supplemented with QSI are unable to spread disease to *A. cervicornis*. 16S rDNA sequence data suggest that QSI addition inhibits WBD-causing bacteria including Flavobacteriaceae, Vibrionaceae, Alteromonadaceae, Colwelliaceae, and Rhodobacteraceae while helping to maintain a healthy microbiome rich in *Endozoicomonas*. Moreover, QSI only affects disease-associated species; the addition of QSI to a healthy microbiome does not significantly change the population structure. As a result, QSI has potential conservation implications as the methods presented here have potential to be modified to prevent and combat coral disease on the reef.
Acknowledgements

We thank Sarah Gignoux-Wolfsohn, Erik Holum, and Tarik Gouhier for bioinformatics and statistical guidance, as well as Amanda Dwyer for lesioning more corals than she cares to remember. We also thank the Smithsonian Tropical Research Institute for field and lab support. Collection permits were provided by Autoridad Nacional del Ambiente (ANAM) SE/A-9-16. This work was supported by National Science Foundation Award #1458158.
Literature Cited


Table 3.1. PERMANOVA of Bray-Curtis dissimilarities between coral-associated bacterial communities from Times 1 and 2.

**Time 1**

<table>
<thead>
<tr>
<th>Source</th>
<th>Df</th>
<th>Sum Sq.</th>
<th>Mean Sq.</th>
<th>F-value</th>
<th>R²</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disease Exposure</td>
<td>1</td>
<td>0.887</td>
<td>0.887</td>
<td>5.668</td>
<td>0.0899</td>
<td>0.002</td>
</tr>
<tr>
<td>QSI</td>
<td>1</td>
<td>1.248</td>
<td>1.248</td>
<td>7.973</td>
<td>0.126</td>
<td>0.001</td>
</tr>
<tr>
<td>Disease Exposure:QSI</td>
<td>1</td>
<td>0.845</td>
<td>0.845</td>
<td>5.394</td>
<td>0.0856</td>
<td>0.003</td>
</tr>
<tr>
<td>Residuals</td>
<td>44</td>
<td>6.889</td>
<td>0.156</td>
<td>NA</td>
<td>0.698</td>
<td>NA</td>
</tr>
<tr>
<td>Total</td>
<td>47</td>
<td>9.869</td>
<td>NA</td>
<td>NA</td>
<td>1</td>
<td>NA</td>
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</tbody>
</table>

**Time 2**

<table>
<thead>
<tr>
<th>Source</th>
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<th>Sum Sq.</th>
<th>Mean Sq.</th>
<th>F-value</th>
<th>R²</th>
<th>p-value</th>
</tr>
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<tbody>
<tr>
<td>Disease State</td>
<td>1</td>
<td>0.950</td>
<td>0.950</td>
<td>5.298</td>
<td>0.0836</td>
<td>0.002</td>
</tr>
<tr>
<td>QSI</td>
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<td>1.566</td>
<td>8.736</td>
<td>0.138</td>
<td>0.001</td>
</tr>
<tr>
<td>Disease Exposure:QSI</td>
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<td>0.956</td>
<td>0.956</td>
<td>5.331</td>
<td>0.0841</td>
<td>0.002</td>
</tr>
<tr>
<td>Residuals</td>
<td>44</td>
<td>7.888</td>
<td>0.179</td>
<td>NA</td>
<td>0.694</td>
<td>NA</td>
</tr>
<tr>
<td>Total</td>
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<td>11.340</td>
<td>NA</td>
<td>NA</td>
<td>1</td>
<td>NA</td>
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</table>
Figure 3.1. Incidence of WBD infection in healthy *A. cervicornis* exposed to WBD bacterial communities incubated with QSI. FSW and WBD pools were incubated with FSW and QSI for five hours. Aquaria were then converted to closed systems and healthy *A. cervicornis* were dosed with one of the four treatments: (1) –WBD/–QSI (2) –WBD/+QSI (3) +WBD/–QSI and (4) +WBD/+QSI. After dosage, the corals were closely monitored for WBD signs. Incidence of infection was analyzed using Likelihood Ratio Tests (LTR) for significance of GLM terms. Mean ± SE shown.
Figure 3.2. Relative abundance of coral-associated bacterial communities sorted taxonomically by family for the four treatments at Time 1 (12 hours post-dosage). The percent abundance of families whose incidence reached ten percent of the total OTU count in at least one sample are shown. Rare families are grouped as “Other”.

![Graph showing relative abundance of coral-associated bacterial communities sorted taxonomically by family for the four treatments at Time 1 (12 hours post-dosage).](image-url)
Figure 3.3. Relative abundance of coral-associated bacterial communities sorted taxonomically by family for the four treatments at Time 2 (24 hours post-dosage). The percent abundance of families whose incidence reached ten percent of the total OTU count in at least one sample are shown. Rare families are grouped as “Other”.

![Figure 3.3](image-url)
Figure 3.4. Principal coordinate analysis (PCoA) plots of dissimilarities between coral-associated bacterial communities from the four treatments. Black points represent –WBD/–QSI corals, blue points represent –WBD/+QSI corals, red points represent +WBD/–QSI corals, and purple points represent +WBD/+QSI corals. (A) PCoA of Time 1 (12 hours post-dosage) communities; no physical WBD signs are apparent (B) PCoA of Time 2 (24 hours post-dosage); early WBD signs apparent in +WBD/–QSI corals.
Figure 3.5. Boxplots representing the Shannon indices (alpha diversity) of the coral-associated bacterial communities from the four treatments. The median Shannon index and the upper and lower quartiles are shown for each treatment. (A) Shannon indices at Time 1 (12 hours post-dosage); no physical WBD signs are apparent (B) Shannon indices at Time 2 (24 hours post-dosage); early WBD signs apparent in +WBD/–QSI corals. Means not sharing the same letter are significantly different (Tukey HSD, p < 0.001).
Figure 3.6. Significantly differentially abundant OTUs for the interaction between disease exposure and QSI addition. All OTUs shown have an average expression of greater than 100 across all samples. OTUs are organized by family between the +WBD/–QSI (left) treatment and the +WBD/+QSI (right) treatment at (A) Time 1 and (B) Time 2.
Figure 3.7. Number of OTUs belonging to (A) Vibrionaceae (B) Flavobacteriaceae (C) *Endozoicomonas* and (D) Halomonadaceae across the four treatments across the three time points.
## Supplementary Information

**Table 3.S1.** Likelihood Ratio Tests table for aquaria-based experiment.

<table>
<thead>
<tr>
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<th>Df</th>
<th>Pr(&gt;Chisq)</th>
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<tbody>
<tr>
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<td>5.546</td>
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<td>0.0185</td>
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<tr>
<td>QSI</td>
<td>13.258</td>
<td>1</td>
<td>0.000271</td>
</tr>
<tr>
<td>Disease Exposure:QSI</td>
<td>2.376</td>
<td>1</td>
<td>0.123</td>
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**Table 3.S2.** ANOVA table for Shannon indices (alpha diversity) for Times 0, 1, and 2.

**Time 0**

<table>
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<tr>
<th>Source</th>
<th>Df</th>
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<th>Mean Sq.</th>
<th>F-value</th>
<th>p-value</th>
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</thead>
<tbody>
<tr>
<td>Disease Exposure</td>
<td>1</td>
<td>0.145</td>
<td>0.145</td>
<td>1.740</td>
<td>0.194</td>
</tr>
<tr>
<td>QSI</td>
<td>1</td>
<td>0.011</td>
<td>0.0107</td>
<td>0.127</td>
<td>0.723</td>
</tr>
<tr>
<td>Disease Exposure:QSI</td>
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<td>0.001</td>
<td>0.0011</td>
<td>0.013</td>
<td>0.910</td>
</tr>
<tr>
<td>Residuals</td>
<td>44</td>
<td>3.680</td>
<td>0.0836</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

**Time 1**

<table>
<thead>
<tr>
<th>Source</th>
<th>Df</th>
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<th>Mean Sq.</th>
<th>F-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disease Exposure</td>
<td>1</td>
<td>4.838</td>
<td>4.838</td>
<td>24.08</td>
<td>1.31e-05</td>
</tr>
<tr>
<td>QSI</td>
<td>1</td>
<td>3.857</td>
<td>3.857</td>
<td>19.20</td>
<td>7.20e-05</td>
</tr>
<tr>
<td>Disease Exposure:QSI</td>
<td>1</td>
<td>3.295</td>
<td>3.295</td>
<td>16.40</td>
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<td>Residuals</td>
<td>44</td>
<td>8.841</td>
<td>0.201</td>
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**Time 2**

<table>
<thead>
<tr>
<th>Source</th>
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<th>p-value</th>
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<tr>
<td>Disease Exposure</td>
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<td>6.306</td>
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<td>26.01</td>
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<tr>
<td>QSI</td>
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<td>6.018</td>
<td>24.82</td>
<td>1.02e-05</td>
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<tr>
<td>Disease Exposure:QSI</td>
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<td>6.004</td>
<td>6.004</td>
<td>24.76</td>
<td>1.04e-05</td>
</tr>
<tr>
<td>Residuals</td>
<td>44</td>
<td>10.669</td>
<td>0.242</td>
<td>NA</td>
<td>NA</td>
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</tbody>
</table>

**Table 3.S3.** Significantly differentially abundant OTUs across treatments across time points.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time 0</th>
<th>Time 1</th>
<th>Time 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disease Exposure</td>
<td>0</td>
<td>634</td>
<td>831</td>
</tr>
<tr>
<td>QSI</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Disease Exposure:QSI</td>
<td>0</td>
<td>387</td>
<td>615</td>
</tr>
</tbody>
</table>
Figure 3.S1. Relative abundance of coral-associated bacterial communities sorted taxonomically by family for the six WBD pools. The percent abundance of families whose incidence reached five percent of the total OTU count in at least one sample are shown. Rare families are grouped as “Other”.
Figure 3.S2. Principal coordinate analysis (PCoA) plots of dissimilarities between coral-associated bacterial communities from the four treatments at Time 0 (pre-dosage). Black points represent –WBD/–QSI corals, blue points represent –WBD/+QSI corals, red points represent +WBD/–QSI corals, and purple points represent +WBD/+QSI corals.
**Figure 3.S3.** Relative abundance of coral-associated bacterial communities sorted taxonomically by family for the four treatments at Time 0 (pre-dosage). The percent abundance of families whose incidence reached ten percent of the total OTU count in at least one sample are shown. Rare families are grouped as “Other”.
A metatranscriptomic analysis of differential gene expression between healthy versus white band disease-infected Acropora cervicornis microbiomes

Abstract
Coral disease is one of the greatest threats facing reefs around the globe. In the Caribbean, white band disease (WBD) has nearly eradicated two of the region’s most important hermatypic species: Acropora cervicornis and Acropora palmata. Although recent studies have started to elucidate the microbial population structure of bacterial communities on diseased corals, the genetic responses during infection utilized by disease-associated microbes is almost entirely unknown. Here we compare gene expression between healthy versus WBD-infected A. cervicornis microbiomes focusing on genes belonging to the putative WBD-associated pathogenic groups Vibrio and Flavobacteriaceae. 16S rRNA sequencing revealed a significant effect of disease state and coral genotype on coral-associated bacterial populations. Metatranscriptomic results identified 444 differentially expressed genes between healthy and WBD-infected coral microbiomes. We also show that disease-associated Vibrios rely on DNA repair and the SOS response while disease-associated Flavobacteriaceae rely on lipid biosynthesis to maintain infection.
**Introduction**

Many marine ecosystems harbor diverse microbial communities. The coral holobiont – a community of organisms that comprise a healthy coral – includes the coral animal, photosynthetic dinoflagellates, and associated microbial species (Knowlton and Rohwer 2003, Rosenberg et al. 2007). Compared to the well-documented mutualism between corals and their photosynthetic algae, much less is known about the relationship between a coral and its bacterial microbiome (Knowlton and Rohwer 2003). Although many details regarding this association remain uncharacterized, it is known that breakdowns in the relationships between coral and microbes often lead to or contribute to coral disease (Bourne et al. 2009, Garcia et al. 2013, Harvell et al. 2007, Muller and van Woesik 2012).

Recent decades have seen a sharp rise in coral epizootics, which have become increasingly responsible for the decline of coral reef health worldwide (Aronson and Precht 2001, Harvell et al. 2007). However, despite their scope and severity, the etiology and pathology of most coral diseases remain unknown (Sutherland et al. 2004). White band disease (WBD) is a classic example of a widespread and devastating yet poorly understood epidemic. Since its original outbreak in 1979, WBD has nearly eradicated Caribbean acroporids, decimating the populations of two of the region’s most important reef-building corals (Aronson and Precht 2001). As a direct result of WBD, both *Acropora cervicornis* (staghorn coral) and *Acropora palmata* (elkhorn coral) are classified as critically endangered on the IUCN Red List of Threatened Species (Randall and van Woesik 2015).
WBD belongs to a group of related coral diseases known as “white syndromes,” which are classified through a shared phenotype of rapid tissue loss as the diseases progresses along the surface of the coral (Luna et al. 2010). WBD is highly transmissible both via the water column onto injured individuals as well as through animals reservoirs (Gignoux-Wolfsohn et al. 2012, Kline and Vollmer 2011). Although a specific etiological agent has yet to be identified, several bacterial genera have been associated with the disease including Rickettsiales (Casas et al. 2004), Flavobacteriales (Gignoux-Wolfsohn and Vollmer 2015), and *Vibrio* (Gil-Agudelo et al. 2006, Kline and Vollmer 2011, Sweet et al. 2014). Recent research suggests that many coral diseases, including WBD, can be attributed to opportunistic pathogenesis of resident coral-associated bacteria (Lesser et al. 2007, Ritchie 2006). Several studies have shown that diseased corals undergo a shift in microbial community composition from one composed of commensals and symbionts to one dominated by characteristically opportunistic species and known marine pathogens (Ritchie 2006, Thurber et al. 2009).

Anthropogenic stressors, especially rising seawater temperatures, contribute to the destabilization of the coral-bacterial relationship (Aronson and Precht 2001, Harvell et al. 2007, Muller and van Woesik 2009, Muller and van Woesik 2012, Ritchie 2006, Rosenberg and Ben-Haim 2002, Thurber et al. 2009). Sustained environmental change places undue stress on the coral animal, thus compromising any immune response and increasing the animal’s susceptibility to infection (Ritchie 2006). Indeed, the emergence and perpetuation of WBD has been coupled with intensifying climate-driven thermal stress on Caribbean reefs (Randall and van Woesik 2015). This finding lends further credence to the compromised-host hypothesis (Lesser et al. 2007), which states that coral disease may not arise from exposure to novel pathogens but rather
the weakened ability of corals to combat systemic infection (Lesser et al. 2007, Muller et al. 2008, Muller and van Woesik 2009). In this scenario, stressed corals become increasingly vulnerable to an overgrowth of opportunistic pathogens, which outcompete commensal microbes, leading to disease.

A major challenge in studying coral disease is the high diversity of the coral microbiome often consisting of thousands of distinct microbial OTUs (Gignoux-Wolfsohn and Vollmer 2015). As a result, culture-dependent techniques often fall short in obtaining meaningful information regarding coral bacteria community composition. One great advantage of next-generation sequencing (NGS) is the ability to accurately profile complex environmental samples by shotgun sequencing bulk DNA. A number of studies have published coral-associated bacterial metagenomes (Garcia et al. 2013, Thurber et al. 2009) and 16S datasets (Gignoux-Wolfsohn and Vollmer 2015, Pantos and Bythell 2006, Sunagawa et al. 2009) as a means to identify potential pathogens responsible for coral disease. One problem with these DNA-based, community structure approaches is that they do not account for gene expression and instead measure the relative abundances of microbial taxa or microbial genes. The relative presence or absence of certain species does not necessarily correlate to a microbial species’ genetic influence on pathogenicity. For example, Thurber et al. 2009, determined that thermally stressed corals did not yield a statistically significant increase in their abundance of Gammaproteobacteria. However, metabolically, *Vibrios* were responsible for the discrepancy in gene abundance between heat-stressed and control corals. This low-abundance genus drove the metabolic shifts in thermally stressed corals and controlled virulence-associated genes like chemotaxis, motility,
catalases, and efflux pumps (Thurber et al. 2009). Thus, relatively uncommon species may be a driving force behind the transmission and progression of coral disease.

In this study, we focus on coral-associated bacterial gene expression in order to characterize the microbial communities on healthy versus diseased corals. We collected healthy and WBD-infected corals and used next-generation 16S rDNA metagenomic as well as metatranscriptomic sequencing to analyze the genetic differences between the microbiomes of healthy and WBD-infected *A. cervicornis*. Specifically, we focus on the behavior of two taxa – the genus *Vibrio* and the family Flavobacteriaceae – known to be associated with WBD-infected corals.

**Methods**

*Coral collection and homogenization*

All corals were sampled in February 2014 from Crawl Cay (9° 14’ 00” N, 82° 08’ 00” W) in Bocas del Toro, Panama. Paired samples of healthy and WBD-infected *A. cervicornis* fragments were collected from six colonies displaying both a healthy and a WBD phenotype; an asymptomatic branch and diseased branch were both sampled from the same coral colony. Paired samples were used in order to minimize the potential influence of coral genotype on coral-associated microbes. Coral fragments were then transported to the Smithsonian Tropical Research Institute in separate containers. Within two hours of collection, each of the 12 *A. cervicornis* fragments was separately homogenized in 10 mL 0.2 um-filtered seawater with sterile glad beads. The resulting tissue homogenate was filtered at 5 um to obtain the bacterial fraction.
Preserving nucleic acids from coral-associated bacteria

For each of the 12 fragments, two mL of each bacterial fraction were added to two 1.5 mL tubes and centrifuged at 13,000 rpm for three minutes to pellet the bacteria. Supernatant was removed. Half of the bacterial pellets were re-suspended in 300 uL TRI Reagent (Molecular Research Center) and flash frozen in liquid nitrogen. The other half was re-suspended in DNA Buffer (Fukami et al. 2004) for subsequent 16S analysis.

RNA extraction and mRNA purification

Total bacterial RNA was extracted from TRI Reagent according to manufacturer instructions and solubilized in 15 uL TE buffer. Bacterial mRNA (and ribosomal subtraction) was then isolated using the MICROBExpress Bacterial mRNA Enrichment Kit (Ambion) and stored in TE buffer. mRNA concentrations were measured using the Qubit fluorometer (Life Technologies).

Illumina library preparation and bioinformatics

A cDNA library was created from purified bacterial mRNA using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs). Quantity and quality of libraries were checked with the Qubit fluorometer and the Bioanalyzer (Agilent Technologies). Multiplexed cDNA Illumina 250 bp paired-end libraries were then sequenced on the Illumina HiSeq 2500 platform at Harvard University. Raw sequencing reads were filtered based on a quality score of 30 with Trimmomatic (Bolger et al. 2014) and paired end reads were aligned using FLASH (Magoc and Salzberg 2011) according to Westreich et al. 2016. The trimmed and aligned sequences were submitted for annotation to the MG-RAST (Metagenomic Rapid Annotations using Subsystems Technology) server (Meyer et al. 2008).
**Differential gene expression**

Both organism and functional annotations were downloaded for each of the 12 metatranscriptomic libraries. Using the SAMSA pipeline, organism and function annotations were summarized into a sorted count by abundance for each metatranscriptome (Westreich et al. 2016). Lingering eukaryotic and ribosomal contamination was removed based on the MG-RAST annotation. Differentially expressed genes between healthy and WBD-infected coral-associated microbiomes were determined using the fitZig function from the R package metagenomeSeq, which computes the weighted fold-change estimates and t-statistics (Paulson et al. 2013a, Paulson et al. 2013b). This function is based on a zero-inflated Gaussian mixture model and the metagenomeSeq package relies on the function topTable in the R package limma (Ritchie et al. 2015). Disease state was defined as a main effect while coral genotype was defined as a batch effect (healthy and diseased samples were paired). Only genes that appeared in three or more samples with ten or more reads were included in the analysis.

Using the SAMSA pipeline, genes associated with the genus *Vibrio* and the family Flavobacteriaceae were isolated from each microbiome sample (Westreich et al. 2016). This feature uses the MG-RAST annotation results to screen for all IDs in the functional files that match certain taxonomy in the corresponding organism file. Differentially expressed *Vibrio* and Flavobacteriaceae genes were determined using the aforementioned fitZig function. Only genes that appeared in three or more samples ten or more times were included in the analysis.
Statistical analysis

*Vibrio* and Flavobacteriaceae-associated gene counts were normalized using the metagenomeSeq method which accounts for sparsity due to undersampling (Paulson et al. 2013a, Paulson et al. 2013b). Comparisons between healthy and WBD-infected microbiomes were visualized using principal coordinate analysis (PCoA) applying the Hellinger transformation – which gives low weights to low abundance species – using the function decostand in the R package Vegan (Oksanen et al. 2007). Community level differences between healthy and diseased corals were determined by PERMANOVA of Bray-Curtis dissimilarities using the Adonis function in the R package Vegan.

16S library preparation, bioinformatics, and statistical analysis

Total DNA was extracted from the 14 samples using the BioSprint 96 DNA Blood Kit (Qiagen). The V4 hypervariable region of the 16S rRNA gene was amplified and sequenced (Fadrosh et al. 2014). A separate PCR was performed for each sample using a unique combination of barcoded primers (minus the spacers) and Phusion High-Fidelity DNA Polymerase (New England Biolabs). The SequaPrep Normalization Plate Kit (Thermo Fisher Scientific) was used to normalize PCR products and Agencourt AMPure XP magnetic beads (Beckman Coulter) were used to concentrate and then pool the samples. The resulting multiplexed libraries were sequenced on the Illumina 2500 HiSeq platform. While 250 bp paired-end V3-V4 libraries were originally sequenced, a lack of overlapping reads led to the demultiplexing of single read sequences using a custom Python script. OTUs were clustered at 97% similarity and singletons were removed using the open reference picking script from QIIME (Caporaso et al. 2010) and
UCLUST against the SILVA database (Quast et al. 2013). OTU taxonomy was assigned using BLAST against the SILVA database.

16S OTU counts were normalized using metagenomeSeq. Comparisons between healthy and WBD-infected microbiomes were visualized using principal coordinate analysis (PCoA) applying the Hellinger transformation using the function decostand in the R package Vegan. Community level differences between healthy and diseased corals were determined by PERMANOVA of Bray-Curtis dissimilarities using the Adonis function in the R package Vegan.

Results

16S results
All 12 coral-associated bacterial samples used in the metatranscriptomic analysis were sequenced for 16S. 97% clustering yielded 3,064 OTUs. Bacterial population PCoA plots show healthy and diseased microbiomes clustering away from each other (Figure 1). PERMANOVA analyses corroborate the results of the PCoA (PERMANOVA: F = 2.550, R² = 0.1468, p = 0.047) as disease state has a significant effect (Table 1). Coral genotype (batch effect) also has a highly significant effect on the coral-associated bacterial populations (PERMANOVA: F = 5.8237, R² = 0.3352, p = 0.001) (Table 1). Coral genotypes (colony number) two, three, and four are dominated by Hahellaceae (Endozoicomonas) OTUs despite disease state (Figure 2). Healthy corals contain a significant number of Halomondadaceae (Figure 2). WBD-infected corals contain a significant number of Blattabacteriaceae (order Flavobacteriales) (Figure 2). In general, WBD-infected microbiomes contain a larger number of Vibrio and Flavobacteriaceae OTUs than did their healthy counterparts (Figure 3).
**Metatranscriptome results**

A total of 23,347 genes were left after removal of eukaryotic and ribosomal contamination as well as any genes that mapped to a “hypothetical” protein (Table 2); further parsing to include only those genes that occurred in three or more samples with ten or more total reads left 6,760 genes (Table 2). Of the 6,760 genes, metagenomeSeq identified 1,771 as differentially expressed between healthy and WBD microbiomes (FDR p < 0.05) (Figure 5A, Table 2). In all, 444 of the differentially expressed genes had a log2 fold change of greater than $|2|$, a cut-off typically considered to be biologically relevant (Figure 5A, Table 2) (McCarthy and Smyth 2009). 235 of these genes were upregulated in WBD microbiomes while 209 were upregulated in healthy microbiomes (Figure 5A, Table 2). The top 100 differentially expressed genes with the greatest variance (standard deviation) are plotted on a heatmap (Figure 4A). These top 100 genes included 22 genes that map to the family Vibrionaceae and 3 genes that map to the class Flavobacteria (Supplementary Table 1). Principal coordinate analysis (PCoA) plots of the 6,760 genes show no difference between healthy and diseased microbiomes (Figure 6A). PERMANOVA analyses corroborate the results of the PCoA (PERMANOVA: $F = 0.806, R^2 = 0.073, p = 0.901$) (Table 1).

Because of the greater total abundances of *Vibrio* and Flavobacteriaceae on WBD-infected corals (Figure 3) – and their associated with WBD in past studies – the total number of genes was then split by these taxa certain taxa. 7,558 genes belong to a *Vibrio* and this group was again parsed to include only those genes that occurred in three or more samples ten or more times. 1,240 genes were retained, 94 of which are differentially expressed between healthy and WBD-associated microbiomes (Figure 5B, Table 2). Differentially expressed genes are plotted on a heatmap
Twenty-nine *Vibrio* genes have a log2 fold change of greater than |2| and 15 are upregulated in WBD microbiomes while 14 are upregulated in healthy microbiomes (Figure 5B). PCoA plots show little difference between healthy and diseased microbiome gene expression (Figure 3B). PERMANOVA analyses corroborate the results of the PCoA (PERMANOVA: $F = 1.104$, $R^2 = 0.097$, $p = 0.257$) (Table 2). The 29 *Vibrio* genes with a log2 fold change of greater than |2| were characterized by gene ontology (GO) terms for molecular function (Table 3, Figure 7A) and biological process (Table 3, Figure 8A).

For Flavobacteriaceae, 1,729 genes matched this family, 168 of which were found in three or more samples ten or more times (Table 2). Fifty-four Flavobacteriaceae genes were differentially expressed (Figure 5C) and these are shown in a heatmap (Figure 4C). Seven genes have a significant log2 fold change with 5 upregulated in WBD and 5 upregulated in healthy microbiomes (Figure 5C). Again, PCoA plots show no significant clustering (Figure 6C) and PERMANOVA supports this result (PERMANOVA: $F = 1.042$, $R^2 = 0.095$, $p = 0.362$) (Table 1). The seven Flavobacteriaceae genes with a log2 fold change of greater than |2| were characterized by gene ontology (GO) terms for molecular function (Table 4, Figure 7B) and biological process (Table 4, Figure 8B).

### Discussion

*Uncommon species may be driving disease*

Surprisingly, coral disease state does not have a significant effect on bacterial gene expression (Table 1). The discrepancy in clustering between whole metatranscriptome (Figure 6A, Table 1) and 16S metagenome (Figure 1, Table 1) datasets suggests that a relatively small number of
expressed genes are contributing to the WBD phenotype. A large number of similar genes from like bacterial species – regardless of disease state – potentially masks the genes involved in WBD infection. In the metatranscriptomic data, 444 out of 23,347 bacterial genes were differentially expressed at log2 fold change greater than |2| between healthy and diseased coral microbiomes (Table 2). Since gene expression rather than taxonomy of the originating organism was the focus of this study, we aggregated the MG-RAST annotations by gene name. As a result, differentially expressed genes from the full taxonomy dataset belong to multiple organisms. Even so, of the genes plotted in the heatmap (Figure 4A) – the top 100 with the greatest variance – around 20% are *Vibrio* and three belong to the class Flavobacteria (Supplementary Table 1). Moreover, based on the 16S data, both of these taxa are more abundant in WBD-infected corals (Figure 3). *Vibrio* and Flavobacteraeae have been repeatedly associated with WBD-infected *A. cervicornis* (Gignoux-Wolfsohn and Vollmer 2015, Gil-Agudelo et al. 2006), including chapters 2 and 3 of this dissertation, yet these groups are relatively uncommon when looking at the 16S data (Figure 2). As a result, we narrow our search to putative WBD pathogens in order to find WBD-associated genes.

*Vibrio genes upregulated in WBD microbiomes – the SOS response*

The 29 differentially expressed *Vibrio* genes with a log2 fold change > |2| are diverse with regards to categorization via GO terms (Table 3) for molecular function (Figure 7A) and biological process (Figure 8A). WBD-associated genes generally involve the modification of nucleic acids. Seven of the 15 genes upregulated in diseased microbiomes are characterized by the molecular functions of DNA or RNA binding (Figure 7A, Table 3). DNA-directed RNA polymerase and tRNA-specific 2-thiouridylase MnmA are involved in transcription and
translation but genes such as RecA, RuvB, and DNA polymerase II are involved in the DNA
damage/SOS response (Figure 5A, Table 3). RecA is the global regulator for the bacterial SOS
response, during which the cell cycle is stopped in order to correct DNA damage (Singer 1989).
RecA is also associated with increased protection against UV radiation as well as antibiotic
resistance (Ching et al. 2017, Rapa et al. 2014). DNA repair initiated by RecA increases
spontaneous mutations which often result in the emergence of antibiotic resistant mutants (Rapa
et al. 2014). Furthermore, RecA is found in known Vibrio pathogens including multiple strains of
V. parahaemolyticus (Gonzalez-Escalona et al. 2015) and is upregulated during V. cholerae
infections (LaRocque et al. 2005).

RuvB is also critical to bacterial DNA repair as it resolves the Holliday junction created during
homologous recombination. Along with RecA, RuvB is involved in the SOS response (Sanchez-
Alberola et al. 2012) and is upregulated in V. cholerae infections (LaRocque et al. 2005). DNA
polymerase II is also found within the SOS regulon (Iwasaki et al. 1990, Sanchez-Alberola et al.
2012) and contains exonuclease proofreading capabilities aimed at DNA repair more so than
replication.

Other notable disease-associated Vibrio genes include DNA-binding protein HU-alpha (hupA)
and dimethyladenosine transferase (ksgA) (Table 3). HupA is a heme receptor and is necessary
for heme utilization as a source of iron (Oh et al. 2009). As iron is necessary for cell survival and
multiplication, HupA is required for pathogenesis by the marine pathogen V. vulnificus (Oh et al.
2009) and is upregulated when V. vulnificus is exposed to increased temperatures (Oh et al.
2009) This suggests that HupA contributes to the opportunistic pathogenesis of V. vulnificus
which fits with the finding that WBD transmission increases when sea surface temperatures rise (Randall and van Woesik 2015). KsgA is an rRNA methyl transferase that plays a role in antibiotic resistance according to the GO term database for biological processes. Inactivation of KsgA in E. coli leads to kasugamycin resistance (Ochi et al. 2009, van Buul and van Knippenberg 1985). Though kasugamycin resistance via mutation of KsgA has never been demonstrated in a Vibrio, KsgA is highly conserved across all microorganisms (Ochi et al. 2009).

*Vibrio genes downregulated in WBD microbiomes – response to oxidative stress*

Based on their molecular functions, many of the *Vibrio* genes downregulated in diseased microbiomes are involved in general aerobic metabolism. Five of these 14 genes are oxioreductases, enzymes that catalyze the transfer of electrons from one molecule to another (Figure 4A, Table 3). One interesting gene from this set is catalase-peroxidase HPI, a protein that belongs to a family of enzymes involved in breaking down hydrogen peroxide, a toxic compound produced during aerobic respiration (Table 3). Peroxidases and related catalases are often upregulated in many pathogenic bacteria during the infection process including in the marine pathogen *Vibrio parahaemolyticus* (Chen et al. 2016).

Another notable *Vibrio* gene downregulated in WBD microbiomes is a putrescine transporter, a hydrolase (Figure 4A, Table 3). Putrescine is a toxic organic chemical compound produced during the breakdown of amino acids. Polyamines like putrescine may serve as a form of communication between pathogenic bacteria, including known pathogens like *V. cholerae, V. parahaemolyticus*, and *V. vulnificus* (Karatan et al. 2005). Additionally, putrescine and related
compounds may be involved in biofilm formation as polyamines were shown to increase surface accumulation in *V. cholerae* and swarming in *Proteus mirabilis* (Karatan et al. 2005). Intriguingly, similar to peroxidase, putrescine may also have antioxidant properties and reduce the effects of oxidative stress (Baharoglu et al. 2013).

Based on the candidate genes downregulated in WBD *A. cervicornis* microbiomes, *Vibrio* behavior within healthy microbiomes is largely characterized by response to oxidative stress. This behavior is likely a defense against reactive oxygen species (ROS) production by the coral host, a well-conserved innate immune response to invading pathogens (Anderson et al. 2016, Libro et al. 2013, Wright et al. 2015). It is well-established that ROS production is upregulated in corals experiencing thermal stress and infectious disease (Wright et al. 2015). WBD-associated pathogens – such as *Vibrios* – have been shown to trigger phagocytosis in infected corals (Libro et al. 2013). Coral ROS are generated within host phagosomes in order to kill engulfed pathogens (Libro et al. 2013).

*Flavobacteriaceae genes upregulated in WBD microbiomes – lipid metabolism and antibiotic resistance*

Of the five Flavobacteriaceae genes upregulated in WBD microbiomes, two are involved in lipid metabolism and two contribute to antibiotic resistance via transmembrane transport (Figure 8B, Table 3). Both phosphomannomutase (capsule biogenesis/degradation) and acyl-CoA dehydrogenase (lipid metabolism) are enzymes involved in the production of capsules and lipopolysaccharide (LPS) (Figure 8B, Table 3). Capsule and LPS are associated with virulence in many species of gram-negative pathogenic bacteria (Kusecek et al. 1984, Moxon and Kroll
Bacterial capsules aid in the avoidance of phagocytosis by the host (Daffe and Etienne 1999) and LPS allow for the secretion of various virulence factors (Kulp and Kuehn 2010). In the known pathogen *Pseudomonas aeruginosa*, phosphomannomutase is central to the formation of alginate capsule and LPS, both of which facilitate host/pathogen interaction by enabling bacterial adhesion to host cells (McCarthy et al. 2005). Fish pathogen *Flavobacterium columnare* also utilizes capsule and LPS to enable adhesion during the infection process (Dumpala et al. 2010, Zhang et al. 2006). LPS is also essential to the formation of outer membrane vesicles, ubiquitous in gram-negative bacteria, especially in pathogens (Ellis and Kuehn 2010). These vesicles function as a delivery system for numerous virulence factors including toxins, protein adhesins, and enzymes (Ellis and Kuehn 2010).

Along with specialized lipids, Flavobacteriaceae may utilize antibiotic pumps to mount an attack against their coral hosts. Both the outer membrane efflux protein and hydrophobe/amphiphile efflux-1 are involved in the general transport of small molecules across the outer membrane of gram-negative bacteria (van Bambeke et al. 2000, Zgurskaya et al. 2011). These molecules often include toxins or other virulence factors secreted as part of the infection process (van Bambeke et al. 2000, Zgurskaya et al. 2011). Interestingly, these two antibiotic pumps align with the upregulation of lipid metabolism since transmembrane channels are typically hydrophobic (van Bambeke et al. 2000). LPS and capsule comprise virulence factors and their vesicles that are secreted from the cell via efflux proteins. Not only do these channels facilitate infection, there is evidence that both of these proteins contribute to bacterial antibiotic resistance (Perrin et al. 2010, Poole et al. 1993), adding another barrier to host immunity.
Flavobacteriaceae genes downregulated in WBD microbiomes – heat shock proteins

Only two Flavobacteriaceae genes with a log2 fold change > |2| were upregulated in healthy microbiomes: dnaK and succinyl-CoA synthetase (Table 3). These genes code for proteins that are known immunogenic molecules of several species of pathogenic bacteria including the fish pathogen *Flavobacterium columnare* (Lange et al. 2016, Liu et al. 2012). DnaK (GO biological process: stress response) is synonymous with the well-studied 70 kilodalton heat shock protein (HSP70), a molecular chaperone produced by cells in response to stressful conditions (Figure 8B) (Farr and Kogoma 1991). Similar to the healthy-associated *Vibrio* genes, DnaK also provides protection against oxidative stress, a main immune response in corals (Chakrabarti et al. 1999). Interestingly, in addition to protecting the bacterial cell from stress and host immunity, DnaK contributes to bacterial virulence and has been shown to increase pathogenesis in the fish pathogen *Edwardsiella tarda* (Dang et al. 2011). Stressful conditions are likely to arise immediately post-infection, when the host’s defenses are strongest. As such, DnaK may simultaneously function as a virulence factor promoting infection and as a defense against the coral immune response.

Healthy versus diseased microbiomes

The overall insignificant effect of disease state on bacterial gene expression (Figure 6, Table 1) implies that most bacteria behaviors are similar between healthy and diseased corals. This may be partially explained by the strong effect of coral genotype on bacterial residents. However, based on the differentially expressed genes, the similar behavior between healthy and diseased microbiomes may also be attributed to the fact that relatively few genes dominate the WBD response. In fact, many of the downregulated genes in WBD microbiomes are counterintuitive
since *Vibrio* oxidative stress genes and Flavobacteriaceae heat shock genes are virulence factors. It is possible that the coral host is simply impeding infection by targeting damaging bacterial genes. Coral immunity relies heavily on ROS (Libro et al. 2013) which targets bacterial proteins and DNA. It is also possible that the healthy (i.e. asymptomatic) sections of the sampled corals may have shown early signs of bacterial infection. Future studies should start with completely asymptomatic corals and track WBD through early and late stages.

*Quorum sensing potential of differentially expressed genes*

Quorum sensing (QS) may also play a role in the regulation of WBD-associated genes. Demonstrated in the previous chapter, *Vibrio* and Flavobacteriaceae growth – and consequently WBD transmission – is arrested by quorum sensing inhibitor. Of the differentially expressed genes identified using metatranscriptomics, *Vibrio* RecA may be controlled by quorum sensing as it is upregulated along with other SOS response-associated genes in wildtype enterohemorrhagic *E. coli* compared to the QS-deficient mutant (Sperandio et al. 2001). Likewise, QS systems in *P. aeruginosa* have also been shown to control the expression of catalase and other genes related to oxidative stress response (Hassett et al. 1999). Putrescine, a cell-cell signaling molecular in its own right (Karatan et al. 2005), has also been shown to fall under the influence of QS in human cells (Kristiansen et al. 2008). As for lipid-associated Flavobacteriaceae genes, there is evidence for QS control of both LPS and capsule production in *P. aeruginosa* (Wagner et al. 2003). LPS, capsular polysaccharides, and phosphomannomutase are upregulated in QS-controlled pathogenesis pathways in *V. vulnificus* (Shin et al. 2007). Moreover, in addition to toxins, the highly conserved outer membrane efflux pumps also facilitate the secretion of homoserine lactones involved in QS (Poole 2001).
Conclusion

This chapter attempts to step beyond the existing literature and elucidate the bacterial genes involved in WBD infection of *A. cervicornis*. Our findings show that coral genotype has an unexpectedly strong effect on its microbial residents. As a result, pinpointing WBD-associated bacterial genes was challenging; relatively few genes drive the disease phenotype. Focusing on known WBD-associated taxa – *Vibrio* and Flavobacteriaceae – we establish that *Vibrios* rely on the SOS response and Flavobacteriaceae utilize lipid virulence factors and antibiotic pumps to infect the coral host. Interestingly, there exist many similarities between healthy and disease-associated differentially expressed genes. Both healthy and WBD-associated genes from both taxa are involved in bacterial pathogenicity. Additionally, many genes upregulated in both disease states may be controlled, in part, by quorum sensing pathways.

Acknowledgements

We would like to thank Sam Westreich for bioinformatics and coding assistance, Dr. Tarik Gouhier for statistical guidance, Dr. Yunrong Chai for sharing his depth of knowledge on bacterial genes, and Dr. Sarah Gignoux-Wolfsohn for introducing me to Amazon Web Services, offering sage advice, and being a general sounding board for my ideas. We also thank the Smithsonian Tropical Research Institute, particularly Sebastian, for field and lab support. Collection permits were provided by Autoridad Nacional del Ambiente (ANAM) SE/A-108-13. This work was supported by National Science Foundation Award #1458158.
Literature Cited


Table 4.1. PERMANOVA of Bray-Curtis dissimilarities between healthy and WBD-associated differentially expressed bacterial genes – for all taxa, *Vibrio*, and Flavobacteriaceae genes – and bacterial communities.

**All Taxa**

<table>
<thead>
<tr>
<th>Source</th>
<th>Df</th>
<th>Sum Sq.</th>
<th>Mean Sq.</th>
<th>F-value</th>
<th>R^2</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disease State</td>
<td>1</td>
<td>0.3149</td>
<td>0.3149</td>
<td>0.8065</td>
<td>0.0734</td>
<td>0.901</td>
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<td>Genotype</td>
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<td>0.4623</td>
<td>0.4624</td>
<td>1.1840</td>
<td>0.1077</td>
<td>0.110</td>
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<tr>
<td>Residuals</td>
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<td>0.8189</td>
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</tr>
<tr>
<td>Total</td>
<td>11</td>
<td>4.2912</td>
<td>NA</td>
<td>NA</td>
<td>1</td>
<td>NA</td>
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**Vibrio**

<table>
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<th>R^2</th>
<th>p-value</th>
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<tr>
<td>Disease State</td>
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<td>0.4855</td>
<td>1.1036</td>
<td>0.0972</td>
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<td>0.5495</td>
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<td>Residuals</td>
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**Flavobacteriaceae**

<table>
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<th>Mean Sq.</th>
<th>F-value</th>
<th>R^2</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disease State</td>
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<td>0.4475</td>
<td>1.0422</td>
<td>0.0947</td>
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<td>0.4147</td>
<td>0.9660</td>
<td>0.0878</td>
<td>0.632</td>
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<td>Residuals</td>
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<td>0.8176</td>
<td>NA</td>
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<td>Total</td>
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</table>

**16S**

<table>
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<th>F-value</th>
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<td>0.001</td>
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<td>1</td>
<td>NA</td>
</tr>
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</table>

Table 4.2. Summary of metatranscriptome results.

<table>
<thead>
<tr>
<th></th>
<th>All Taxa</th>
<th>Vibrio</th>
<th>Flavobacteriaceae</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total Genes</strong></td>
<td>23,347</td>
<td>7,558</td>
<td>1,729</td>
</tr>
<tr>
<td><strong>Filtered Genes</strong></td>
<td>6,760</td>
<td>1,240</td>
<td>168</td>
</tr>
<tr>
<td><strong>DE Genes</strong></td>
<td>1,771</td>
<td>94</td>
<td>54</td>
</tr>
<tr>
<td>**LogFC &gt;</td>
<td>2</td>
<td>Genes**</td>
<td>444</td>
</tr>
<tr>
<td><strong>Healthy vs WBD Genes</strong></td>
<td>209</td>
<td>235</td>
<td>14</td>
</tr>
</tbody>
</table>

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Table 4.3. Differentially expressed *Vibrio* genes with a log2 fold change > |2|.

<table>
<thead>
<tr>
<th>Gene</th>
<th>GO: Molecular Function</th>
<th>GO: Biological Process</th>
<th>Upregulated</th>
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<tbody>
<tr>
<td>2-amino-3-ketobutyrate coenzyme A ligase</td>
<td>Acyltransferase</td>
<td>Biosynthetic process</td>
<td>Healthy</td>
</tr>
<tr>
<td>Adenosine deaminase</td>
<td>Hydrolase</td>
<td>Nucleotide metabolism</td>
<td>Healthy</td>
</tr>
<tr>
<td>Aldo/keto reductase</td>
<td>Oxioreductase</td>
<td>Unknown</td>
<td>WBD</td>
</tr>
<tr>
<td>Aspartate carbamoyltransferase regulatory subunit</td>
<td>Zinc ion binding</td>
<td>pyrimidine nucleotide biosynthesis</td>
<td>WBD</td>
</tr>
<tr>
<td>Aatalase/peroxidase HPI</td>
<td>Oxioreductase</td>
<td>Response to oxidative stress</td>
<td>Healthy</td>
</tr>
<tr>
<td>Citrate synthase type II</td>
<td>Transferase</td>
<td>TCA cycle</td>
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</tr>
<tr>
<td>Cytoplasmic dynein 1 heavy chain 1</td>
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<td>Unknown</td>
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<td>Dimethyladenosine transferase</td>
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<td>WBD</td>
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<td>DNA-binding protein HU-alpha</td>
<td>DNA binding</td>
<td>DNA condensation</td>
<td>WBD</td>
</tr>
<tr>
<td>DNA-directed RNA polymerase</td>
<td>DNA binding</td>
<td>Transcription</td>
<td>WBD</td>
</tr>
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<td>Fructose bisphosphatase II</td>
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<td>Glucosamine-fructose-6-phosphate aminotransferase</td>
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<td>Holliday junction DNA helicase RuvB</td>
<td>DNA binding</td>
<td>DNA repair; SOS response</td>
<td>WBD</td>
</tr>
<tr>
<td>L-proline glycerine betaine ABC transport ATP-binding protein proV</td>
<td>ATPase activity</td>
<td>Glycine betaine transport</td>
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<tr>
<td>L-threonine 3-dehydrogenase</td>
<td>Oxioreductase</td>
<td>Threonine catabolic process</td>
<td>Healthy</td>
</tr>
<tr>
<td>Peptide ABC transporter; periplasmic peptide-binding protein</td>
<td>Peptide transporter activity</td>
<td>Transmembrane transport</td>
<td>Healthy</td>
</tr>
<tr>
<td>Putrescine transporter: periplasmic-binding component of ABC superfamily</td>
<td>Hydrolyase</td>
<td>Transport</td>
<td>Healthy</td>
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<tr>
<td>RecA protein</td>
<td>DNA binding</td>
<td>DNA repair; SOS response</td>
<td>WBD</td>
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<td>Ribonucleotide reductase of class Ia (aerobic) alpha subunit</td>
<td>Oxioreductase</td>
<td>DNA replication</td>
<td>Healthy</td>
</tr>
<tr>
<td>Serine/threonine transporter SstT</td>
<td>Amino acid:sodium symporter activity</td>
<td>Amino acid transport</td>
<td>WBD</td>
</tr>
<tr>
<td>SSU ribosomal protein S1p</td>
<td>RNA binding</td>
<td>Translation</td>
<td>WBD</td>
</tr>
<tr>
<td>Succinate dehydrogenase</td>
<td>Electron carrier activity</td>
<td>TCA cycle</td>
<td>WBD</td>
</tr>
<tr>
<td>Succinate-semialdehyde dehydrogenase NADP+</td>
<td>Oxioreductase</td>
<td>Aminobutyric acid catabolic process</td>
<td>Healthy</td>
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<tr>
<td>Thiamine biosynthesis protein Thil</td>
<td>tRNA adenyllytransferase activity</td>
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<td>WBD</td>
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<td>Thymidine phosphorylase</td>
<td>Glycosyltransferase</td>
<td>DNA damage stimulus</td>
<td>WBD</td>
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<tr>
<td>Trans-2-enoyl-CoA reductase</td>
<td>Oxioreductase</td>
<td>Fatty acid biosynthesis</td>
<td>Healthy</td>
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<tr>
<td>tRNA-specific 2-thiouridylylase MnM</td>
<td>RNA binding</td>
<td>tRNA processing</td>
<td>WBD</td>
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<tr>
<td>Valyl-tRNA synthetase</td>
<td>Aminoacyl-tRNA editing activity</td>
<td>Protein biosynthesis</td>
<td>Healthy</td>
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Table 4.4. Differentially expressed Flavobacteriaceae genes with a log2 fold change > 2.

<table>
<thead>
<tr>
<th>Flavobacteriaceae</th>
<th>GO: Molecular Function</th>
<th>GO: Biological Process</th>
<th>Upregulated</th>
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<tbody>
<tr>
<td>Acyl-CoA dehydrogenase</td>
<td>Oxioreductase</td>
<td>Lipid metabolism</td>
<td>WBD</td>
</tr>
<tr>
<td>Glu/Leu/Phe/Val dehydrogenase</td>
<td>Oxioreductase</td>
<td>Amino acid metabolism</td>
<td>WBD</td>
</tr>
<tr>
<td>Hydrophobe/amphipile efflux-1 (HAE1) family protein</td>
<td>Transport</td>
<td>Transmembrane transport</td>
<td>WBD</td>
</tr>
<tr>
<td>Molecular chaperone DnaK</td>
<td>Chaperone</td>
<td>Stress response</td>
<td>Healthy</td>
</tr>
<tr>
<td>Outer membrane efflux protein</td>
<td>Transport</td>
<td>Antibiotic resistance</td>
<td>WBD</td>
</tr>
<tr>
<td>Phosphomannomutase</td>
<td>Isomerase</td>
<td>Capsule biogenesis/ degradation</td>
<td>WBD</td>
</tr>
<tr>
<td>Succinyl-CoA synthetase subunit beta</td>
<td>Ligase</td>
<td>TCA cycle</td>
<td>Healthy</td>
</tr>
</tbody>
</table>
Figure 4.1. Principal coordinate analysis (PCoA) plots of dissimilarities between coral-associated bacterial communities derived from 16S analyses. Black symbols represent healthy-associated genes and red symbols represent WBD-associated genes. Shapes represent coral genotype (colony number).
**Figure 4.2.** Relative abundance of coral-associated bacterial communities sorted taxonomically by family for the 12 microbiome samples. The top six bars represent healthy-associated communities and the bottom six bars represent WBD-associated communities. The percent abundance of families whose incidence reached five percent of the total OTU count in at least one sample are shown. Rare families are grouped as “Other”.

![Bar chart showing relative abundance of coral-associated bacterial communities sorted taxonomically by family for the 12 microbiome samples. The top six bars represent healthy-associated communities and the bottom six bars represent WBD-associated communities. The percent abundance of families whose incidence reached five percent of the total OTU count in at least one sample are shown. Rare families are grouped as “Other”](image-url)
Figure 4.3. Healthy versus WBD-associated OTU counts by coral genotype for (A) *Vibrio* and (B) Flavobacteriaceae.
Figure 4.4. Heatmaps of differentially expressed genes. Yellow bars represent healthy-associated microbiomes and green bars represent WBD-associated microbiomes. The log2 of gene counts are represented. (A) The first panel displays the top 100 differentially expressed genes with the greatest variance between healthy and WBD microbiomes across all taxa. (B) The second panel displays the 94 differentially expressed genes associated with *Vibrio*. (C) The third panel displays the 54 differentially expressed genes associated with Flavobacteriaceae.
**Figure 4.5.** Volcano plots of the filtered genes from (A) all taxa (B) *Vibrio* and (C) Flavobacteriaceae. All points represent genes that occurred in three or more samples ten or more times. Blue points represent genes that are differentially expressed. Red points represent genes that are differentially expressed and have a log2 fold change > 2.
**Figure 4.6.** Principal coordinate analysis (PCoA) plots of dissimilarities between bacterial genes from (A) all taxa (B) *Vibrio* and (C) Flavobacteriaceae. Black symbols represent healthy-associated genes and red symbols represent WBD-associated genes. Shapes represent coral genotype (colony number).
Figure 4.7. Healthy and WBD-associated genes organized by GO term for molecular function for the (A) 29 Vibrio genes and (B) the seven Flavobacteriaceae genes with a log2 fold change > 2.
Figure 4.8. Healthy and WBD-associated genes organized by GO term for biological process for the (A) 29 *Vibrio* genes and the (B) seven Flavobacteriaceae genes with a log2 fold change $> |2|$. 


## Supplementary Information

**Table 4.S1.** Taxonomy of the top 100 differentially expressed genes with the greatest variance.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Taxonomy</th>
<th>Upregulated</th>
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</thead>
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<tr>
<td>1-deoxy-D-xylulose 5-phosphate reductoisomerase</td>
<td>Unknown</td>
<td>Healthy</td>
</tr>
<tr>
<td>1-deoxy-D-xylulose 5-phosphate synthase</td>
<td>Alteromonas macleodii</td>
<td>Healthy</td>
</tr>
<tr>
<td>1-deoxyxyulose-5-phosphate synthase</td>
<td>Vibrio harveyi</td>
<td>Healthy</td>
</tr>
<tr>
<td>3-deoxy-manno-octulosonate cytidylyltransferase</td>
<td>Unknown</td>
<td>WBD</td>
</tr>
<tr>
<td>3-hydroxyacyl-CoA dehydrogenase NAD-binding</td>
<td>Xanthobacter autotrophicus</td>
<td>WBD</td>
</tr>
<tr>
<td>4-alpha-glucanotransferase</td>
<td>Vibrio coraliilyticus</td>
<td>Healthy</td>
</tr>
<tr>
<td>5-methyltetrahydrofolate--homocysteine methyltransferase</td>
<td>Vibrio cholerae</td>
<td>Healthy</td>
</tr>
<tr>
<td>5-nucleotidase</td>
<td>Vibrio orientalis</td>
<td>Healthy</td>
</tr>
<tr>
<td>ABC transporter ATP binding protein</td>
<td>Vibrio tasmaniensis</td>
<td>Healthy</td>
</tr>
<tr>
<td>acetyl-CoA carboxylase beta subunit</td>
<td>Bermanella marisubri</td>
<td>Healthy</td>
</tr>
<tr>
<td>Alanine dehydrogenase</td>
<td>Blastopirellula marina</td>
<td>WBD</td>
</tr>
<tr>
<td>amino acid/peptide transporter</td>
<td>Hyphomicrobium denitrificans</td>
<td>WBD</td>
</tr>
<tr>
<td>arginine ABC transporter permease protein ArtM</td>
<td>Ferrimonas balearica</td>
<td>Healthy</td>
</tr>
<tr>
<td>ATP-dependent Clp protease adaptor protein ClpS</td>
<td>Marinobacter algicola</td>
<td>WBD</td>
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<tr>
<td>ATP-dependent helicase HrpA</td>
<td>Vibrio</td>
<td>Healthy</td>
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<tr>
<td>bifunctional N-acetylglucosamine-1-phosphate uridylyltransferase/glucosamine-1-phosphate acetyltransferase</td>
<td>Vibrio parahaemolyticus</td>
<td>Healthy</td>
</tr>
<tr>
<td>biotin synthase</td>
<td>Propionibacteriaceae</td>
<td>Healthy</td>
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<td>casein kinase 1 epsilon</td>
<td>Danio rerio</td>
<td>WBD</td>
</tr>
<tr>
<td>catalase/peroxidase HPI</td>
<td>marine gamma proteobacterium</td>
<td>Healthy</td>
</tr>
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<td>cell division protein FtsY</td>
<td>Vibrio sp. 16</td>
<td>WBD</td>
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<td>chaperone DnaJ</td>
<td>Gramella forsetii</td>
<td>WBD</td>
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<td>chorismate mutase/prephenate dehydratase</td>
<td>Colwellia psychrerythraea</td>
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<td>Cytidylate kinase</td>
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<td>cytochrome c-type biogenesis protein CcmF</td>
<td>Flavobacteriales bacterium ALC-1</td>
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<td>cytochrome c-type protein TorY</td>
<td>Vibrio harveyi</td>
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<td>DNA ligase</td>
<td>Marinobacter adhaerens</td>
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<tr>
<td>Elongation FacTor family member (eft-2)</td>
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<td>ferredoxi</td>
<td>Thioalkalivibrio sp.</td>
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<td>Tribolium castaneum</td>
<td>WBD</td>
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<td>formyltetrahydrofolate deformylase</td>
<td>Pseudomonas entomophila</td>
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<tr>
<td>fumarate reductase flavoprotein subunit</td>
<td>Vibrio campbellii</td>
<td>Healthy</td>
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<td>galactose/methyl galactoside transporter ATP-binding protein</td>
<td>Escherichia coli</td>
<td>Healthy</td>
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<td>Glutamate synthase (ferredoxin)</td>
<td>Nocardiopsis dassonvillé</td>
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<td>Oligotropha</td>
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<td>State</td>
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<td>Dethiosulfovibrio peptidovorans</td>
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<td>L-threonine 3-dehydrogenase</td>
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<td>multifunctional tRNA nucleotidyl transferase/23-cyclic phosphodiesterase/2nucleotidase/phosphatase</td>
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<td>myosin-10</td>
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<td>Magnetospirillum magneticum</td>
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<td>Flavobacteria bacterium MS024-2A</td>
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<td>Microscilla marina</td>
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<td><strong>Vibrio coralliilyticus</strong></td>
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<td><strong>Vibrio</strong></td>
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<td><strong>Pseudoalteromonas tunicata</strong></td>
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<td><strong>signal peptide prediction</strong></td>
<td><strong>Vibrio vulnificus</strong></td>
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<td><strong>signal transduction protein</strong></td>
<td><strong>Haella chejuensis</strong></td>
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<td><strong>similar to high density lipoprotein-binding protein vigilin</strong></td>
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<td><strong>WBD</strong></td>
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<td><strong>sodium ion-translocating decarboxylases</strong></td>
<td><strong>Chlorobium phaeobacteroides</strong></td>
<td><strong>Healthy</strong></td>
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<td><strong>Spermidine/putrescine-binding periplasmic protein</strong></td>
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<td><strong>SRY (sex determining region Y)-box 9</strong></td>
<td><strong>Saccharophagus degradans 2-40</strong></td>
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<td><strong>Vibrio fischeri</strong></td>
<td><strong>Healthy</strong></td>
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<td><strong>Coleofasciculus chthonoplastes</strong></td>
<td><strong>WBD</strong></td>
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<td><strong>TRAP-typemannitol/chloroaromatic compound transporter large permease</strong></td>
<td><strong>Haella chejuensis</strong></td>
<td><strong>Healthy</strong></td>
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<td><strong>tRNA delta(2)-isopentenylpyrophosphate transferase</strong></td>
<td><strong>Vibrio orientalis</strong></td>
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<td><strong>tRNA modification GTPase TrmE</strong></td>
<td><strong>Photorhabdus luminescens</strong></td>
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<td><strong>tubuli</strong></td>
<td><strong>Alteromonadales</strong></td>
<td><strong>WBD</strong></td>
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<td><strong>type I restriction-modification system DNA-methyltransferase subunit M</strong></td>
<td><strong>Unknown</strong></td>
<td><strong>Healthy</strong></td>
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<td><strong>UDP-glucose pyrophosphorylase</strong></td>
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<td><strong>Healthy</strong></td>
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<td><strong>Homo sapiens</strong></td>
<td><strong>Healthy</strong></td>
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<td><strong>Desulfovibrio alaskensis</strong></td>
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<td><strong>YaeC family lipoprotein</strong></td>
<td><strong>Paracoccus denitrificans</strong></td>
<td><strong>WBD</strong></td>
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<td><strong>zinc finger CCCH type containing 12D</strong></td>
<td><strong>Alcanivorax borkumensis</strong></td>
<td><strong>WBD</strong></td>
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</table>
Dissertation Conclusions

Research summary
This dissertation provides insight into the microbial ecology and genomics of white band disease (WBD) in *Acropora cervicornis*. Our findings include a description of the population structure and gene expression profiles of coral-associated bacterial communities from healthy and WBD-infected corals. Much of this work is viewed through the lens of quorum sensing (QS) as a mechanism for bacterial virulence and pathogenicity.

Throughout this dissertation we focus on bacteria that are associated with WBD infection, namely those taxa that are more abundant on diseased corals. In each of the three chapters presented in this dissertation, we examine the relationship between two taxa of putative coral pathogens – *Vibrios* and Flavobacteria – and WBD (Garcia et al. 2013, Gignoux-Wolfsohn and Vollmer 2015, Gil-Agudelo et al. 2006, Harvell et al. 2007, Luna et al. 2010, Thurber et al. 2009). Using culture-based methods in chapter 2 we determined that both of these groups are more abundant on diseased corals (Certner and Vollmer 2015). In chapter 3 we use 16S rRNA sequencing to reaffirm the connection between WBD and these taxa. Specifically, we show that the families Vibrionaceae and Flavobacteriaceae are dominant components of the disease microbiome but exist in far lower abundances on healthy corals. Finally, in chapter 4 we use metatranscriptomic analyses to examine the gene expression of healthy versus WBD-associated microbial communities with a special focus on *Vibrios* and Flavobacteriaceae.
This dissertation also explores the role of QS in the transmission and progression of WBD. In chapter 2, we show adding the AI-1 autoinducer N-hexanoyl-DL-homoserine lactone to healthy *A. cervicornis* microbiomes results in a disease vector capable of transmitting WBD to asymptomatic corals (Certner and Vollmer 2015). This finding demonstrates that QS-controlled virulence pathways contribute to the opportunistic pathogenicity of resident coral microbes and thus WBD infection. This study also hypothesizes that the putative coral pathogens *Vibrio* and Flavobacteria may be influenced by QS. We know from culture-based methods that both of these taxa are more abundant on diseased corals. As it is well known that *Vibrios* rely on QS to control a variety of behaviors including virulence (Hammer and Bassler 2003, Henke and Bassler 2004), it is likely that resident coral-associated *Vibrios* were stimulated by the addition of autoinducer. QS in Flavobacteria is less studied but certain pathogens from this class produce autoinducers similar to the acyl homoserine lactone used in this chapter (Dobretsov et al. 2007, Romero et al. 2010).

Chapter 3 aims to provide greater evidence that QS exacerbates WBD transmission through culture-independent methods. This chapter also looks to establish a relationship between the aforementioned candidate pathogens and QS. Unlike chapter 2, this study attempts to inhibit rather than induce QS pathways. An acyl homoserine lactone antagonist, or QS inhibitor (QSI) was added to WBD-associated microbiomes. These QSI-supplemented diseased microbiomes lost the ability to transmit disease to healthy corals in aquaria. 16S rRNA metagenomic sequencing of these corals (as well as control infected corals) looks at the population structure of bacterial species associated with healthy, QSI-supplemented, and diseased corals. The QSI-
supplemented rescued corals had a microbial community structure very similar to those found on untreated healthy *A. cervicornis*. This result indicates that QSI has the ability to inhibit the pathogenicity of WBD microbiomes. Vibrionaceae and Flavobacteriaceae abundances on these QSI-supplemented corals were very low, similar to their numbers on untreated corals. Conversely, WBD-infected corals contained significantly elevated levels of Vibrionaceae and Flavobacteriaceae. This result provides strong evidence that these taxa contribute to WBD infection; inhibition of these taxa by QSI prevented their proliferation and thus pathogenicity. Corals rescued by the addition of QSI contained high abundances of the putative coral symbiont *Endozoicomonas* (Bayer et al. 2013, Yang et al. 2010) as well as Halomonadaceae indicating that QSI addition helps to maintain the populations of potentially beneficial taxa. These groups were also found in high abundances on untreated healthy corals but were virtually absent on WBD-infected corals. This finding adds to the emerging hypothesis that certain bacterial taxa act as general coral symbionts.

Chapter 4 moves beyond population structure to focus on the gene expression of WBD-causing bacteria. This study isolated the microbial communities from paired healthy and WBD-infected *A. cervicornis* and conducted RNA sequencing to establish bacterial behavior from each environment. 16S rRNA metagenomic sequencing was also applied to the same microbiomes so as to compare bacterial population versus gene expression. Based on the number of differentially expressed genes from the full dataset, we determined that relatively few genes contribute to WBD infection. The top 100 differentially expressed genes with the greatest variance from this dataset contained a large number of *Vibrio* genes as well as several Flavobacteria genes. Additionally, the 16S data shows that both groups make up a notable portion of the WBD-
associated microbiomes. As a result, we decided to once again focus on our putative WBD pathogens in order to establish gene expression patterns between healthy versus disease microbiomes. WBD-associated *Vibrio* genes were characterized by their involvement in the bacterial SOS response and antibiotic resistance. The SOS response is responsible for DNA repair (Singer 1989), during which spontaneous mutagenesis often occurs (Rapa et al. 2014) leading to increased bacterial pathogenicity. WBD-associated Flavobacteriaceae were characterized by their involvement in lipid metabolism and antibiotic resistance. Capsule and lipopolysaccharide biosynthesis genes were upregulated in disease-associated Flavobacteriaceae, two mechanisms linked to virulence in gram-negative bacteria (Kusecek et al. 1984, Moxon and Kroll 1990) including known Flavobacteria pathogens (Dumpala et al. 2010, Zhang et al. 2006). An outer membrane efflux pump was also associated with diseased Flavobacteriaceae, a transmembrane pathway generally utilized for the secretion of toxins and other small molecules (Zgurskaya et al. 2011).

**Future directions and conservation implications**

This dissertation brings us closer to understanding the bacterial taxa and virulence pathways behind WBD-infection in *A. cervicornis*. However, these studies also inspire a number of additional experiments, especially with regards to QS. Future work should attempt to isolate bacterial autoinducers from WBD-associated microbiomes in order to determine which QS-controlled virulence pathways are being activated during the infection process. In chapter 2, we employ one acyl homoserine lactone widely utilized by several gram-negative species (Churchill and Chen 2010). However, there exist many different classes of autoinducers including oligopeptides used by gram-positive species and AI-2, a furanosyl borate diester thought to be a
universal signal used for interspecies communication (Miller and Bassler 2001). Along with 16S data, an overview of disease-associated autoinducers will paint a more accurate picture of disease-causing bacteria and their virulence mechanisms.

Future metatranscriptomic work should also incorporate coral genotype as an effect along with disease state. In chapter 4 we saw a significant effect of coral colony on its bacterial residents in the 16S data. Rather than a paired healthy and diseased sampling strategy, future experiments should incorporate multiple replicates of each disease state from a single coral colony. By better controlling for inter-colony variation, genes actually associated with WBD will be become clearer. Metatranscriptomic studies should also incorporate time series during the sampling process. In our experiment we compared microbiomes from healthy corals and late-stage infection corals. In chapter 3, we see a progression of disease-associated taxa as the WBD infection matures. It is probably that the genes involved in initial infection are different than the genes associated with late-stage WBD. A sampling strategy that includes a time series would allow for the separation of bacterial genes involved with opportunistic infection and bacterial genes involved in disease progression or secondary infection.

This dissertation uncovers several opportunities for conservation of A. cervicornis. Through our work we provide strong evidence for the importance of QS in WBD infection. We show that inhibiting QS-controlled pathways prevents WBD transmission and we also identify candidate genes involved in the infection process. Large-scale use of QSI may benefit WBD-impacted reefs if it can be applied in a targeted manner to disease lesions. QSI may also be used in nurseries or coral aquaculture facilities, where the environment is more easily controlled, to
protect vulnerable coral propagules and outplants while they mature in high-density aquaria. Identification of WBD-associated genes also creates a potential for the use of targeted antibiotics. Our metatranscriptomic analysis revealed a number of *Vibrio* and Flavobacteriaceae genes upregulated in disease microbiomes. Blocking the virulence pathways initiated by these genes may render harmless two powerful disease-associated taxa, thus preventing WBD infection.
Literature Cited


