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Quorum Sensing Signals Produced by Heterotrophic Bacteria in Black Band Disease (BBD) of Corals and Their Potential Role in BBD Pathogenesis

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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

QUORUM SENSING SIGNALS PRODUCED BY HETEROTROPHIC BACTERIA IN
BLACK BAND DISEASE (BBD) OF CORALS AND THEIR POTENTIAL ROLE IN
BBD PATHOGENESIS

A dissertation submitted in partial fulfillment of

the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

BIOLOGY

by

Chinmayee D. Bhedi

2017

To: Dean Michael R. Heithaus
College of Arts, Sciences and Education

This dissertation, written by Chinmayee D. Bhedi, and entitled Quorum Sensing Signals Produced by Heterotrophic Bacteria in Black Band Disease (BBD) of Corals and Their Potential Role in BBD Pathogenesis, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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Date of Defense: June 30, 2017

The dissertation of Chinmayee D. Bhedi is approved.

Dean Michael R. Heithaus
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Andrés G. Gil
Vice President for Research and Economic Development
and Dean of the University Graduate School

Florida International University, 2017

DEDICATION

This dissertation is dedicated to my parents, Aai and Baba, and my husband Mayuresh.

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ABSTRACT OF THE DISSERTATION
QUORUM SENSING SIGNALS PRODUCED BY HETEROTROPHIC BACTERIA IN
BLACK BAND DISEASE (BBD) OF CORALS AND THEIR POTENTIAL ROLE IN
BBD PATHOGENESIS

by

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Florida International University, 2017

Miami, Florida

Professor Laurie L. Richardson, Major Professor

Black band disease (BBD) of corals is a temperature dependent, highly virulent, polymicrobial disease affecting reef-building corals globally. The microbial consortium of BBD is primarily comprised of functional physiological groups that include photosynthetic cyanobacteria, sulfate reducers, sulfide oxidizers and a vast repertoire of heterotrophic bacteria. Quorum sensing (QS), the cell-density dependent communication phenomenon in bacteria, is known to induce expression of genes for a variety of virulence factors in diseases worldwide. Microbes capable of QS release signals such as acyl homoserine lactones (AHLs) and autoinducer-2 (AI-2), which coordinate microbial interaction. The focus of the present study was to investigate the presence and potential role of QS in BBD pathogenicity, utilizing culture dependent and independent methodologies. Isolates across coral health states including BBD, were screened for production of QS signals, and AHL and AI-2 production capabilities were analyzed via LC-MS/MS. The effect of temperature on AHLs was also examined. Additionally, antimicrobial production capabilities of isolates were tested. BBD metagenomes were

utilized to screen for sequences related to QS, antimicrobial synthesis, and antimicrobial resistance genes. BBD isolates represented a significantly higher proportion of isolates capable of producing QS signals in comparison to healthy coral isolates. Several AHLs produced by coral derived bacterial cultures were identified, and three AHLs, specifically 3OHC4, 3OHC5 and 3OHC6, showed a significant increase in production at an elevated temperature of 30 °C, which correlates with increased BBD incidence on reefs with increasing water temperature. Most of the BBD cultured isolates were identified as vibrios. Several sequences related to QS, antimicrobial synthesis and resistance genes were detected in the BBD metagenomes. Based on the findings of this study, a model for potential microbial interactions amongst BBD heterotrophs, centered around QS, is proposed. Taken together, the findings from this study provide a clearer understanding of the potential role of QS in BBD, and serve as the basis for further studies aimed at elucidating the pathogenesis of an intricate coral disease.

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CHAPTER 1

INTRODUCTION

The worldwide deterioration of coral reef ecosystems has been attributed to several factors such as global warming, ocean acidification, over-fishing, and pollution (Sutherland et al. 2004; Hoegh-Guldberg et al. 2007; Carpenter et al. 2008; Hernandez-Agreda et al. 2017). Over the past few decades, it is being increasingly recognized that coral diseases are aggravating the decline of coral diversity and coral cover (Ainsworth et al. 2010; De'ath et al. 2012). Corals are known to exist in symbiotic associations with bacteria, viruses, algae, protozoa, and dinoflagellates, often labeled collectively as the coral holobiont (Rowan 1998; Rohwer et al. 2002). As proposed in the coral probiotic hypothesis, the dynamic nature of the symbiotic associates of the coral animal facilitates efficient adaptation of the coral holobiont to environmental changes (Reshef et al. 2006). The surface mucopolysaccharide layer (SML) of corals is a prime source of nutrients and organic carbon to resident microbes (Ritchie and Smith 2004). Several reports have studied the diversity of bacteria inhabiting healthy corals, indicating evident shifts in microbial inhabitants after environmental stress (Ritchie 1995; Rohwer et al. 2001; Cróquer et al. 2013).

Black band disease (BBD) was the earliest documented coral disease, in the early 1970s, and is now recognized as one of the most virulent infections affecting gorgonian and scleractinian species of corals (Antonius 1973; Garrett and Ducklow 1975; Antonius 1981; Rützler et al. 1983; Richardson 2004). Since its discovery in the Caribbean, it has subsequently been reported on reefs of the Red Sea, Indo-Pacific, Great Barrier Reef (GBR), Philippines, Hawai'i, Indonesia, and Indian Ocean and is currently identified as a globally distributed disease (Edmunds 1991; Page and Willis 2006; Kaczmarzsky 2006; Barneah et al. 2007; Aeby et al. 2015; Johan et al. 2015; Sere et al. 2016). The disease

manifests as a dark, migrating mat on the surface of a coral colony, covering a distance of 1 mm to up to 3 cm per day (Rützler et al. 1983; Kuta and Richardson 1997). Tissue lysis by this biofilm-like mat leads to coral death, exposing the coral skeleton, which is then colonized by algae. BBD has been widely accepted to be of polymicrobial nature, with no single pathogen involved (Carlton and Richardson 1995; Richardson 2004). The highly variable microbial consortium of BBD is largely comprised of phycoerythrin-rich cyanobacteria, sulfide oxidizers, sulfate reducers, fungi, archae, and a vast population of organoheterotrophs (Garrett and Ducklow 1975; Ducklow and Mitchell 1979; Ramos-Flores 1983; Rützler et al. 1983; Sato et al. 2013). The volumetric mass of the band is dominated by photosynthesizing cyanobacteria that include *Leptolyngbya*, *Geitlerinema* and *Roseofilum reptotaenium* (Myers et al. 2007; Casamatta et al. 2012), with the latter found in BBD on reefs worldwide. Vertical gradients of sulfide, light and oxygen/anoxia are present in the band (Kuta and Richardson 1996; Page and Willis 2006). Within the band itself, sulfate reducers produce sulfide while photosynthesis by cyanobacteria releases oxygen. These reactions lead to the formation of a sulfide-rich anoxic base and an oxygenated mat surface, along with a sulfide/oxygen interface that migrates vertically (Carlton and Richardson 1995; Richardson et al. 2001).

Numerous investigators have studied the variable BBD heterotrophic community; yet, this is the least understood of the physiological groups of BBD in relation to pathogenicity (Cooney et al. 2002; Frias-Lopez et al. 2002; Frias-Lopez et al. 2004; Sekar et al. 2006; Sekar et al. 2008; Richardson et al. 2016). Black band disease clone libraries have revealed the presence of alphaproteobacteria, verrucomicrobia, deltaproteobacteria, bacteroidetes and firmicutes as dominant BBD heterotrophs (Sekar et al. 2006). Cooney

et al. (2002) have reported the additional occurrence of epsilonproteobacteria, *Bacillus/Clostridium* (BC) and *Cytophaga-Flexibacter-Bacteroides* (CFB) group bacteria within BBD. A meta-analysis of 87 clone libraries of BBD spanning 16 species of corals found alphaproteobacteria to be the most varied group, with a sequence matching *Roseovarius crassostreae*, the second most repeated sequence amongst BBD heterotrophs (Miller and Richardson 2011). Clone libraries have also reported the presence of *Ferrimonas* sp. in the BBD consortium (Sekar et al. 2006; Sekar et al. 2008). Despite studies indicating the presence of specific bacteria in BBD, the precise interaction of these microbes with each other and role in disease initiation and progression has yet to be elucidated.

As with most coral diseases, BBD incidence on reefs is influenced by a variety of ecological factors such as temperature, light intensity, the presence of nutrients, water depth, and anthropogenic factors (Kuta and Richardson 1996; Page and Willis 2006; Voss and Richardson 2006b). Many studies have reported a positive correlation between increased temperature and BBD abundance as well as severity (Antonius 1981; Kuta and Richardson 1996; Voss and Richardson 2006a). Under the premise of global warming resulting from climate change, increased disease prevalence and intensity with high temperatures is of specific concern to coral populations susceptible to BBD (Sato et al. 2015). Warm temperatures have been proposed to decrease host immunity while concurrently increasing bacterial virulence (Harvell et al. 2002; Harvell et al. 2007). Such a change in microbial pathogenicity may be triggered by cell signaling phenomena such as quorum sensing.

Quorum sensing is a cell density mediated microbial communication facilitated by signaling molecules (Nealson et al. 1970; Nealson and Hastings 1979; Bassler 1999). When a precise 'quorum' of bacterial cells is achieved, bacteria regulate gene expression of a variety of characters such as biofilm formation, conjugation, and competence capabilities, increased and specialized nutrient uptake strategies, and antimicrobial production and resistance abilities (Bassler et al. 1994; Taga and Bassler 2003; Waters and Bassler 2005b; Jayaraman and Wood 2008; Bandara et al. 2012; Rutherford and Bassler 2012). One or more of the above-described characteristics often enable the conversion of a non-pathogenic bacterial strain to a pathogenic one (Jayaraman and Wood 2008). Quorum sensing in most Gram-negative bacteria is facilitated by secretion of small molecular weight signaling compounds characterized as acyl homoserine lactones (AHLs) (Fuqua et al. 2001). These signaling compounds are comprised of a fatty acid side chain attached to a homoserine (lactone) ring. By permutations and combinations of side chain groups and saturation, bacteria are capable of manufacturing up to hundreds of AHL variants (Fuqua et al. 2001; Hmelo 2017). In polymicrobial assemblages, the precise variants of signaling molecules generate bacterial responses for inducing gene expression of specific pathogenic characters. Interspecies communication in bacteria is achieved by another set of signaling molecules called autoinducer 2 (AI-2), produced by both Gram-positive as well as Gram-negative bacteria (Greenberg et al. 1979; Bassler et al. 1994). Due to the ubiquitous signaling nature of this compound, it is often designated as the universal signaling molecule (Jayaraman and Wood 2008). The chemical nature of QS signaling molecules makes them vulnerable to ecological factors such as pH, oxygen, and temperature (Decho et al. 2009; Decho et al. 2010; Decho et al.

2011). Specific environmental variations, such as alkaline milieus, often render signaling molecules like AHLs, incapable of functioning (Decho et al. 2009).

Recent advances in reporting microbial interactions within the coral holobiont have indicated the synthesis of AI-2 as well as AHLs by microbes (Tait et al. 2010; Alagely et al. 2011; Golberg et al. 2011; Hunt et al. 2012; Ransome 2013). Proteobacterial populations, including vibrios, are well-known producers of AHLs in the marine environment (Visick and Fuqua 2005; Hmelo 2017). Along with QS, antimicrobial production as a microbial interaction phenomenon has also been studied in microbes of healthy as well as diseased corals (Mao-Jones et al. 2010; Hunt et al. 2012; Kvennefors et al. 2012; Glasl et al. 2016). Both QS and antimicrobial interactions are estimated to influence the structuring and organization of microbes inhabiting the coral SML. In polymicrobial diseases such as BBD, the impact of microbial exchanges on configuration and operation of the disease community can be of unique significance. Accordingly, the present study examines the interactions amongst BBD heterotrophic bacteria primarily through the lens of quorum sensing and signaling molecules. The present dissertation includes both laboratory (microbiological and analytical) and metagenomic data to obtain a strong understanding of microbial collaborations influencing BBD pathogenicity.

Chapter two begins by inspecting the AHL and AI-2 production capabilities of microbes across healthy and BB-diseased coral health states. Isolates of bacteria positive for both categories of signaling molecules are screened and subjected to identification (of AHLs) and quantification (of AI-2) via LC-MS/MS. Part of the data presented in this chapter have been published in Zimmer et al. 2014.

Chapter three has been published in FEMS Microbiology Ecology (Bhedi et al., 2017) and investigates the role of temperature on AHL production by BBD microbes. AHL production is compared across health states of corals infected with BBD. AHLs most significantly varied with temperature are identified via LC-MS/MS and all AHL producers are revealed to be vibrios. This chapter also examines the QS sequences annotated in BBD metagenomes.

Chapter four reports the AHL and antimicrobial production capabilities of microbes across coral health states of BBD. This chapter also compares BBD and other publicly available non-diseased coral metagenomes to evaluate QS and antimicrobial synthesis as well as antimicrobial resistance sequences in BBD metagenomes. The proteobacterial population across metagenomes is also evaluated in this chapter.

Chapter five summarizes the implications of the findings from this dissertation and proposes a model of microbial interaction including establishment and maintenance of the virulent BBD heterotrophic community, centered primarily around quorum sensing.

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CHAPTER 2

INVESTIGATING THE PRODUCTION OF ACYL HOMOSERINE LACTONE (AHL) AND AUTOINDUCER-2 (AI-2) MOLECULES BY BLACK BAND DISEASE HETEROTROPHS

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Abstract

Black band disease (BBD) is a destructive disease of corals manifested as a dark band migrating over the coral colony, lysing coral tissue, and leaving behind bare coral skeleton. The polymicrobial mat is composed of cyanobacteria, sulfate reducers, sulfide oxidizers, and a wide variety of heterotrophic bacteria. Several studies have pointed towards the potential for the heterotrophs to be significant contributors to the pathogenicity of BBD. In the present study, heterotrophic bacteria from black band disease mat, the surface mucopolysaccharide layer (SML) of healthy coral and healthy part of the BBD infected coral were isolated and tested for their potential to produce quorum sensing signals (QSSs). Specifically, isolates were analyzed via LC-MS/MS for production of acyl homoserine lactones and (*S*)-4-5-dihydroxy-2,3-pentandione (DPD), the chemical precursor for autoinducer-2 (AI-2). Twelve bacterial isolates across coral health states showed production of AHLs, while nine produced DPD. The detection of these compounds indicated the active production of QSSs by heterotrophs in black band disease of corals. The potential influence of these QSSs in the organization and pathogenicity of the BBD community requires further investigation.

Introduction

Over the last three decades, global coral reef deterioration has been caused by several factors including global warming, ocean acidification, anthropogenic factors, water quality deterioration, over-fishing (Hoegh-Guldberg et al. 2007; Harborne et al. 2017), and the foremost contributor to this decline has been coral disease. Black band disease is one such devastating coral disease, plaguing reef-building corals globally. The disease mat is a consortium of several operative groups of microbes that move across the coral

colony, lysing the coral tissue at a rate of 3 mm per day (Rützler et al. 1983). The collection of functional players of the black band mat includes photoautotrophic cyanobacteria (Rützler et al. 1983), primarily, *Roseofilum reptotaenium* (Casamatta et al. 2012), sulfate reducers such as *Desulfovibrio* sp. (Antonius 1981; Richardson 1996; Viehman et al. 2006), sulfide oxidizers such as *Beggiatoa* sp. (Ducklow and Mitchell 1979), marine fungi (Ramos-Flores 1983), archae (Sato et al. 2013), and a diverse assembly of heterotrophic bacteria (Ducklow and Mitchell 1979; Cooney et al. 2002; Frias-Lopez et al. 2004; Sekar et al. 2008). Black band disease heterotrophic bacteria have been proposed to include potential primary pathogens (Cooney et al. 2002; Frias-Lopez et al. 2002; Sekar et al. 2006). A meta-analysis of 16 scleractinian coral species across 87 BBD samples from the literature revealed the alpha- and gammaproteobacteria to be the most diversely represented groups amongst the BBD heterotrophic population (Miller and Richardson 2011). Bacteria in such large and diverse assemblages have been known to interact with one another in an intricate manner, to not only coexist but also potentially communicate with one another to exploit the environment for self-perpetuation. One such phenomenon of bacterial interaction is quorum sensing.

The first reported indication of a cell density dependent ‘activator substance’ causing competence of bacterial cells was described in the early 1960s (Tomasz and Hotchkiss 1964). Autoinduction in *Vibrio fischeri* was described as the cause of bioluminescence in the 1970s (Nealson et al. 1970; Nealson and Hastings 1979). ‘Quorum sensing’ (QS) as a term was coined much later in the 1990s (Fuqua et al. 1994). The unique phenomenon of bacterial communication was revealed to be facilitated by small molecular weight molecules called quorum sensing signals (QSSs) that are released into the environment

by bacterial cells upon reaching a certain cell number or ‘quorum’. Several types and sub-types of QSSs produced by prokaryotes as well as eukaryotes have since been discovered and described in the literature. Communication via QSSs in bacteria induces gene expression of a variety of functional characteristics such as biofilm formation, motility, swarming, competence, production of virulence factors, antibiotics etc. (Williams 2007; Hmelo 2017). Such characteristics enable bacteria to adapt and evolve in concordance with the external environment, by conferring ecological advantages when competing in a harsh and complex milieu.

One of the most common and abundantly produced categories of prokaryotic QSSs is the acyl homoserine lactones (AHLs), produced by Gram-negative bacteria. These molecules serve to facilitate intra-species signaling amongst the bacteria producing these compounds. The chemical structure of an AHL molecule is comprised of a lactone ring, with an acyl side chain varying in chain length (C4-C20), saturation, and addition of functional groups (Decho et al. 2011). So far, hundreds of AHL variant molecules have been discovered (Decho et al. 2011), which enable bacteria to correspond precisely in polymicrobial environments.

The first evidence for interspecies communication was unearthed in the bacterium *Vibrio harveyi* (Greenberg et al. 1979), where the bioluminescence of the bacterium was found to be a function of a special signal molecule called AI-2 (Bassler et al. 1994). Since then, gene expression of an inordinate variety of prokaryotic and eukaryotic genes has been attributed to the AI-2 signaling system. The most common synthase-receptor complex in bacterial cells capable of producing and detecting AI-2 is LuxS-LuxP; however other receptor proteins such as LsrB and RbsB have also been recognized.

Apart from being a QSS, AI-2 is a byproduct of an integral biochemical reaction series in living cells called the activated methyl cycle (AMC). The AMC cycle is responsible for the recycling of the primary methyl-donating compound in living cells, namely *S*-adenosylmethionine (SAM) (Schauder et al. 2001). The synthase, LuxI, utilizes SAM along with acyl substrates in the cell to manufacture AHL autoinducer molecules. The compound, SAM, undergoes a series of three reactions as part of the AMC, to be ultimately converted into (*S*)-4,5-dihydroxy-2,3-pentandione (DPD), the linear, unstable form of AI-2, by the synthase protein, LuxS (Bandara et al. 2012; Pereira et al. 2013).

Acyl homoserine lactones (AHLs) and AI-2 are the two most pertinent signal molecules produced by marine bacterial QS systems (Hmelo 2017). Bacteria in the phylum Proteobacteria are the most predominant synthesizers of AHLs in the bacterial world (Visick and Fuqua 2005). The diverse proteobacterial population within BBD makes investigating QSS production capabilities of the heterotrophic bacterial population within BBD and across coral health states pertinent as well as intriguing. In the present study, bacteria from BBD as well as healthy corals were tested for production of AHLs and DPD, the precursor molecule of AI-2. The results presented in this chapter have been published as part of Zimmer et al. (2014). Additional data (AHL and DPD detection in *Vibrio coralliilyticus* BAA-450, DPD detection and quantitation of all cultures over eight additional time-points, t=1, 2, 3, 4, 5, 6, 7, 8 hrs) are also presented here.

Materials and Methods

Sample collection and isolation

Black band disease (BBD) mat, SML from apparently healthy coral (HSML) and SML from apparently healthy part of BBD infected coral (BSML) were collected as previously

described (Zimmer et al. 2014; Bhedi et al. 2017). Samples were collected with sterile, needleless syringes (60 ml), from the Water Factory reef site in Curaçao (12° 06.799' N, 68° 57.662' W) from *Pseudodiploria strigosa* in February 2013 and from *Colpophyllia natans* at Horseshoe Reef, Florida Keys (25° 08.362' N, 80° 17.641' W) in September 2012. Samples in syringes were held in zip lock bags containing seawater at ambient temperatures in coolers until transfer to the laboratory at Florida International University, Miami, FL. The BBD mat was then rinsed in sterilized artificial seawater (ASW) to reduce seawater microbial contamination.

Bacteria from BBD were isolated by performing a dilution series up to 10^{-6} in sterilized ASW followed by plating and isolation of bacteria on Difco Marine Agar (MA) plates and sterile seawater tryptone agar. The biomass from the BBD mat was also streaked directly onto both media. Plates were incubated at room temperature (25 °C) and colonies were chosen on the basis of unique colony morphology and other characteristics (Zimmer et al. 2014).

***Chromobacterium violaceum* CV026 assay**

A total of 46 bacterial BBD isolates were tested for detection of AHLs using the genetically modified *Chromobacterium violaceum* CV026 reporter strain. A bioassay with the reporter strain was conducted as described by (McClellan et al. 1997) with slight modification (Zimmer et al. 2014; Bhedi et al. 2017) for testing the production of short and medium chain AHLs by the selected cultures. A 100 µl of freshly grown *C. violaceum* CV026 culture (18hrs at 35 rpm) was added to 5 ml of molten Luria Bertani (LB) medium (containing 0.7% agar) to create an overlay onto LB plates (1.5% agar). Test cultures grown on plates for 24-48 hrs were spotted onto the overlaid plates. Sterile

LB broth was spotted as a negative control onto each test plate while 0.01 μ l of synthetic C6-AHL was used as a positive control (50 mM in ethanol). All assays were replicated thrice (Zimmer et al. 2014).

Extraction and LC-MS/MS detection of AHLs

Of the 46 BBD isolates tested, two strains tested positive in the CV026 assay. These two cultures along with nine isolates from Zimmer (2012) that also tested positive for QS signal production via CV026 and other QS signal bioreporter strains were used for LC-MS/MS analysis (Zimmer et al. 2014). Additionally, *Vibrio coralliilyticus* BAA-450 was investigated for its AHL production capacity.

The 12 cultures (6 BBD, 2 BSML, 3 HSML isolates and *V. coralliilyticus* BAA-450) were tested for AHLs via LC-MS/MS as described previously (Zimmer et al. 2014), at University of Tennessee, Knoxville in the laboratory of Dr. Shawn Campagna. Bacterial cultures were grown in duplicate for 24 hrs at 25 °C and 200 rpm in half strength marine broth (MB). After 24 hrs, aliquots of 10 ml were pipetted out and filtered through nylon filters (0.22 μ m), in duplicate, to create four replicates per bacterial isolate tested. After passing the filtrate through a separatory funnel and washing the funnel with 1 ml water, 5ml of acetic acid in ethyl acetate was added twice to extract the filtrate. Organic layers were then combined and filtered post drying with MgSO₄. Concentration of the residual filtrate was achieved via vacuum and the residual oil was added to 300 μ l of acetic acid in ethyl acetate in an autosampler vial for processing via LC-MS/MS (Zimmer et al. 2014).

Extracted AHLs were processed and analyzed as described (Zimmer et al. 2014) employing the AHL detection method originally documented by (May et al. 2012). Ten μ l of the extract from the autosampler vials was injected onto a C18 reverse-phase core

shell column (5 μm , 100 \AA , 100 mm \times 2 mm). 0.1% acetic acid in water and 0.1% acetic acid in acetonitrile were used for separation, at a 200 $\mu\text{l min}^{-1}$ flow rate. Eluent was passed into a TSQ Quantum Ultra Triple Stage Quadrupole mass spectrometer using electrospray ionization. Multiple reaction monitoring (MRM) in a positive ion mode was used for detection. The AHLs were identified based on their precedence from literature and LC retention times. MSconvert algorithm (Proteowizard) was employed to convert .RAW files to .mzML (Kessner et al. 2008). Detection of peak intensities and generation of extracted ion chromatograms for each MRM was achieved using MAVEN (Melamud et al. 2010). Peaks were envisioned via an extraction window of 200 ppm and percentages of the AHLs were calculated from the integrated areas (Zimmer et al. 2014).

Detection and quantitation of DPD

The compound, DPD, was analyzed by methods described previously (Zimmer et al. 2014) using a documented protocol with some modifications (Campagna et al. 2009) at $t=24$ hrs, at University of Tennessee, Knoxville. Eppendorf tubes containing 10 μl of ^{13}C labeled DPD (used as the internal standard) were used for adding 300 μl of the sample culture in order to achieve a final ^{13}C -DPD concentration of 500 nM. After vortexing the contents in Eppendorf tubes, bacterial cells were pelleted out by centrifuging at 16.1 rcf for 1.5 min. 260 μl of the supernatant was pipetted out into Eppendorf tubes containing 25 μl of a DPD derivatizing solution (5 mg/ml) (Campagna et al. 2009). This was followed by vortexing the solution and resting for 45-60 min. Ethyl alcohol was used to extract the resulting solution twice. The extracts were combined and aliquoted into autosampler vials and stored at 4 $^{\circ}\text{C}$ until further analysis (Zimmer et al. 2014).

Samples over eight additional time-points were taken and analyzed for the present study (t=1, 2, 3, 4, 5, 6, 7, 8 hrs).

DPD analysis by LC-MS/MS

DPD analysis by LC-MS/MS was carried out as described in (Zimmer et al. 2014) at University of Tennessee, Knoxville. Ten μl of extracted sample was injected onto a C18 Kinetex column (5 μm , 100 \AA , 100 mm \times 2.1 mm). Separation was achieved at a flow rate of 200 $\mu\text{l min}^{-1}$ via an isocratic gradient of 5% HPLC grade acetonitrile and 95% of 0.1% acetic acid in HPLC grade water. The subsequent eluent was passed through a TSQ Quantum Ultra Triple Stage Quadrupole mass spectrometer using electrospray ionization. Similar to AHL detection, multiple reaction monitoring selected via positive mode was used for DPD detection (Campagna et al. 2009). Specifically, 381 m/z -202 m/z and 382 m/z -203 m/z were used as parent m/z -fragment m/z ratios and used for endogenous DPD- ^{13}C -DPD. The collision energy used for both was 43 (Zimmer et al. 2014).

Peaks were integrated with Xcalibur (Thermo Electron) and the concentration of DPD was analyzed by comparing peak areas of DPD in each sample with ^{13}C -DPD and multiplying the concentration of the internal standard. Additionally, a correction factor accounting for presence of natural isotopes was also employed (Campagna et al. 2009; Zimmer et al. 2014).

Results

AHL detection across isolates via LC-MS/MS

A total of 12 bacterial cultures were tested for AHLs via LC-MS/MS. Of these 12

isolates, the AHL detection results of 11 isolates have been reported in (Zimmer et al. 2014). The results of the AHLs detected via LC-MS/MS are depicted as percentages in Figure 1. C6 was the most abundantly and commonly produced AHL across all isolates tested. A total of five cultures out of 12 produced C6, while four isolates produced the next most abundant AHL, 3OC4. Both C14 and 3OHC10 were produced by three isolates each. The non-hydroxylated counterpart of 3OHC10, i.e. C10, was produced by two isolates. Similarly, AHLs 3OHC4, C19:1 and C20 were also produced by two isolates. The rest of the AHLs, specifically 3OHC18, 3OHC6:1, 3OHC5 and 3OC6, were each produced by one isolate.

C6 was the highest produced AHL by all isolates producing it. The production of C6 by isolates ranged from 69-92%. BBD-CUR-3M8 was the highest producer of this AHL at 92%. 3OC4, the next most common AHL, was the most abundantly produced AHL by two out of four of its producing isolates and its percentage production ranged from 11-52%. The ranges for the next most abundantly produced AHL, 3OHC10, varied between 9% and 75% amongst three of its producing isolates. C14, which was also produced by three isolates, ranged in production between 19-45%. C10 was produced by HSML-FTL-9i at 70% with C10 being its most abundant AHL while the other C10 producer, BSML-FTL-61, contributed to its AHL production at 26%. 3OHC4 was produced by two isolates at 20% and 24% each while C19:1 ranged in its production between 12-19%. The longest chain AHL, 3OC20, was produced at 5% each by two isolates. The rest of the AHLs were produced just once across all 12 isolates.

One particular isolate from the apparently healthy part of the BBD infected coral, BSML-FTL-7l, produced 26 different AHLs that ranged in amounts between 0.6% and

14%. This was the only isolate tested which did not have any dominant AHL produced. All other isolates also produced several AHLs similarly in lesser amounts depicted as ‘others’ in Figure 1.

DPD detection and quantitation

Figure 2 shows DPD quantitation of the isolates tested across time-points (t=1 to t=8 hrs) at one-hour intervals and a final time point at t=24. Nine of the 12 isolates tested showed production of DPD. At t=1, all isolates had similar concentrations of DPD. Along successive time-points, BBD-FLK-1M2 increased DPD production to the greatest extent followed by BBD-CUR-3M8. Out of the twelve isolates, four isolates (BBD-FLK-1M2, BB-CUR-3M8, HSML-FTL-9c, *V. coralliilyticus* BAA-450) showed a consistent increase in DPD concentration upto t=8, followed by a marked decrease leading upto t=24. The remaining eight isolates showed a consistent increase upto t=8 and maintained the DPD concentration upto t=24.

Discussion

The results of the present study revealed, for the first time, the production of AHL and DPD molecules by cultured isolates from BBD heterotrophs (Zimmer 2012; Zimmer et al. 2014). Production of QSSs has been previously investigated in a number of other marine environments that include healthy corals (Van Houdt et al. 2007; Tait et al. 2010; Golberg et al. 2011; Ransome et al. 2014), diseased and bleached corals (Tait et al. 2010) sponges (Mohamed et al. 2008) and marine snow (Gram et al. 2002). The precise function of these QS systems within these marine environments, however, is not known. Within ocean waters, QSSs face several challenging environmental conditions with some being acutely unsuitable for signaling functions. For example, QSSs produced by

planktonic prokaryotic or eukaryotic cells are subjected to extensive dilution by mass transfer (Boyer and Wisniewski- Dyé 2009). This dilution increases the ‘calling distance’ amongst bacteria (Gantner et al. 2006) making it difficult for them to respond and activate QS-regulated genes. AHLs are particularly sensitive to alkaline environmental conditions as they undergo hydrolysis and are hence unable to function as a cue for microbes (Yates et al. 2002; Decho et al. 2009). AHLs with oxo- substitutions on the third carbon, as were detected in this study (3OC4, 3OC6, 3OC20), are capable of undergoing a condensation reaction to form tetrameric acids, that are inept of signaling capabilities (Kaufmann et al. 2005). Considering the oxygenated nature of the pelagic zone, AHLs may undergo these reactions and render themselves incapable of signaling.

Within the black band mat, the pH, redox potential and sulfide concentrations undergo diel fluctuations in concurrence with photosynthetic processes during the day and respiration during the night (Carlton and Richardson 1995; Zimmer et al., 2014; Sato et al. 2015). As a result of nocturnal accumulation of fermentation products within the mat, the pH in the mat drops, creating a more suitable environment for AHLs to function (Carlton and Richardson 1995; Decho et al. 2009). The diverse repertoire of AHLs detected, as produced by heterotrophs from the mat, may indicate the differential use of AHLs in conjunction with the diel chemical fluctuations within the mat. For instance, due to their higher structural stabilities, long chain AHLs may be employed more predominantly during the day when the pH is more alkaline (Decho et al. 2011). Redox flux may also possibly attenuate signaling capabilities of QSSs due to generation of reactive oxygen species created due to interaction of organic matter with sunlight (Cámara et al. 2002). Contradictory to the speculative fates of QSS molecules in the

ocean, studies have confirmed the relative stability of AHLs in sea-water (Hmelo and Van Mooy 2009). Additionally, the biofilm like properties of the mat may assist in concentrating the QSSs within BBD, facilitating a sufficient quorum of bacterial cells for signaling functions.

DPD was detected for all of the BBD isolates that were tested in this study. AI-2 has been implicated to be the universal signaling molecule allowing interspecies signaling amongst microbes (Miller and Bassler 2001). Although, in the present study, only heterotrophs were tested for their AI-2 production, it is reasonable to contemplate a role of interspecies communication amongst the different functional groups of prokaryotes within BBD via AI-2. The intricate and interconnected network of microbes is most likely necessary for disease proliferation in this polymicrobial disease. As was previously mentioned, AI-2 is the byproduct of a chemical reaction series of SAM in the AMC cycle (Schauder et al. 2001). Since SAM is the chief methyl donor for reactions involving growth and development within cells, production of AI-2 may be considered a fair indication of the metabolic standing of a cell (Xavier and Bassler 2003; Parveen and Cornell 2011).

The major AHL producers amongst marine bacteria across corals have been alpha- and gammaproteobacteria, specifically *Vibrio* sp. and members of the *Roseobacter* clade (Cude and Buchan 2013; Milton 2006). In agreement with these results, the QSS producing isolates from this study were all found to be alpha- and gammaproteobacteria (see Zimmer et al. 2014). While QSSs were detected in cultures isolated across all coral health states, a higher number of bacteria from BBD (six isolates) produced QSS as compared to HSML (three isolates) and BSML (two isolates) health states. AHLs were

detected across all three coral health state isolates, while DPD was detected in BBD and HSML isolates only.

The presence of QSSs in bacteria isolated from BBD leads us to postulate the conceivable responsibilities of QS within BBD. QS has been shown to be involved in gene expression of biofilm formation and or/maintenance (Irie and Parsek 2008). The biofilm-like characteristics of the BBD mat may be contributed by exopolysaccharides secreted by QS bacteria. QS may aid in initiation and/or maintenance of the virulence factors in the mat community required for progression of this disease mat.

BBD heterotrophs include a large population of vibrios (Arotsker et al. 2015), which are known to produce QSSs. Vibrios are also known to produce metalloproteases responsible for tissue degradation (Arotsker et al. 2009). QS may additionally be involved in the production of antimicrobials that kill probiotic microbes within the SML of healthy corals, creating a niche to be taken over by pathogenic microbes. Certain QS bacterial systems in BBD may also be involved in production of inhibitor substances that interfere with QS systems of healthy coral associated bacteria.

At this point we can only speculate upon the plausible roles for QS in this intricate coral disease. However, the results of this study pave the way for further exploration of QS within BBD, in an effort to comprehend the mechanism of pathogenicity of this globally distributed, devastating coral disease.

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Figure 2-1: Production of AHLs in percentages across isolates as analyzed via LC-MS/MS. ‘Others’ included AHLs that were produced at levels less than 14% of the total amount

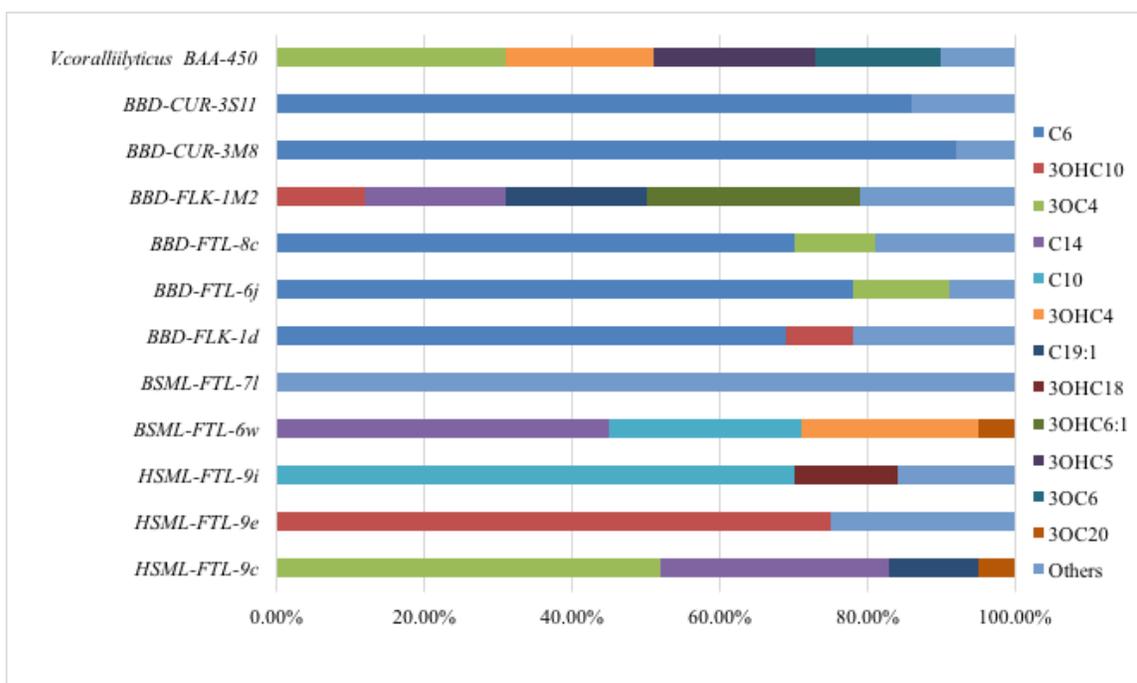
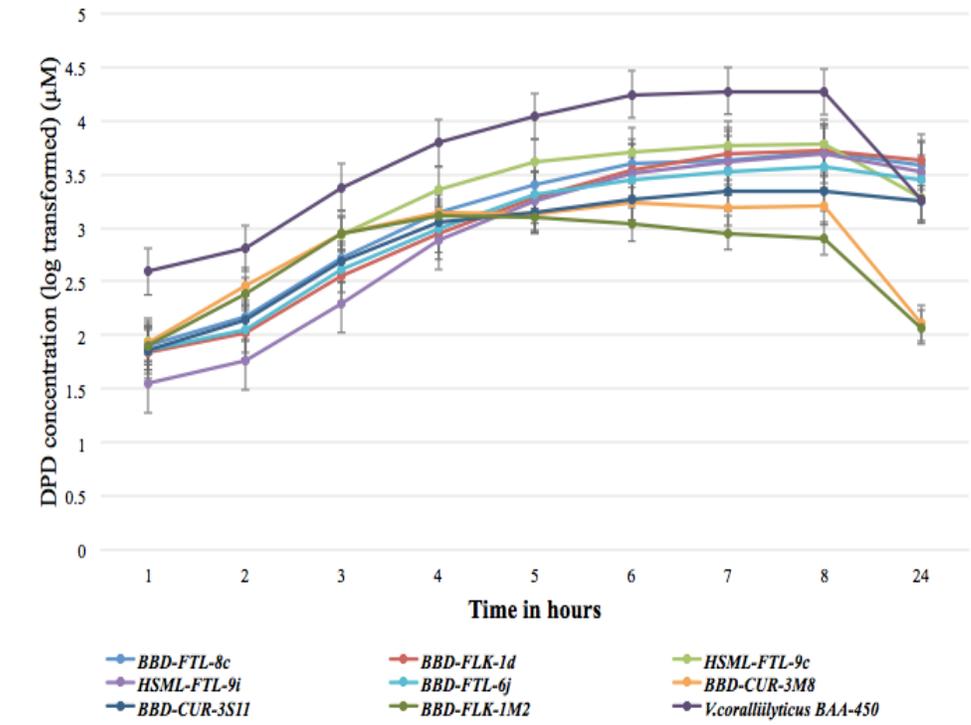


Figure 2-2: DPD detection and quantitation across isolates over time as detected using LC-MS/MS. DPD concentration measured at 0 hour was subtracted from samples and all values were log₁₀ transformed. Samples were taken at 0, 2, 4, 6, 8, and 24 hrs.



CHAPTER 3

ELEVATED TEMPERATURE ENHANCES SHORT TO MEDIUM CHAIN ACYL HOMOSERINE LACTONE PRODUCTION BY BLACK BAND DISEASE VIBRIOS

This study has been published as is:

Bhedi CD, Prevatte CW, Lookadoo MS, Waikel PA, Gillevet PM, Sikaroodi M, Campagna SR, Richardson LL (2017) Elevated temperature enhances short to medium chain acyl homoserine lactone production by black band disease associated vibrios.

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Abstract

Black band disease (BBD) of corals is a horizontally migrating, pathogenic, polymicrobial mat community which is active above a temperature threshold of 27.5° C on the reef. Bacterial isolates from BBD, the surface mucopolysaccharide layer (SML) of healthy corals, and SML of healthy areas of BBD infected corals were tested for production of short to medium chain acyl homoserine lactones (AHLs) using the *Chromobacterium violaceum* CV026 reporter strain. Of 110 bacterial isolates tested, 19 produced AHLs and 15 of these were from BBD. Eight AHLs were identified using LC-MS/MS, with 3OHC4 the most commonly produced, followed by C6. AHL-producing isolates exposed to three temperatures (24°, 27°, 30° C) revealed that production of three AHLs (3OHC4, 3OHC5, and 3OHC6) significantly increased at 30° C when compared to 24°C. 16S rRNA gene sequencing revealed that all of the AHL producing BBD isolates were vibrios. Metagenomic data of BBD communities showed the presence of AHL (and autoinducer-2) genes, many of which are known to be associated with vibrios. These findings suggest that quorum sensing may be involved in BBD pathobiology and community structure due to enhanced production of quorum sensing signal molecules (AHLs) above the temperature threshold of this globally distributed coral disease.

Introduction

There has been a significant decline in coral cover and diversity on tropical and subtropical reefs worldwide and one of the foremost contributing factors has been coral disease (Bourne *et al.* 2009). Disease could affect the coral animal itself or one or more of its associates, including bacteria, fungi, endolithic algae, zooxanthallae, protozoa, and other partners, collectively termed the coral holobiont (Rowan 1998; Rohwer *et al.* 2002).

The diverse and host specific coral-associated microbial community is harbored within the tissue, skeleton, and protective surface mucopolysaccharide layer (SML) secreted by the coral epidermal mucus cells (Johannes 1967; Ritchie and Smith 2004). Interactions within this microbial community have been proposed to be cooperative as well as antagonistic in nature and its members have been proposed to be potentially pathogenic and/or probiotic (Rosenberg *et al.* 2007; Teplitski and Ritchie 2009).

The composition and taxonomy of microbial communities of the SML layer of apparently healthy corals have been well researched (Ritchie and Smith 1995; Rohwer *et al.* 2001; Frias-Lopez *et al.* 2002, 2004; Beleneva *et al.* 2005; Sekar *et al.* 2006; De Castro *et al.* 2010; Cróquer *et al.* 2013; Krediet *et al.* 2013; Lins-de-Barros *et al.* 2013; Lee *et al.* 2015; Glasl *et al.* 2016). Such studies have shown that there is a shift in the heterotrophic microbial population within the SML when corals are stressed, including in the case of disease (Ritchie and Smith 1995; Frias-Lopez *et al.* 2002; De Castro *et al.* 2010; Mao-Jones *et al.* 2010; Cróquer *et al.* 2013).

One of the most destructive and intricate coral diseases is black band disease (BBD) (Richardson 2004). It is also one of the most widely distributed diseases, affecting sixty-four species of corals and exhibiting coral host and geographic specificity (Frias-Lopez *et al.* 2003; Sutherland *et al.* 2004; Voss *et al.* 2007; Miller and Richardson 2011). The disease manifests as a dark pigmented (phycoerythrin) band volumetrically dominated by a dense population of the gliding, filamentous cyanobacterium *Roseofilum reptotaenium* (Casamatta *et al.* 2012; Richardson *et al.* 2014), and consists of a highly variable polymicrobial consortium of microbes that contains four known functional groups: photoautotrophs (cyanobacteria) (Rützler *et al.* 1983), sulfate-reducing bacteria (Garrett

and Ducklow 1975), sulfide-oxidizing bacteria (Ducklow and Mitchell 1979), and heterotrophs (Garrett and Ducklow 1975). By far, the heterotrophic bacteria have been found to be the most taxonomically rich and varied community within the disease consortium (Miller and Richardson 2011). Several research groups have proposed that this diverse set of microbes includes potential primary, as well as secondary, pathogens (Cooney *et al.* 2002; Frias-Lopez *et al.* 2002; Sekar *et al.* 2006, 2008; Mao-Jones *et al.* 2010).

A number of studies have targeted BBD heterotrophic bacteria. Bacterial 16S rRNA gene clone libraries demonstrated shifts in bacterial ribotypes during transitions from cyanobacterial patches (CP) shown to develop into BBD on corals on the Great Barrier Reef (GBR) (Sato *et al.* 2010). In these studies, α -proteobacteria-affiliated sequences were dominant in CP libraries, whereas γ -proteobacterial ribotypes became more abundant after transition to the BBD community (Sato *et al.* 2010). The CP-to-BBD transition has only been seen on reefs of the GBR. In other studies, α -proteobacteria were found to be the most diversely represented group of bacteria as determined by a meta-analysis of clone libraries produced from 87 BBD samples collected on reefs world-wide (Miller and Richardson 2011).

BBD most actively infects corals when the sea water temperature rises above 27.5 °C (Edmunds 1991; Kuta and Richardson 1996; Voss and Richardson 2006; Sato *et al.* 2015). Since bacterial species and communities have stringent tolerance thresholds regarding the environment (including temperature), any change in surroundings surpassing a threshold may lead to a perturbation of the delicate relationship with a host. Environmental factors can also lead to decreased host health and fitness, and an increase

in temperature has been shown to cause a switch from a symbiotic to a pathogenic role in some host-associated microbes (Webster *et al.* 2008). In several cases this transition has been shown to be controlled by quorum sensing (Jayaraman and Wood 2008).

Quorum sensing is a type of pheromone-based cell to cell communication system that is often density regulated and that allows bacteria to converse with the aid of chemically synthesized signal molecules called autoinducers (AIs) (Nealson *et al.* 1970; Nealson and Hastings 1979). It has been noted that QS systems in vibrios often integrate information, including environmental parameters, with cell density (Lyell *et al.* 2013). Quorum sensing as a phenomenon is responsible for regulating a wide range of bacterial behaviors that include virulence, bioluminescence, biofilm maintenance and maturation, motility, symbiosis, antibiotic synthesis, exoenzyme production, and swarming (Eberhard *et al.* 1981; Pirhonen *et al.* 1993; Zhang *et al.* 1993; Eberl *et al.* 1996; Davies *et al.* 1998; Bassler 1999).

Different bacteria synthesize different AIs and release them into the environment. After extracellular accumulation of AI molecules reaches a threshold (the "quorum" in quorum sensing), bacteria regulate their behavior to coordinate expression of specific genes to adapt and thrive in that specific environment. This is achieved by inducing production of an array of physiological and functional adaptations (De Kievit and Iglewski 2000; Miller and Bassler 2001; March and Bentley 2004; Jayaraman and Wood 2008; Dobretsov *et al.* 2009; Li and Tian 2012; Lee and Zhang 2014).

Gram negative bacteria capable of quorum sensing primarily produce acyl homoserine lactone (AHL) signaling molecules which have a fatty acid (acyl) side chain attached to a homoserine lactone ring (Fuqua *et al.* 2001). The side chain varies on the

basis of length, saturation and presence/absence of functional groups, creating a suite of AHL molecules that bacteria use to communicate in complex environments (Fuqua *et al.* 2001). While some bacteria synthesize unique AHLs, many different bacteria can also synthesize the same AHL, allowing for intra- and inter-species signaling and potential coordination between different bacterial genera as well as species (Miller and Bassler 2001). Various factors such as temperature, pH, oxygen availability, and redox state have been known to affect QS regulation and QS regulatory gene expression (Decho *et al.* 2010; Frederix and Downie 2011). Some of these factors also affect the AHL molecules themselves. For example, as the side chain length increases, the solubility of the AHL molecule decreases while the stability increases (Yates *et al.* 2002; Decho *et al.* 2009). In some cases the structural integrity of the homoserine lactone ring has been observed to be reversibly susceptible to alkaline conditions, affecting long chain AHLs to a lesser extent (Voelkert and Grant 1970; Yates *et al.* 2002; Decho *et al.* 2009). This is significant in terms of environmental influence since lactone ring hydrolysis disables the AHL molecule from signaling (Decho *et al.* 2009).

Recent studies have begun to investigate the role of QS within the coral holobiont (Van Houdt *et al.* 2007; Tait *et al.* 2010; Alagely *et al.* 2011; Golberg *et al.* 2011; Hunt *et al.* 2012; Kimes *et al.* 2012; Tello *et al.* 2012; Ransome *et al.* 2014; Zimmer *et al.* 2014) as well as other marine invertebrates (Britstein *et al.* 2016). Kimes *et al.* (2012) tested the effect of temperature on AHL production by coral-associated vibrios, and showed that different strains of vibrios varied their AHL production with temperature. Yates *et al.* (2002) had previously shown that increasing temperature increased the rate of homoserine lactone ring opening, resulting in reduction in the production of intact AHLs.

Healthy and diseased coral-associated bacterial isolates, particularly vibrios, were found to produce both AHLs and AI-2 (Tait *et al.* 2010), and the coral pathogen *Serratia marcescens* was shown to utilize AHL-QS systems for regulating a range of virulence factors, including biofilm formation (Van Houdt *et al.* 2007). AHLs, mostly short and medium chain length, were recently shown to be produced by 6% of BBD bacterial isolates tested, as well being present in all samples of freshly collected BBD mat (Zimmer *et al.* 2014).

The nature of the complex polymicrobial BBD is one in which BBD microbial groups interact with one another as well as bacteria living in the coral SML, tissue and skeleton. To assess a potential role of quorum sensing in BBD etiology, both laboratory (physiological and analytical) and genetic approaches can be used. While this study focused on use of physiological and analytical methods, we recognize that metagenomics is also a powerful approach that can potentially reveal which quorum sensing genes are present in the BBD community, and which specific QS signal molecules are likely to be expressed as the result of genes present for complete biosynthetic pathways. A number of whole genome sequencing studies have been carried out to study coral metagenomics (Wegley *et al.* 2007; Dinsdale *et al.* 2008), coral stress conditions (Vega Thurber *et al.* 2008, 2009), to identify specific microbes both inhabiting a coral host (Marhaver *et al.* 2008; Carlos *et al.* 2013; Lesser and Jarett, 2014) and associated with coral diseases such as white plague (Garcia *et al.* 2013) and bleaching (Littman *et al.* 2011). However, to our knowledge, no metagenomics study has addressed the potential role of QS in BBD.

This study extends the research on BBD to examine the variation of QS (AHL) molecules produced by laboratory cultures of BBD and coral-associated bacteria in

response to environmental (temperature) changes, including a comparison of AHLs produced across different coral health states. As mentioned above, BBD is typically observed in summers when sea water temperatures exceed 27.5 °C (Kuta and Richardson 1996; Voss and Richardson 2006). Therefore, we examined AHL production at temperatures close to, above, and below this threshold. Metagenomics data produced from BBD samples were also assessed to determine the presence and nature of quorum sensing genes in the BBD mat to supplement and interpret the results from the culture dependent methods.

Materials and Methods

Field sampling and isolation of bacteria

Samples of black band disease (BBD), apparently healthy SML from a BBD infected coral (BSML) and SML from an apparently healthy coral (HSML) were collected from colonies of *Montastraea cavernosa* located on Algae Reef in the northern Florida Keys, USA (N 25' 08.799' W 80' 17.579'). Sterile needleless 60 ml syringes were used for sampling. BBD mat and SML were suctioned off of the coral host with slight agitation, after which the syringes were capped. BSML samples were collected at a minimum distance of 10 cm from the BBD mat. HSML samples were collected from an adjacent (healthy) colony of the same species. Once on the boat, syringes with samples were held in coolers containing sea water at ambient temperature and transported to the shore. BBD mat and SML were then transferred to sterilized artificial sea water (ASW) to minimize contamination from sea water bacteria.

Isolation of bacteria from three coral health states

Heterotrophic bacteria were isolated from samples of each of the three coral health conditions. Dilution series (10^{-1} to 10^{-6}) of BBD, HSML and BSML samples were prepared using filtered sea water and incubated at both room temperature and 30°C under a 12 hours light:12 hours dark regime. 100 μl from dilution tubes 10^{-3} , 10^{-4} , 10^{-5} and 10^{-6} were plated every day for a period of seven days and incubated at 30°C . Direct streaking from the original mat and SML samples was also carried out. All plating was onto Difco marine agar (MA) and sea water tryptone (SWT) agar plates (0.5% tryptone, 0.3% glycerol, 0.3% yeast extract, 1.5% agar in 70% sea water) (Boettcher and Ruby 1990), in triplicate. Uniquely distinct colonies were chosen based on colony color and morphology followed by streaking for purification via plating onto the same medium.

***Chromobacterium violaceum* CV026 AHL reporter strain assay**

QS signal production capabilities were tested using the reporter strain *Chromobacterium violaceum* CV026 using an agar overlay method with some modifications (McClellan *et al.* 1997, Zimmer *et al.* 2014). This strain is used to detect the presence of short to medium chain AHLs, particularly C4-AHL, 3-oxo-C4-AHL, C6-AHL, 3-oxo-C6-AHL, C8-AHL and 3-oxo-C8-AHL (McClellan *et al.* 1997; Steindler and Venturi 2007), and was used based on earlier results (Zimmer *et al.* 2014) in which it was determined that BBD isolates produce mainly short/medium chain length AHLs. Test isolates were grown overnight (18 hrs) on MA at room temperature (25°C). The *C. violaceum* CV026 biosensor strain was also grown overnight in 5 ml Luria Bertani (LB) broth at 30°C with shaking at 35 rpm. Test plates consisted of a basal layer of LB medium overlaid with 5 ml of molten LB with 100 μl of the freshly grown overnight

broth culture of *C. violaceum* CV026. For a positive control 0.01 µl of 50 mM synthetic *N*-hexanoyl-L-homoserine lactone solution in ethanol was used while sterile LB broth served as negative control. All assays were carried out in triplicate. All results were analyzed statistically using the proportion t-test for significance ($p \leq 0.05$).

AHL production and temperature variation

Isolates that tested positive for production of AHLs using the *C. violaceum* CV026 assay were further tested for the effect of temperature on AHL production by exposing isolates to three different temperatures (24 °C, 27 °C and 30 °C). For each temperature, 25 ml aliquots of freshly made half strength marine broth (MB) were prepared in 125 ml Erlenmeyer flasks. Half strength MB was used in order to avoid clogging of the LC column (see below) due to particulate matter in this growth media. Flasks were inoculated in duplicate with 500 µl of culture freshly grown (overnight) at each respective temperature (24 °C, 27 °C and 30 °C) with shaking (250 rpm). One ml of sample was then aliquoted every two hours from each flask at $t = 0, 2, 4, 6, 8, \text{ and } 10$ hours. At every time point for every sample, OD was measured (600 nm) and 500 µl of sample was added to an eppendorf tube containing deuterated AHL internal standards that consisted of (D₂)C₄, (D₂)3OHC₄, (D₂)C₆, (D₂)C₃OC₆, (D₂)C₇, (D₂)C₈, (D₂)C₁₂, (D₂)3OHC₁₂:1 and (D₂)C₁₄ that were synthesized as previously described in (May *et al.* 2012). This was done in duplicate to generate a total of four samples per isolate per time point per temperature. The eppendorf tubes were then centrifuged for 1 min at 20,000 ×g to spin down bacterial cells, after which 500 µl of the supernatant were placed in autosampler vials and kept at -80 °C until further processing.

LC-MS/MS analysis of QS signals

AHL extraction and analysis were carried out using an LC-MS/MS method optimized for AHL recognition (May *et al.* 2012) using previously reported methods (Zimmer *et al.* 2014) with slight modifications. Briefly, each sample was kept in an autosampler (Thermo Electron Surveyor) at 4°C before injection of 10 µl of the sample onto a reverse-phase C18 core-shell column (5 µm pore size, 100 Å particle size, 100 mm x 2 mm or 2.1 µm pore size, 100 Å particle size, 100 mm x 2 mm). A gradient of 0.1% acetic acid in water and 0.1% acetic acid in acetonitrile at a 200 µl min⁻¹ flow rate was used to elute and separate the analytes, which were then introduced into a TSQ Quantum Ultra Triple Stage Quadrupole mass spectrometer using electrospray ionization. Multiple reaction monitoring (MRM) in positive ion mode was used for compound detection, annotation and identification. Specifically, the method screened for 54 unique parent m/z–fragment m/z pairs based on neutral loss of the acyl chain giving common 102 m/z fragment corresponding to the common homoserine lactone of all AHLs. The method includes masses corresponding to chain lengths ranging from 4 to 20 carbons with the possibility of one hydroxyl or ketone at the 3-position as well as one double bond in the chain. Note: MRM alone is unable to differentiate between compounds with the same parent m/z-fragment m/z pairs (e.g., 3OC6-HSL and C7-HSL). To circumvent this, further chemical (such as LC retention time) and biological (precedence for the compound in the literature) information was used to annotate structures for AHLs for which standards were not available as previously reported (Zimmer *et al.* 2014). Unique parent m/z-fragment m/z pairs were assessed to confirm the presence of AHL molecules.

Using Proteowizard's MSconvert algorithm, the .RAW files were converted to

.mzML (Kessner *et al.* 2008). MAVEN was used to detect and report peak intensities and to generate extracted ion chromatograms (EICs) for each MRM (Melamud *et al.* 2010). To envision the peaks, an EIC extraction window of 200 ppm was used and the area for each AHL was integrated, which was further used to detect relative abundance and percentage of each detected AHL.

16S rRNA gene sequencing of isolates

The genomic DNA of cultured bacterial isolates was extracted using the FastDNA Spin Kit for Soil, according to the manufacturer's protocol or alternatively by adding an individual purified colony in 100 µl of sterile phosphate buffered saline (PBS) heating at 95 °C for 10 min followed by centrifugation for 10 min at 5000 g (modified from Zimmer *et al.* 2014). PCR amplification of DNA was carried out using the universal bacterial primers 27F 5'-AGA GTT TGA TCM TGG CTC AG-3' and 1492R 5'-TAC GGY TAC CTT GTT ACG ACT T-3' in a Peltier Thermal Cycler. Amplified bacterial 16S rDNA was cleaned using the ExoSAP-IT PCR cleanup kit followed by sequencing with an ABI Prism™ 3100 genetic analyzer at the DNA Core Facility at Florida International University using the BigDye® Terminator. Sequence trimming and cleaning, followed by alignment and assembly was carried out using DNA Baser Sequence Assembler (Zimmer *et al.* 2014). The sequences (1300-1500 bp) were then queried using the BLAST queuing system (Altschul *et al.* 1990) in order to find their closest relatives from NCBI GenBank. Sequences have been submitted to GenBank database under the accession numbers KX146440- KX146449 and KX353776-KX353778.

Phylogenetic tree

All 16S rRNA sequences of isolates used in the present study and their closest hits in NCBI GenBank were aligned using MUSCLE (www.ebi.ac.uk/Tools/msa/muscle/) (Edgar *et al.* 2004). The aligned FASTA sequences of isolates and their closest relatives were manually edited using M7 Alignment explorer featured in MEGA version 4 (Tamura *et al.* 2007). A maximum likelihood phylogenetic analysis was carried out with bootstrap analysis using MEGA (Tamura *et al.* 2007).

Metagenomics

Samples of BBD for metagenomics were collected from BBD infected *Colpophyllia natans* (Horseshoe Reef, northern Florida Keys, August 2013) and *Pseudodiploria strigosa* (Curaçao, off shore of the CARMABI research station, May 2013). Samples were collected with sterile 10 ml syringes and placed in RNA-later, frozen at -80°C and sent for whole genome sequencing. A paired end sequencing was performed in a single lane on the Illumina HiSeq. The paired ends were sequenced with an insert size of 500 bp. We obtained an average of 12 million reads per sample with a read length of 150 base pairs. The metagenomics datasets were uploaded onto the Metagenomics RAST (MG-RAST) server (<http://metagenomics.anl.gov/>). MG-RAST is a public resource for the automatic phylogenetic and functional analysis of metagenomes (Meyer *et al.* 2008). The presence of QS genes was assessed by using subsystems with MG-RAST (Max. e-value cutoff = 10⁻⁵, Min. % identity cutoff = 60%, Min. alignment length cutoff = 40 amino acids) with averages calculated as per Wilke *et al.* 2013.

Results

Bacterial isolates and detection of short to medium chain AHLs

A total of 110 pure cultures of heterotrophic bacteria were isolated from BBD infected and healthy coral colonies (Table 1). Of these, 36 were from the BBD mat, 36 from the apparently healthy part of the BBD infected coral (BSML), and 38 from an apparently healthy coral (HSML). Each isolate was tested for QS signal production using the *Chromobacterium violaceum* CV026 bioassay. Among the 110 isolates, 19 tested positive in the QS assay, consisting of 15 BBD isolates and two each from HSML and BSML (Table 1). HSML and BSML isolates had similar percentages of isolates that tested positive for QS, 5.3% (2 out of 38) and 5.6% (2 out of 36) respectively. The percentage of QS positive isolates from BBD in comparison was approximately seven times higher at 41.7% (15 of 36). When comparing QS producing isolates between BBD vs. HSML and BBD vs. BSML, a significant ($p \leq 0.05$) difference in proportions was detected.

Variety and abundance of AHLs across different temperatures

Isolates that tested positive for AHL signal production were investigated further (Tables 2 and 3). Data are reported for 13 of the 15 BBD isolates and one of the two BSML isolates that tested positive in the *C. violaceum* assay since these three isolates did not grow during the additional experiments. The isolates tested were identified by 16S rRNA gene sequencing (Table 2) and analyzed using LC-MS/MS to identify specific AHLs (Table 3). All of the isolates that were able to be grown for sequencing were found to be *Vibrio* species (Table 2 and Figure 1). It should be noted that although several

Vibrio harveyi and *Vibrio* spp. are listed in Table 2, they are different strains with different 16S rRNA gene sequences.

AHLs produced by the isolates were identified and quantified across three temperatures (24 °C, 27 °C and 30 °C) across six time points (t = 0, 2, 4, 6, 8, and 10 hours). Data (Table 3) are reported in amounts of relative abundance of AHLs per isolate, with relative amounts also indicated in Figure 1. 3OHC4 was found to be produced by the highest number of isolates (14 out of 16), followed by C6 (6 out of 16), 3OHC5 and 3OHC6 (each 5 out of 16). Comparing relative quantities (per isolate), the production of 3OHC4 was approximately 10 times more than that of the next most abundantly produced AHL, C6 (data not shown), followed by 3OHC5 and 3OHC6. The longest chain AHL (3OC12) detected in this study was produced by two isolates (isolates 15 and 16), both from HSML. C5, 3OC5:1 and 3OHC8 were the rarest, each with one isolate producing it in combination with other AHLs.

Of the 16 isolates, one isolate produced only one AHL, identified as C6 (Table 3). Eleven isolates produced two AHLs and four isolates produced three AHLs. The two HSML isolates produced the same three AHLs (3OHC4, 3OHC6 and 3OC12) but in varying percentages. Most of the AHL production by the sole BSML isolate tested was contributed by 3OHC4 (99.2%).

At each sampling, the OD for each isolate was measured to assess any correlation between AHL production and the stage of growth of that isolate. There was no correlation between AHL production and growth phase (data not shown).

Table 4 compiles statistically significant results ($p \leq 0.05$) from univariate ANOVA analysis of all isolates, and the AHLs produced by each isolate, based on comparing

temperatures. In each case, all AHLs produced were pooled for each isolate, and AHL production values across all time-points were taken together across all replicates. Isolates with non-significant differences in AHL production across temperature, and temperature comparisons that were non-significant for a particular AHL, are not shown in Table 4. AHLs 3OHC4, 3OHC5, 3OHC6, and 3OC5:1 had significant differences within all three temperature comparisons for 10 of the 16 AHL positive isolates. All isolates that produced 3OHC4 and 3OHC5 showed significantly higher production at 30°C as compared to 24 °C, while 3OHC6 did so inconsistently across the isolates that produced this AHL. 3OC5:1 had a significant difference only when comparing temperatures 24 °C vs. 27°C (Table 4) with production higher at 24 °C; however, it is noteworthy that this result pertained to only one isolate that produced this AHL (isolate no. 2).

Table 5 shows relative AHL production among isolates (all time-points pooled) when comparing production at 24 °C vs 30 °C (below and above the temperature threshold of BBD on the reef). Only data that were significant ($p \leq 0.05$) are shown, representing six of the 16 tested. Of these six, five were from BBD and one from BSML (isolate no. 14). In each case, AHL production was significantly higher at 30 °C when compared to 24 °C. The elevated production above the BBD temperature threshold was seen for three AHLs, with 3OHC5 by far the most common.

Comparison of short to medium chain AHLs across three coral health states

BBD isolates, in addition to constituting the sample group with the highest number of QS producing isolates (Table 2), produced the largest variety of AHLs when compared to isolates from the other two coral health states (Table 6). AHLs 3OHC4 and 3OHC6 were produced by isolates from all three health states. These AHLs were also

two of the three that varied significantly in terms of temperature (Table 5). As noted above, 3OHC4 was the most commonly produced AHL among all isolates (Table 3).

Metagenomics

Table 7 presents all proteins and their ascribed functions associated with QS genes that were identified using MG-RAST within the BBD metagenome samples from the Florida Keys and Curaçao. As is evident, many of the identified QS genes were found to be those associated with vibrios. The *luxI*, *rhIL* and *luxR* genes identified under the subset of ‘AHL autoinducer QS’ are not associated with any particular bacterial genus.

QS associated functional sequences were differentially found within the two BBD metagenomes. Both *luxI* and *luxR* genes associated with AHL synthesis were found in both samples, while *rhIL*, another AHL synthase, was found to be present only in the Florida Keys BBD metagenome. Four *Vibrio*-associated QS genes were found exclusively in the Florida Keys metagenome, specifically *cqsA*, *luxP*, *luxM* and *hapR*. Other QS genes (*luxI*, *luxR*, *luxO*, *luxS* and *uvrY*) were found in both metagenomes. A particular *N*-homoserine lactone hydrolase (associated with QS in *Yersinia*) was uniquely present in the Curaçao metagenome. One *Pseudomonas* associated QS gene was exclusively present in either of the two metagenomes (*vfr* in Curaçao metagenome and *rhIR* in Florida Keys metagenome). The results of the QS genes in the BBD metagenomes correlated with the identification of the AHL-producing isolates used in this study (mainly vibrios) in that a high number of *Vibrio*-associated QS genes were present in the metagenomes.

Discussion

One of the most interesting findings of this study was the fact that, by far, isolates from BBD had a significantly higher proportion of members that produced AHLs when compared to isolates from the SML of apparently healthy and BB-diseased corals. Only four of 74 (5.4%) isolates from BSML and HSML combined tested positive using the reporter strain whereas 15 of 36 (41.7%) of BBD isolates did so. This shows that short to medium chain AHL-producing isolates are more active in BBD bacterial communities as compared to SML bacterial communities. Overall, BBD is a pathogenic polymicrobial consortium and bacteria living in the coral SML are believed to have probiotic characteristics. Both of these types of microbial communities (pathogenic and probiotic) have been proposed to involve QS on a functional basis.

A second very interesting result of this study was that 10 of the 16 short to medium chain AHL producing isolates exhibited a significant difference in AHL production in terms of temperature. For six of the ten isolates there was significantly increased AHL production at the higher temperature (30 °C) compared to the lower temperature (24 °C). Five of the six isolates that varied AHL production significantly above and below the temperature threshold of BBD (27.5°C) were all isolates from the BBD community. Three (3OHC4, 3OHC5 and 3OHC6) of the eight AHLs detected in this study showed this pattern. Interestingly, the production of 3OHC5 consistently increased from 24 °C to 27 °C to 30 °C across all five isolates that produced it, and for two of the five isolates producing this AHL, all three temperature comparisons were statistically significant. Considering the fact that 3OHC5 consistently increased at higher temperatures, it is

plausible to hypothesize a role of some importance for this AHL in the etiology of this temperature dependent coral disease.

Previous studies of AHL production in terms of temperature and disease revealed varying effects for different AHLs. For example, AHL production by some sub-species of *Erwinia*, which causes soft rot in plants above a threshold temperature of 20 °C, was increased at 34.5 °C compared to 28 °C, whereas production decreased at the elevated temperature for other, non-pathogenic sub-species of the same genus (Hasegawa *et al.* 2005). *Pecobacterium atrosepticum*, which causes soft rot in potatoes at and above 20 °C, produces four AHLs, all of which increased from 4 °C to 24 °C, but decreased at 28 °C (Latour *et al.* 2007). More recently, it was shown that the concentration of eight AHLs produced at 16 °C by *Allivibrio salmonicida*, which causes cold water vibriosis in Atlantic salmon, was reduced to less than 5% in concentration at 6 °C (Hansen *et al.* 2015). These findings, along with the results of this study, implicate temperature as an environmental factor that influences the role of QS in disease.

AHLs are affected by other environmental factors in addition to temperature. These include pH, redox state and oxygen concentration (Horswill *et al.* 2007; Frederix and Downie 2011). Alkaline pH causes degradation of AHLs by rendering the molecule incapable of signaling (Yates *et al.* 2002). In general, it has been shown that the longer the AHL chain length, the more stable the molecule is to external factors and hence, lower the turnover rate (Yates *et al.* 2002; Hmelo and Van Mooy 2009). The pH of sea water is around 8.2, hence one can argue that marine AHL producing bacteria must have long chain AHLs to be capable of signaling in an alkaline pH (Huang *et al.* 2008). However, several studies have found that both short and long chain AHLs are produced

by bacteria in marine environments (Gram *et al.* 2002; Taylor *et al.* 2004; Wagner-Döbler *et al.* 2005; Mohamed *et al.* 2008; Tait *et al.* 2010; Alagely *et al.* 2011; Golberg *et al.* 2011; Biswa and Doble 2013; Ransome *et al.* 2014; Zimmer *et al.* 2014). It may be that the small chain AHLs are produced at high quantities to counteract breakdown due to external factors. Decho *et al.* (2009) proposed that differential degradation of AHLs likely affects signal reception by bacteria, and that this could result in variation of chronological windows in which signaling can occur.

A greater abundance of AHLs has also been observed in buffered media as compared to unbuffered media, which could be the result of increased pH as a growing culture becomes more alkaline (Yates *et al.* 2002). Although buffered media were not utilized in the present study, growth periods were short (on the order of hours) and samples were extracted and immediately frozen at - 20 °C to minimize any breakdown and/or degradation of AHL molecules.

In terms of BBD, the results of this study showed that temperature had an effect on the relative abundance of AHLs produced by BBD isolates with more AHL produced above the BBD temperature threshold. It may be that pH is involved as well, as pH fluctuates widely within BBD over each 24 hour period. The pH in the band can decrease to 7.2 during the night due to release of CO₂ during respiration and increase to 8.2 during the day due to CO₂ uptake during photosynthesis (Carlton and Richardson 1995; Decho *et al.* 2009; Glas *et al.* 2012). Yates *et al.* (2002) showed a pH dependent lactonolysis of AHLs that was higher at 37 °C than at 22 °C. Sea water temperatures above 27.5°C promote BBD infections in corals. Healthy SML has an acidic pH of 5.5 (Philips, 1963), and within the BBD mat the average pH recorded is 7.5 (Glas *et al.* 2012). Sea water pH

fluctuates between 8-8.2. Hence theoretically, coral-associated AHLs could be most stable in healthy SML, followed by BBD mat and lastly the surrounding sea water. However, an increase in surrounding sea-water temperature could cause pH and temperature to influence each other in a way that they start affecting the stability of AHLs (as was shown in Yates *et al.* 2002). Furthermore, as shown in Decho *et al.* (2009), the half-life of most AHLs significantly reduces over a pH of 8.2. As the pH of sea water varies between 8-8.2, some influence of sea water pH on stability of AHLs on a coral, healthy or diseased, cannot be denied. However, in the case of corals, the effect of the immediate environment likely has a greater influence on the stability of AHLs rather than sea water pH.

In our study we did not investigate the effect of pH or temperature on AHLs as we did not isolate (purify) the AHLs. They were identified, using analytical techniques, solely for the purpose of determining their presence and identification. Experiments targeting the effects of pH and temperature on AHLs purified from BBD are well worth doing in future studies.

All of the isolates that produced AHLs in this study were identified to be *Vibrio* species, which is interesting in that many vibrios exhibit QS. *Vibrio* species are also known pathogens in several coral diseases. For example, *Vibrio coralliilyticus* and *Vibrio shiloi* both cause coral bleaching (Kushmaro *et al.* 1996, 1997; Ben-Haim *et al.* 2003), *Vibrio harveyi* and other *Vibrio* species have been implicated in white syndrome disease (Sussman *et al.* 2008; Luna *et al.* 2010) and *V. alginolyticus* is a proposed pathogen of yellow blotch/band disease (Cervino *et al.* 2008). Several studies have found *Vibrio* species to be present in higher abundance in diseased corals as compared to healthy

corals (Tait *et al.* 2010; Arotsker *et al.* 2015; Tout *et al.* 2015) and vibrios have been proposed to be, in general, opportunistic pathogens of corals (Bourne and Munn 2005), including BBD (Arotsker *et al.* 2009). Several vibrios that have caused disease in corals have done so at a temperature of above 26 °C, close to the temperature threshold of BBD. In our study all six of the BBD isolates that produced significantly more AHLs at 30°C compared to 24°C were vibrios. The vibrios could be either opportunistic colonizers or opportunistic pathogens. Arotsker *et al.* (2009) showed that between 26-28 °C vibrios increase their proteolytic activity in the black band mat, either by increasing the number of proteases produced per bacterial cell or by enhancing the activity rate of the previously manufactured proteases. This observation led to a speculation that the proteolytic activity of the mat is possibly augmented by temperature increase due to increased activity of the *Vibrio* spp. (Arotsker *et al.* 2009). Proteases may assist infiltration of the coral tissue, followed by lysis, thus enhancing disease progression (Ben-Haim and Rosenberg 2002; Arotsker *et al.* 2009).

The presence of many well-known and common QS genes in the BBD metagenome dataset indicates a very high potential for active quorum sensing within the black band mat. Both AHL and AI-2 genes were found to be present in the BBD metagenome, many of which are *Vibrio* associated, which agrees with the high proportion of vibrios among the BBD isolates tested. Overall, a greater variety of QS genes were exclusively present in the Florida Keys metagenome.

In summary, our findings reveal that temperature has a significant effect on the production of specific short to medium length quorum sensing signal molecules (AHLs) produced by isolates of BBD bacteria and that production of three of the eight AHLs

detected is increased above the BBD temperature threshold. Notably the production of 3OHC5 increased from the lower temperatures to the higher temperatures without exception. The significance and role of this particular AHL in BBD initiation and/or advancement requires further investigation. Furthermore, we found that when comparing QS signal production by BBD bacteria vs. bacteria isolated from coral SML, the proportion of QS sensing by BBD bacteria was significantly higher with a greater variety of AHL production. Our results further imply a particular importance of BBD vibrios in the functioning of BBD community quorum sensing, both in the laboratory experiments using bacterial isolates and in our investigation into the BBD metagenome. Whether vibrios and specific quorum sensing signal production capabilities are directly involved in BBD disease etiology necessitates further study targeting specific mechanisms of BBD pathobiology that are linked to QS.

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Table 3-1: Bacterial isolates from three coral health states that were positive for QS signal production assayed using the *C. violaceum* CV026 biosensor strain.

Isolate source	No. of isolates positive in the CV026 QS assay (%)	Total no. of isolates tested
BBD	15 (41.7)	36
BSML	2 (5.6)	36
HSML	2 (5.3)	38
Total	19 (17.3)	110

All isolates were from the coral species *M. cavernosa*. BBD = black band disease; BSML = apparently healthy SML from the BBD-infected colony; HSML = SML from a healthy, nearby colony

Table 3-2: Identification of AHL producing isolates used in this study based on 16S rRNA gene sequencing.

StrainNo.	Isolate	Source of isolate	Genbank accession number	Closest relative in Genbank	% similarity of relative	Accession number of closest relative in GenBank
1	5-BBD-S1	BBD	KX353776	<i>Vibrio harveyi</i> strain QH141026D	99	KU245732
2	5-BBD-S5	BBD	-	-	-	-
3	5-BBD-S8	BBD	KX146443	<i>Vibrio harveyi</i> strain X11XC19	99	KM881533
4	5-BBD-S11	BBD	KX146444	<i>Vibrio</i> sp. PMS13	99	EU372931
5	5-BBD-S21	BBD	-	-	-	-
6	5-BBD-S22	BBD	KX146442	<i>Vibrio harveyi</i> strain QH141026D	99	KU245732
7	5-BBD-S24	BBD	-	-	-	-
8	5-BBD-M2	BBD	KX146440	<i>Vibrio harveyi</i> strain QH141026D	99	KU245732
9	5-BBD-M5	BBD	-	-	-	-
10	5-BBD-M6	BBD	KX146441	<i>Vibrio harveyi</i> strain QH141026D	99	KU245732
11	5-BBD-M10	BBD	KX146445	<i>Vibrio harveyi</i> strain bb4	99	KC455398
12	5-BBD-M12	BBD	KX146446	<i>Vibrio</i> sp. sw0106-05	100	KT276382
13	5-BBD-M4	BBD	KX146448	<i>Vibrio tubiashii</i> strain T33	99	KP329558
14	5-BSML-S17	BSML	KX146449	<i>Vibrio</i> sp. PaH2.06c	99	GQ406738
15	5-HSML-S16	HSML	-	-	-	-
16	5-HSML-S1	HSML	-	-	-	-

ATCC = American Type Culture Collection. 'M' in isolate designation refers to culture isolated using marine agar medium; 'S' in isolate designation refers to culture isolated using sea water tryptone agar medium. NA = not applicable, '-' = cultures that failed to grow for 16S rRNA gene identification.

Table 3-3: Percentages of AHLs produced by each isolate across three temperatures and six time points (pooled and averaged replicate sample values) as analyzed by LC-MS/MS.

Isolate number	AHLs detected							
	3OHC4	C5	3OHC5	3OC5:1	C6	3OHC6	3OHC8	3OC12
1	2.6%	-	-	-	97.4%	-	-	-
2	-	1.5%	-	2.7%	95.8%	-	-	-
3	-	-	-	-	100%	-	-	-
4	98.7%	-	1.3%	-	-	-	-	-
5	6.5%	-	-	-	93.5%	-	-	-
6	96.4%	-	3.6%	-	-	-	-	-
7	98.3%	-	1.7%	-	-	-	-	-
8	45.4%	-	-	-	54.6%	-	-	-
9	37.9%	-	-	-	62.1%	-	-	-
10	98.6%	-	1.4%	-	-	-	-	-
11	98.6%	-	1.4%	-	-	-	-	-
12	31.2%	-	-	-	-	68.8%	-	-
13	66.7%	-	-	-	-	32.0%	1.3%	-
14	99.2%	-	-	-	-	0.8%	-	-
15	12.9%	-	-	-	-	78.9%	-	8.2%
16	75.7%	-	-	-	-	3.8%	-	20.5%
Number of isolates producing the corresponding AHL (out of 16)	14	1	5	1	6	5	1	2

Table 3-4: Results of univariate ANOVA analysis of pooled AHL production by all isolates based on temperature.

AHL	Temperatures compared	Isolate ID										Overall significance across isolates
		2	4	6	7	10	11	12	13	14	15	
30HC4	24°C vs. 27°C	-	NS	NS	NS	NS	NS	0.001	NS	NS	0.014	0.216
	27°C vs. 30°C	-	NS	NS	NS	NS	NS	0.000	0.045	0.001	0.000	0.000
	24°C vs. 30°C	-	NS	0.001	NS	0.000						
30HC5	24°C vs. 27°C	-	0.029	0.050	0.040	0.004	0.014	-	-	-	-	0.000
	27°C vs. 30°C	-	NS	NS	NS	0.000	0.002	-	-	-	-	0.000
	24°C vs. 30°C	-	0.001	0.001	0.000	0.000	0.000	-	-	-	-	0.000
30HC6	24°C vs. 27°C	-	-	-	-	-	-	0.018	NS	0.002	0.000	0.000
	27°C vs. 30°C	-	-	-	-	-	-	NS	NS	0.007	0.034	0.031
	24°C vs. 30°C	-	-	-	-	-	-	NS	NS	NS	0.001	0.005
30CS:1	24°C vs. 27°C	0.012	-	-	-	-	-	-	-	-	-	0.012

All time points were also pooled across all replicates ($P \leq 0.05$). Comparisons of AHL quantitation were done in pairs (24°C vs. 27°C, 27°C vs. 30°C, 24°C vs. 30°C). '-' = absence of AHL production, NS = non-significant AHL production, +0.000 indicates that the p values were very small. Only data for AHLs that yielded a significant difference per temperature comparison are shown.

Table 3-5: Comparison of AHL production by isolates at 24 °C vs 30 °C.

Isolate no.	3OHC4		3OHC5		3OHC6	
	24°C	30°C	24°C	30°C	24°C	30°C
4	NS	NS	Lower	Higher	-	-
6	NS	NS	Lower	Higher	-	-
7	NS	NS	Lower	Higher	-	-
10	NS	NS	Lower	Higher	-	-
11	NS	NS	Lower	Higher	-	-
14	Lower	Higher	-	-	Lower	Higher

Only AHLs for which there were statistically significant ($P < 0.05$) results are shown. '-' = absence of AHL production, NS = non-significant AHL production.

Table 3-6: Comparison of AHLs produced by isolates in terms of isolate origin across three coral health states.

	Isolate source	
	BBD infected coral (BBD) (n = 13)	SML of healthy coral (HSML) (n = 2)
3OHC4	3OHC4	3OHC4
3OHC6	3OHC6	3OHC6
C5	3OC12	
3OHC5		
3OC5:1		
C6		
3OHC8		

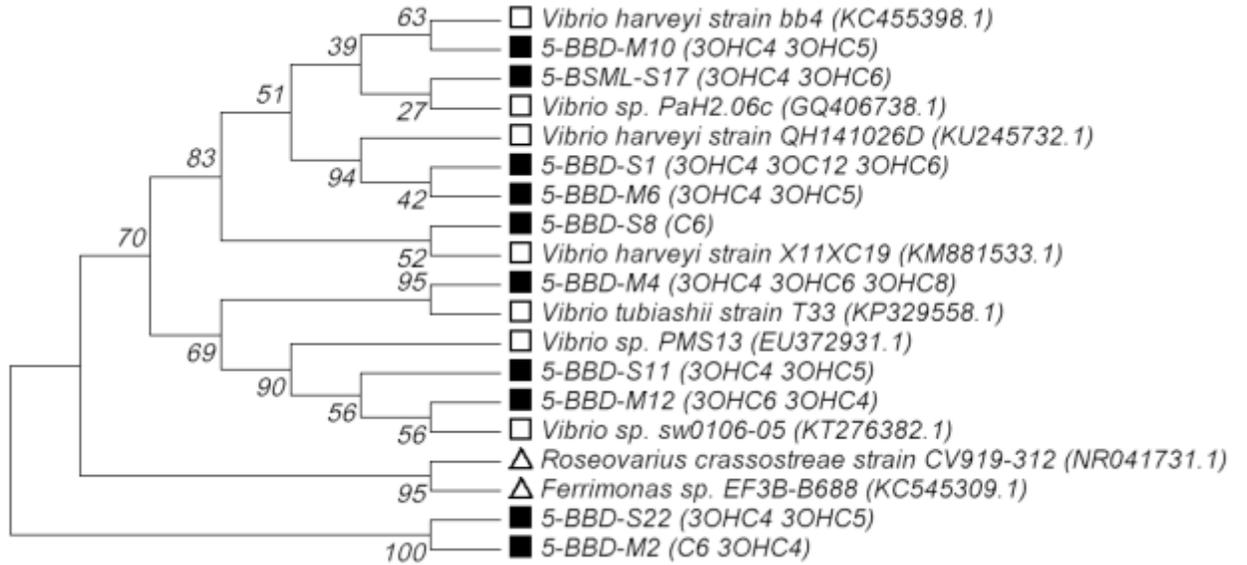
Shaded rows indicate AHLs that were produced by isolates from all three coral health states. Bolded AHLs were observed to have a significant increase in production at 30°C (compared to 24°C) across one or more of their producing isolates.

Table 3-7: Functional annotations of two BBD metagenomes within the subsystem category of Regulation and Cell Signaling in MG-RAST.

Sample	Coral species	Functional annotation of the genetic sequence		Avg. e value	Avg. % identity	Avg. align. length (aa)	No. of hits
Florida Keys	<i>Colpophyllia natans</i>	AHL autoinducer quorum sensing	Autoinducer synthesis protein LuxI	-14.29	80.08	46.38	5
Curaçao	<i>Pseudodiploria strigosa</i>	AHL autoinducer quorum sensing	Autoinducer synthesis protein LuxI	-10	71.14	42.83	5
Florida Keys	<i>Colpophyllia natans</i>	AHL autoinducer quorum sensing	N-Acyl-L-homoserine lactone synthetase RhII	-13.36	80.81	44.41	1
Florida Keys	<i>Colpophyllia natans</i>	AHL autoinducer quorum sensing	Transcriptional activator protein LuxR	-13.26	74.22	47.53	5
Curaçao	<i>Pseudodiploria strigosa</i>	AHL autoinducer quorum sensing	Transcriptional activator protein LuxR	-9.75	72.4	42	3
Florida Keys	<i>Colpophyllia natans</i>	Quorum sensing in <i>Vibrio</i>	8-Amino-7-oxononanoate synthase/CqsA	-18.5	83.15	49.5	2
Florida Keys	<i>Colpophyllia natans</i>	Quorum sensing in <i>Vibrio</i>	Autoinducer 2 sensor kinase/phosphatase LuxQ	-7.67	68.48	41	9
Florida Keys	<i>Colpophyllia natans</i>	Quorum sensing in <i>Vibrio</i>	Autoinducer 2-binding periplasmic protein LuxP precursor	-13.8	82.28	42.94	11
Florida Keys	<i>Colpophyllia natans</i>	Quorum-sensing in <i>Vibrio</i>	N-(3-Hydroxybutanoyl)-L-homoserine lactone synthase LuxM	-19	95.92	49	1
Florida Keys	<i>Colpophyllia natans</i>	Quorum sensing in <i>Vibrio</i>	Quorum-sensing regulator of virulence HapR	-10.21	72.77	44.42	5
Florida Keys	<i>Colpophyllia natans</i>	Quorum sensing in <i>Vibrio</i>	Regulatory protein LuxO	-12.63	75.67	45.78	10
Curaçao	<i>Pseudodiploria strigosa</i>	Quorum sensing in <i>Vibrio</i>	Regulatory protein LuxO	-11.95	74.74	45.92	6
Florida Keys	<i>Colpophyllia natans</i>	Quorum sensing in <i>Vibrio</i>	S-Ribosylhomocysteine lyase/autoinducer-2 production protein LuxS	-12.76	77.39	44.87	32
Curaçao	<i>Pseudodiploria strigosa</i>	Quorum sensing in <i>Vibrio</i>	S-Ribosylhomocysteine lyase/autoinducer-2 production protein LuxS	-13.08	71.85	48.58	18
Florida Keys	<i>Colpophyllia natans</i>	Quorum sensing: autoinducer-2 synthesis	S-Adenosylmethionine synthetase	-17.59	85.16	50.02	256
Curaçao	<i>Pseudodiploria strigosa</i>	Quorum sensing: autoinducer-2 synthesis	S-Adenosylmethionine synthetase	-19.1	83.02	54.46	246
Curaçao	<i>Pseudodiploria strigosa</i>	Quorum sensing in <i>Yersinia</i>	N-Acyl homoserine lactone hydrolase	-18.5	74.58	54	1
Florida Keys	<i>Colpophyllia natans</i>	Quorum-sensing regulation in <i>Pseudomonas</i>	BarA-associated response regulator UvrY	-11.5	68.82	48.71	36
Curaçao	<i>Pseudodiploria strigosa</i>	Quorum-sensing regulation in <i>Pseudomonas</i>	BarA-associated response regulator UvrY	-10.1	72.99	42.81	16
Florida Keys	<i>Colpophyllia natans</i>	Quorum-sensing regulation in <i>Pseudomonas</i>	N-Acyl-L-homoserine lactone synthetase RhII	-13.36	80.81	44.41	1
Curaçao	<i>Pseudodiploria strigosa</i>	Quorum-sensing regulation in <i>Pseudomonas</i>	Vfr transcriptional regulator	-16.5	84.45	47.5	1

Max. e value cutoff = 10^{-5} , min. % identity cutoff = 60%, and min. alignment length cutoff = 40 aa. Average length of fragments obtained was 145 base pairs. Rows that are fully highlighted (gray) represent unique gene products associated with that sample.

Figure 3-1: Maximum likelihood phylogenetic tree based on 16S rRNA gene sequences of isolates used in this study and their closest relatives in GenBank. Shaded squares indicate the isolates used in this study, hollow squares indicate closest relatives of the isolates in GenBank, and hollow triangles indicate sequences that were used for rooting the phylogenetic tree. Values at the nodes are bootstrap values after 1000 resamplings. AHLs produced by the isolates are indicated next to the isolate designation in order of AHL production, from highest to lowest.



CHAPTER 4

EXPLORING THE PRESENCE AND POTENTIAL INFLUENCE OF
SECONDARY METABOLITES PRODUCED BY BLACK BAND DISEASE
HETEROTROPHS WITHIN BBD

Abstract

Black band disease (BBD) of corals, an infectious polymicrobial mat disease, is dominated in biomass by cyanobacteria, accompanied by other functional groups that include sulfate reducers, sulfide oxidizers and an exhaustive collection of heterotrophs. The heterotrophs within BBD have been proposed to harbor primary and secondary potential pathogens. In the present study, culture dependent and culture independent approaches were employed to evaluate the production and implication of two significant secondary metabolites potentially produced by BBD heterotrophs, antimicrobial compounds and quorum sensing (QS) signal molecules. Antimicrobial production by heterotrophs isolated from healthy, diseased and the healthy part of BBD infected corals were tested against two ecologically relevant and possibly significant bacterial strains in BBD, *Roseovarius crassostreae* and *Ferrimonas* sp. Isolates from BBD were also tested for acyl homoserine lactone production, a signaling molecule that enables quorum sensing (QS) amongst bacteria. Additionally, four BBD metagenomes were evaluated for the presence of genes associated with antimicrobial production. Quorum sensing, antimicrobial synthesis, and antimicrobial resistance genes were compared with other non-diseased publicly available coral metagenomes. The proteobacterial populations across all the metagenomes were also compared. In the antimicrobial assays, isolates from the healthy portion of BB-diseased corals, as compared to isolates from healthy corals, displayed greater inhibition against test strains. When comparing metagenomes, both BBD as well as non-diseased coral metagenomes contained comparable gene sequences for ‘Secondary metabolism’ with no significant differences across groups. Isolates from BBD displayed AHL production, which corroborated with the presence of

gene sequences for QS and biofilm formation in BBD metagenomes. A vast repertoire of genes for resistance against antibiotics and toxic compounds was also revealed and an increase in fluoroquinolone resistance genes in BBD metagenomes was detected. The presence of alpha- and gammaproteobacteria was detected in all metagenomes while delta- and epsilonproteobacteria were found only in BBD metagenomes. The observations from this study are aimed at improving the understanding of the structure and community dynamics within the BBD heterotroph population, with the overall goal of elucidating the mechanisms of this intricate coral disease.

Introduction

The global decline in coral reefs as an effect of climate change and coral disease has made the study of coral associated microbes increasingly crucial (Rosenberg and Ben Haim 2002; Weil et al. 2006; Bruno and Selig 2007; Harvell et al. 2007). Black band disease of corals is one such coral disease causing damage to colossal reef building corals (Kuta and Richardson 1997; Richardson 2004). First reported in 1973, (Antonius 1973) the polymicrobial mat BBD constructs a toxic microenvironment on the coral colony with the assistance of photosynthetic cyanobacteria, sulfate reducing and sulfide oxidizing bacteria and a sizeable catalogue of heterotrophic bacteria to eventually cause tissue lysis leading to coral death (Garrett and Ducklow 1975; Ducklow and Mitchell 1979; Rützler et al. 1983; Miller and Richardson 2011). The rate of infection of this highly virulent global disease has a minimum temperature threshold of 27.5 °C (Edmunds 1991; Voss and Richardson 2006a). The heterotrophic population of BBD has often been estimated to shelter potentially significant pathogens capable of structuring bacterial communities

required for formation and progression of the migrating mat community (Cooney et al. 2002; Frias-Lopez et al. 2002; Miller and Richardson 2011).

A vast and varied microbial population resides within the surface mucopolysaccharide layer (SML) of healthy as well as diseased corals (Brown and Bythell 2005). The SML itself is a good source of nourishment for probiotic as well as pathogenic/opportunistic invaders (Kvennefors et al. 2012). Owing to the close vicinity of the water column to coral SML, bacteria from the column are regularly recruited into the mucus, permitting transfers to and from these two habitats (Rohwer et al. 2002; Kuek et al. 2015).

Microbes in the coral SML perform a variety of functions such as occupying the entry niche to prevent overgrowth of undesirable organisms, scavenging limiting nutrients in the oligotrophic environment (Rohwer et al. 2002), and production of secondary metabolites such as antimicrobials and signaling molecules (Hunt et al. 2012; Kvennefors et al. 2012). Since bacteria attached to healthy coral mucus are not subjected to mucus regulated bacterial selection, they could behave opportunistically under stressful conditions such as increased temperatures (Ritchie 2006; Kuek et al. 2015). Such an environmental change causes a shift in coral bacterial community and dynamics (Reshef et al. 2006). A modeling approach by Mao-Jones et al. (2010), demonstrated that exposure to thermal stress shifted the microbial community from being dominated by antibiotic producing microbes to being governed by pathogens, and that long after the stress was removed, the shift in microbial community persisted (Mao-Jones et al. 2010). Glasl et al. (2016) recently demonstrated that exposure of coral colonies to a blend of antibiotics rendered them vulnerable to disease and bleaching, demonstrating the significance of the resident probiotic microbiota of healthy coral SML. Furthermore, multiple studies have presented the potential of healthy and diseased coral microbial

communities to produce quorum sensing (QS) signal molecules (Skindersoe et al. 2007; Tait et al. 2010; Golberg et al. 2011; Alagely et al. 2011; Taylor et al. 2013; Ransome et al. 2014; Munn 2015), suggesting the potential for cell-cell signaling within the coral holobiont. The density dependent signaling phenomenon has been evidenced in a vast selection of bacteria worldwide (Nealson et al. 1970; Nealson and Hastings 1979; Waters and Bassler 2005a), through induction of genes for enhanced bacterial features, including production of antimicrobials, biofilm formation, resistance to antimicrobials and toxic compounds, and induction of virulence genes (Williams et al. 2000; Irie and Parsek 2008; Bandara et al. 2012; Hmelo 2017).

The heterotrophic population within BBD is comprised of numerous classes of proteobacteria including alpha, gamma, epsilon, and delta (Frias-Lopez et al. 2004; Sekar et al. 2006; Sekar et al. 2008; Sato et al. 2017). Several reports have discovered the incidence of an interesting alphaproteobacterium, *Alliroseovarius crassostreae*, consistently associated with the BBD mat (Cooney et al. 2002; Sekar et al. 2006; Sekar et al. 2008; Sato et al. 2010; Miller and Richardson 2011; Miller 2012; Richardson 2012). An OTU with 94-97% identity to the 16S rRNA gene of *A. crassostreae* was disclosed to be the second most abundant OTU across 87 BBD clone libraries (Miller and Richardson 2011). This alphaproteobacterium is the causative agent of Juvenile Oyster Disease (JOD), now known as *Roseovarius* Oyster Disease (ROD), causing conchiolin deposits in juvenile oysters, resulting in mass mortalities (Bricelj et al. 1992; Ford and Borrero 2001; Maloy et al. 2007). The occurrence and role of such a distinctive sequence within BBD advocates further study. Another bacterial species, *Ferrimonas* sp., has also been detected in BBD clone libraries (Sekar et al. 2006). Along with known antimicrobial properties,

several *Ferrimonas* strains are iron reducing. Iron reduction is a known virulence factor (Wooldridge and Williams 1993), making this species of specific interest within the virulent BBD community.

Black band disease progresses as a polymicrobial consortium of several operative participants, wherein the metabolism and by-products of individuals from each functional category affect one another. Cyanobacteria from the mat have displayed inhibition as well as stimulation of growth of heterotrophic bacterial isolates from the BB-diseased mat in in-vitro studies (Gantar et al. 2011). Bacteria from healthy and black band diseased corals were shown to inhibit as well as stimulate growth in varying percentages of isolates when tested against each other (Zimmer et al. 2014). The same study also revealed the AHL and AI-2 producing capabilities of BBD bacterial community (Zimmer et al. 2014). A significantly higher number of bacterial isolates from BBD have been shown to produce AHLs as compared to bacteria isolated from apparently healthy corals (Bhedi et al. 2017).

BBD heterotrophic bacteria, the most phylogenetically diverse group in the disease mat, are likely to exhibit variety in their metabolic functioning, facilitating disease progression. In the present study, the antimicrobial and QS molecule production capabilities of cultured heterotrophs from BBD are explored. Additionally, BBD metagenomes are screened for secondary metabolism sequences including antimicrobial production, quorum sensing and antimicrobial resistance.

Materials and methods

Sample collection and bacterial isolation

Samples of BBD, SML from apparently healthy coral (HSML), and SML from

apparently healthy portions of BBD infected coral (BSML) were collected from *Montastrea cavernosa* (Table 1) as previously used and described in Bhedi et al. 2017. Additionally, in the present study *P. strigosa* isolates (N = 119) were used for AHL screening using a reporter strain (CV026 assay) and LC-MS/MS (AHL detection). Both *P. strigosa* and *M. cavernosa* isolates were used for antimicrobial assays (N = 229) (Table 1). Bacterial cultures from the BBD samples were isolated as explained previously (Bhedi et al. 2017).

Antimicrobial production assays

The antimicrobial production proficiencies of isolates were verified by an agar overlay technique against the strains *Ferrimonas* sp. EF3B-B688 (isolated from BBD) and *A. crassostreae* CV919-312 (ATCC strain) as targets. For antimicrobial testing, Difco MA basal plates (containing 1.5% agar) were overlaid with 5 ml molten MA (containing 0.7% agar) seeded with 100 µl of freshly grown broth culture of either *A. crassostreae* or *Ferrimonas*. Overlaid plates were allowed to dry for 15 min and spot inoculated with freshly grown isolates. Each plate contained a known antimicrobial producing strain as a positive control and sterile Difco Marine Broth (MB) as negative control. The plates were incubated at 25 °C and monitored for zones of inhibition (ZOI) over a period of 48 hrs. To compare results across health states, in each case, ZOI from isolates from BBD as a health state was compared against HSML or BSML via a proportion z-test for significance ($p \leq 0.05$).

Testing for AHL producing isolates

A total of 119 isolates from BBD were tested for production of AHLs with an agar overlay method using *Chromobacterium violaceum* CV026 as described in chapter 3

(‘*Chromobacterium violaceum* CV026 AHL reporter strain assay’ under Materials and Methods) (Bhedi et al. 2017). *R. crassostreae* CV919-312 and *Ferrimonas* sp. EF3B-B688, strains used for the antimicrobial assay were also tested via LC-MS/MS for AHL production.

AHL detection via LC-MS/MS

All isolates positive for production of AHLs via the CV026 assay were analyzed using LC-MS/MS for AHL identification and abundance variation across three temperatures (24, 27 and 30 °C) as described in chapter three (Bhedi et al., 2017) in the laboratory of DR. Shawn Campagna at University of Tennessee, Knoxville.

16S rRNA gene sequencing of AHL producing isolates

16S rRNA gene sequencing was performed on the five isolates that produced AHLs as described in chapter three. Isolates 1 and 2 (Figure 2) were previously described in Zimmer et al. 2014. Isolates 3 and 4 were submitted under the accession numbers KX353778, KX353777 to GenBank. Isolate 5 failed to grow during 16S rRNA gene sequencing.

Metagenomic analysis

Black band disease samples were collected from Horseshoe Reef in the Florida Keys and Curaçao (CARMABI station) from *Colpophyllia natans* (samples 1, 2 and 4) and *Pseudodiploria strigosa* (sample 3) and used for metagenomic analysis. All samples post collection were added into tubes containing RNA later, stored at -80 °C, and sent to the Microbiome Analysis Center to Dr. Patrick Gillevet at George Mason University in Fairfax, Virginia. Metagenomic analysis was carried out as described in chapter three (Bhedi et al., 2017).

Metagenomic analysis from publicly available metagenomes

For comparison of the metagenomes of BB-diseased vs. non-diseased samples, the four BBD samples described above were analyzed and compared with six publicly available metagenomes using MG-RAST (<http://metagenomics.anl.gov/>) (Meyer et al. 2008). ‘Coral atoll samples 1-5’ (4466596.3, 4466597.3, 4466810.3, 4466812.3, 4466844.3 respectively) were used from Dinsdale et al. (2011) (samples with the most coral cover were selected for analysis) and the ‘Apparently healthy coral’ metagenome (4445756.3) was used from Littman et al. (2011) (Dinsdale et al. 2008; Littman et al. 2011). Analyses were compared across levels 1, 2, 3 and 4 categories of subsystems database following a hierarchical classification of annotation (level 4 ‘also denoted as ‘function’ in subsystem). For functional metagenomic analysis throughout the study, the following parameters were used: Databases = Subsystems for functional analysis, RDP for phylogenetic analysis; Maximum e value = 10^{-5} ; Minimum alignment length = 40 aa for protein databases, 90 bp for RNA databases; Minimum percentage identity = 60% for protein databases, 98% for RNA databases.

Results

Antimicrobial production of by isolates from HSML, BSML, and BBD

A total of 229 isolates were tested for antimicrobial production against *Ferrimonas* sp. EF3B-B688 and *Alliroseovarius crassostreae* CV919-312. Of these, 155 were isolated from the black band mat, 38 from HSML and 36 from BSML (Table 1). Twenty-three isolates across health states inhibited *A. crassostreae* CV919-312 (14.8% of isolates tested) (Figure 1). Of these 23 isolates, 14 (9.0%) were BBD isolates, five (13.9%) were from the healthy part of BBD infected coral (BSML) and four (10.5%) were from the

SML of apparently healthy coral (HSML). Inhibition of growth of *Ferrimonas* sp. (EF3B-B688) was caused by 15 (6.6%) of the isolates across all health states. Of the 15 isolates, nine (23.7%) were from HSML, two (5.6%) were from BSML and 4 (2.6%) were from BBD. Sixteen (6.9%) of the isolates inhibited the QS reporter strain, *Chromobacterium violaceum* CV026. Within these 16, nine (23.7%) isolates originated from HSML and seven (19.4%) from BSML. None of the isolates from BBD inhibited the reporter strain.

For *R. crassostreae* CV919-312, both comparisons of BBD vs. HSML and BBD vs. BSML were not significantly different at $p \leq 0.05$. For isolates inhibiting *Ferrimonas* EF3B-B688, BBD vs. BSML was not significant while the comparison between BBD vs. HSML was significant ($p \leq 0.05$) with a greater number of isolates from HSML inhibiting *Ferrimonas* EF3B-B688 as compared to BBD.

AHL production

The results for AHL detection across three temperatures (24, 27 and 30 °C) are presented in Figure 2. Along with five isolates that tested positive in the CV026 assay, AHL production by *R. crassostreae* CV919-312 and *Ferrimonas* sp. EF3B-B688 was also tested. Four AHLs were detected as produced by these seven isolates (Figure 2). C6 was the most frequently produced AHL, followed by 3OHC4. All seven bacterial strains showed some variation with temperature, however none of the variations were statistically significant at $p \leq 0.05$ (data not shown). Isolate five and *Ferrimonas* sp. both produced 3OHC4 dominantly as opposed to the other five bacteria.

Metagenomic analysis

Gene sequences identified in the Subsystems database (level 1) categories of

‘Regulation and cell signaling’ (including genes for QS), ‘Secondary metabolism’ (including genes for antibiotic and antimicrobial synthesis) and ‘Virulence, disease and defense’ (for antimicrobial resistance genes) were compared (Figure 3) across the four BBD samples. In each of the four BBD metagenomes, the gene sequences for ‘Virulence, disease and defense’ were higher than genes for ‘Secondary metabolism’ and ‘Regulation and cell signaling’ categories. The fewest number of gene sequences were observed in the ‘Regulation and cell signaling’ categories across BBD as well as non-diseased metagenomes (data shown only for BBD).

The percentage of gene sequences detected in the ‘Secondary metabolism’ Subsystem category among the BBD and publicly available metagenomes (Figure 4) included those for ‘Bacterial cytostatics, differentiation and antibiotics’ (level 2 category). These were higher in BBD samples two and three as compared to the atoll samples. Gene sequences for ‘Biosynthesis of phenylpropanoids’ were equivalent in BBD-Sample two and Coral atoll-Sample one and less or absent amongst the other samples. The ‘Apparently healthy coral’ sample (Littman et al. 2011) had no gene sequences in the category of ‘Secondary metabolism’ within the MG-RAST Subsystems database. After pooling hits to compare all diseased (four BBD metagenomes) vs. all non-diseased metagenomes (coral atoll samples), sequences under level 1 category of ‘Secondary metabolism’ had no significant difference. However, sequences under level 2 categories of ‘Bacterial cytostatics, differentiation and antibiotics’ and ‘Biosynthesis of phenylpropanoids’ were significantly higher in the BBD metagenomes (z-proportion test, $p \leq 0.05$).

Figure 5 shows the level 1 category of ‘Regulation and cell signaling’ displayed as gene sequences annotated under level 4, also denoted as ‘function’ in subsystems

hierarchical classification. Sequences for *S*-adenosyl methionine synthase (level 4) were most dominant and present across most samples investigated. As with ‘Secondary metabolism’, sequences under ‘Regulation and cell signaling’ were also perceived to be of larger variety in non-diseased metagenomes as compared to BBD metagenomes. Apart from BBD-Sample 3, all BBD metagenomes had *S*-adenosyl methionine synthase sequences in great abundance. Sequences for biofilm synthesis (PgaA, PgaB, PgaC) (level 4) were also noted in a few analyzed metagenomes (Coral atoll-Samples 1, 3, 5).

Figure 6 indicates the vast range of level 3 sequences under level 2 category of ‘Resistance to antibiotics and toxic compounds’, a subcategory under level 1 of ‘Virulence, disease and defense’. Most of the sequences represented were indicative of metal resistance genes including zinc, mercury, copper, cobalt, cadmium and arsenic. Several other sequences represented the ability to breakdown antimicrobials, specifically sequences for resistance to antibiotics such as fluoroquinolones, vancomycin, methicillin, and erythromycin. Four types of multidrug efflux pumps/system sequences were also unveiled. Other than the ‘Apparently healthy coral’ metagenome (Littman et al. 2011), all other metagenomes had sequences for resistance to fluoroquinolones in higher percentages, with BBD metagenomes having a higher percentage of sequences in the category in contrast to coral atoll metagenomes.

Figure 7 shows the taxonomic affiliation and sequence abundance (percentages) of proteobacteria across all analyzed metagenomes using the RDP database. Alpha and gammaproteobacteria were most abundant in all metagenomes. BBD samples additionally contain delta- and epsilonproteobacteria, while these were markedly absent in the other metagenomes.

Discussion

Antimicrobial assays

The results of the antimicrobial assays using the *Ferrimonas* strain showed that the strain was inhibited by a significantly higher number of isolates from HSML (13.9%) as compared to BBD (2.6%). Species from the *Ferrimonas* genus have been previously isolated from the mucus of healthy *Acropora millepora* (Kvennefors et al. 2012). One particular strain, *Ferrimonas* sp. A3B-64-2, isolated from the mucus of a healthy coral, demonstrated the ability to inhibit the pathogen of coral bleaching, *Vibrio shiloi* (Nissimov et al. 2009). *Ferrimonas marina* has been isolated previously from a black band diseased colony of *Siderastrea sideria* (Sekar et al. 2006). BBD metagenomes used in this study also showed presence of several strains of *Ferrimonas* (data not shown). The presence of *Ferrimonas* under both healthy and diseased conditions in corals may indicate its functionality as an opportunistic pathogen in BBD.

Isolates from the BSML community had the highest number of inhibitors of *R. crassostreae* (13.9%), followed by HSML isolates at 10.5% and BBD isolates at 9.0%. Comparing inhibitors across the BBD community, *R. crassostreae* had a greater number of inhibiting isolates than *Ferrimonas*. *R. crassostreae*, apart from being consistently present in BBD clone libraries (Miller and Richardson 2011), has additionally been associated with several other coral diseases, such as white plague-like disease (Pantos et al. 2003), white band disease (Pantos and Bythell 2006) and lately, Australian tropical white syndrome (Godwin et al. 2012). Along with *R. crassostreae*, *R. nubinhibens* has been isolated from BBD infecting *Siderastrea sideria* (Sekar et al. 2006). BBD metagenomes used in this study also showed the presence of *R. nubinhibens* (data not

shown). Several members of marine *Rhodobactereaceae* have demonstrated pathogenic traits, including QS (Cude and Buchan 2013). Both the cases of inhibition against *Ferrimonas* and *R. crassostreae* that are exemplary of isolates from the healthy coral SML/healthy portion of BBD infected coral suggest inhibition of putative pathogenic/opportunistic bacteria.

AHL analysis

From the QS results of this study, it was found that AHL C6 was manufactured by all seven bacterial cultures tested. Of these seven cultures, five bacterial strains produced C6 dominantly, as compared to the other three identified AHLs (3OHC4, C5, 3OHC5). These observations are in agreement with findings from previous studies, where C6 (Zimmer et al., 2014) and 3OHC4 (Bhediet al., 2014) were the predominantly produced AHLs. However, due to the small number of isolates tested, a statistically significant change of AHL production with varying temperature was not detected in this study.

Quorum sensing assisted by AHLs has been symptomatic of pathogenicity in a vast number of bacteria in across various habitats. The precise and targeted functionality of AHLs in BBD pathogenicity remains to be studied further. Nevertheless, the ability of these isolates to produce AHLs suggests a role of some prominence of bacterial cell signaling in BB-disease etiology.

Metagenomic analysis of QS, antimicrobial synthesis, and antibiotic resistance sequences

The percentage of sequences associated with ‘Virulence disease and defense’ was higher than that for ‘Secondary metabolism’ and ‘Regulation and cell signaling’ across BBD metagenomes (Figure 3). Additionally, a great variety of sequences associated with

‘Resistance to antibiotics and toxic compounds’ was detected in BBD as well as non-diseased coral metagenomes. Several of these included sequences for metal resistance encompassing zinc, chromium, mercury, copper, cobalt, cadmium and arsenic. Genes for heavy metal resistance in BBD, particularly metallothionein, were recently revealed in a metagenomic and transcriptomics study (Sato et al. 2017). Some studies have indicated an association between heavy metal resistance and higher antibiotic resistance (Pal et al. 2015). A great variety of antibiotic and antimicrobial resistance gene sequences were also observed in the present study, along with the occurrence of several multidrug efflux pumps, within BBD. In comparison to the coral atoll metagenomes, BBD metagenomes had a greater percentage of sequences for resistance to fluoroquinolones. Gene sequences for fluoroquinolone resistance have been previously been detected in microbes of corals (Wegley et al. 2007). The increased percentage of antibiotic resistance sequences in BBD metagenomes likely benefits the BBD microbial population. Such gene sequences may be active in counteracting antimicrobials produced by the resident probiotic community, and therefore may aid in establishing/maintaining a virulent, antimicrobial resistant mat consortium (Nogales et al. 2011).

Biofilms are known to increase resistance of microbes to antimicrobials and antibiotics (Irie and Parsek 2008). The migrating BBD mat functions analogous to a complex biofilm, where enclosed microniches in the mat biofilm enable concentration of secondary metabolites such as antimicrobials and QS signals. The concentration of these molecules in BBD may influence structuring of the BBD community, speculatively facilitating disease progression. Additionally, horizontal gene transfer of antimicrobial

resistance genes may augment mat virulence, also encouraging black band disease development and/or advancement.

Proteobacterial abundance across metagenomes

Assessment of the types of proteobacteria within the metagenomes revealed the abundance of alpha- and gammaproteobacteria in metagenomes of both corals from atolls and BBD. However, delta- and epsilonproteobacteria were present only within BBD metagenomes. Previous studies have reported higher abundances of deltaproteobacteria sequences in BBD in comparison to healthy coral SML (Sekar et al. 2006; Meyer et al. 2017). In a recent study comparing the progression of lesions of relatively benign cyanobacterial patches (CP) to BBD mat, both delta- and epsilonproteobacterial sequences were reportedly higher in BBD metagenomes in contrast to CP datasets (Sato et al. 2017). The presence of sulfate reducing deltaproteobacteria in BBD likely represents the difference in abundances of these sequences between diseased and non-diseased metagenomes.

Potential implications of secondary metabolites in BBD

This study assessed the antimicrobial and AHL production of cultivable bacteria from healthy and BB-diseased coral health states and evaluated BBD and healthy coral metagenomes for sequences related to production of secondary metabolites and resistance. Production of these secondary metabolites is anticipated to directly affect the microbial community dynamics, structure and organization of BBD. However, the nature of the surrounding reef environment as well as the growth condition of the bacteria can also affect production and interaction of these molecules with each other (Bruhn et al. 2007; Horswill et al. 2007). BBD infects corals at warmer water temperatures, which possibly influences the production and abundance of these secondary metabolites. Bhedi

et al. (2017) recently showed an increase in the abundance of specific AHLs (3OHC4, 3OHC5 and 3OHC6) at higher temperatures by BBD isolates (Bhedhi et al. 2017).

From the metagenomic analysis targeted at antimicrobial synthesis and antimicrobial resistance genes, no significant difference in pooled sequences for ‘Secondary metabolism’ was detected across BBD vs. non-diseased coral metagenomes. However, a significantly higher number of sequences in BBD metagenomes were detected under level 3 categories for production of antibiotics and phenylpropanoids (categories ‘Bacterial cytostatics, differentiation and antibiotics’ and ‘Biosynthesis of phenylpropanoids’). One can contemplate that although the BBD metagenomes showed gene sequences that indicate an ability to produce antimicrobials, these compounds may degrade due to warmer temperatures, as has been shown previously (Mao-Jones et al. 2010; Rypien et al. 2010; Glasl et al. 2016). It would be interesting to examine the effect of temperature on antimicrobial production by experimentally quantifying their abundance while being produced by BBD isolates in an attempt to investigate their fate under ecological conditions mimicking BBD. QS has also been known to induce expression of antimicrobial production and antimicrobial resistance genes in several bacteria (Bandara et al. 2012). Presently, the influence of QS on antimicrobial production in BBD microbes is not known.

Summarizing the results of the present study, the antimicrobial assays indicated that, comparatively speaking, the fewest number of BBD isolates inhibited all three strains, *R. crassostreae*, *Ferrimonas* and *C. violaceum* CV026. Although only three strains were examined, these observations suggest that the BBD community exhibits lesser inhibition than bacteria within coral SML. There were more antimicrobial producing isolates from

HSML and BSML than from BBD, likely important in maintaining the probiotic role of SML bacteria, both in terms of preventing colonization and of killing invaders that successfully colonize this niche. On the other hand, under conditions of temperature stress, the abundance of antimicrobials may decrease (Mao-Jones et al. 2010; Rypien et al. 2010; Glasl et al. 2016), paving way for over-growth of opportunistic pathogens or invasion of new pathogens from the water column.

In the present study, production of QS signals by BBD isolates was detected and this result was shown to correlate with the presence of QS gene sequences in BBD metagenomes. QS in BBD may lead to the abundance of a highly diverse population of opportunistic heterotrophic secondary pathogens and may control the transition of non-pathogenic to pathogenic bacteria via expression of QS controlled pathogenic traits. QS may also facilitate horizontal gene transfer of antimicrobial resistance genes, enabling maintenance of the virulent and toxic mat. In this manner, the production of secondary metabolites, antimicrobial compounds, QS signaling molecules and antimicrobial resistance genes may play interactive roles in structuring the BBD community. Targeted studies need to be undertaken to investigate the precise role of these secondary metabolites in BBD pathogenesis.

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Tables

Table 4-1: Sampling dates, locations and bacterial isolates used in this study. All 229 isolates were used in the antimicrobial assays against *R. crassostreae* CV919-312, *Ferrimonas* sp. EF3B-B688 and *C. violaceum* CV026. All isolates from *P. strigosa* colonies (N=119) were additionally tested for AHL production via *C. violaceum* CV026 agar overlay assay. BBD = black band disease mat sample, HSML= sample taken from apparently healthy coral of the same host species, BSML= sample taken from the apparently healthy part of the BBD infected coral. The QS production capabilities of isolates from *M. cavernosa* have been previously used and described in Bhedi et al. 2017.

Date of sample collection	Coral host	Location	Sample type	No. of isolates	
9/30/2012	<i>Pseudodiploria strigosa</i>	Horseshoe Reef, Florida Keys, FL, USA (N 25°08.362' W 80°17.641')	BBD	8	119
2/23/2013	<i>Pseudodiploria strigosa</i>	Water Factory, Curaçao, Netherlands Antilles (N 12° 06.779' W 68° 57.662')	BBD	44	
5/25/2013	<i>Pseudodiploria strigosa</i>	Water Factory, Curaçao, Netherlands Antilles (N 12° 06.779' W 68° 57.662')	BBD	67	
8/19/2013	<i>Montastraea cavernosa</i>	Algae Reef, Florida Keys, FL, USA (N 25' 08.799 W 80' 17.579)	BBD	36	
			HSML	38	
			BSML	36	
Total number of isolates				229	

Figures

Figure 4-1: Differences in percentages of isolates from three coral health states, black band disease mat (BBD), apparently healthy portion of a BB-diseased coral (BSML) and apparently healthy coral SML (HSML), inhibiting *Ferrimonas* EF3B-B688 and *Roseovarius crassostreae* CV919-312 and the AHL reporter strain *Chromobacterium violaceum* CV026 ($p \leq 0.05$).

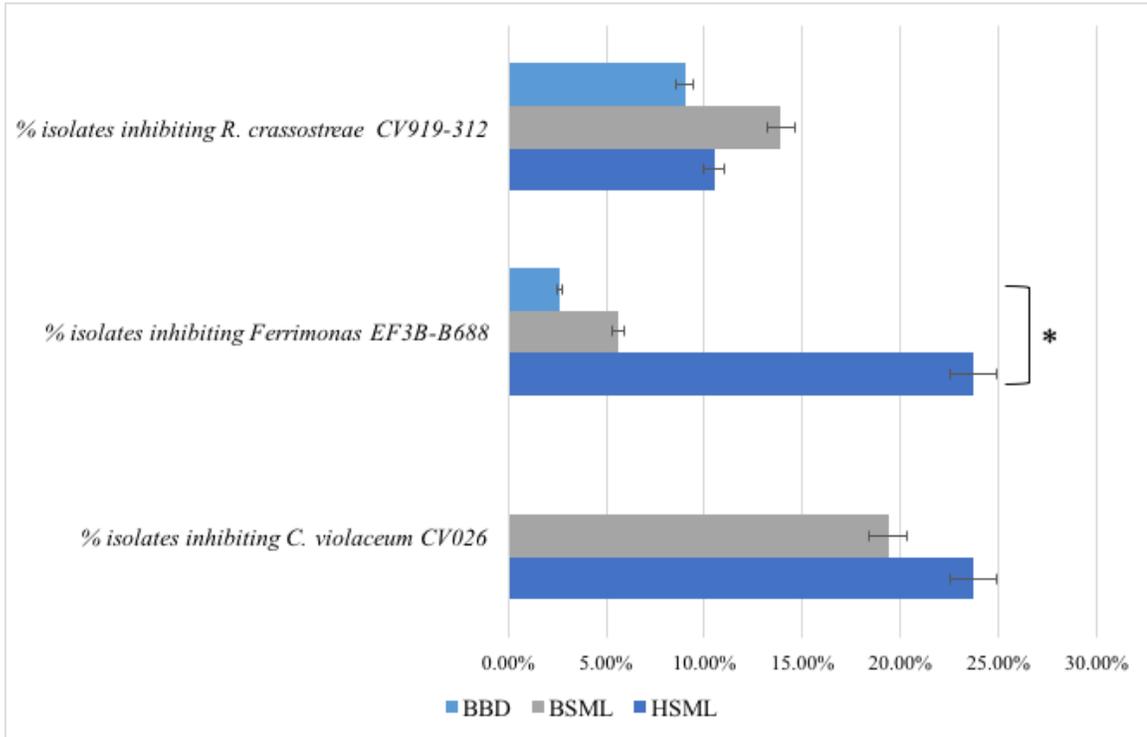


Figure 4-2: AHL production by BBD isolates, *R. crassostreae* CV919-312, *Ferriomonas* EF3B-B688 and influence of temperature on AHL abundance profiles.

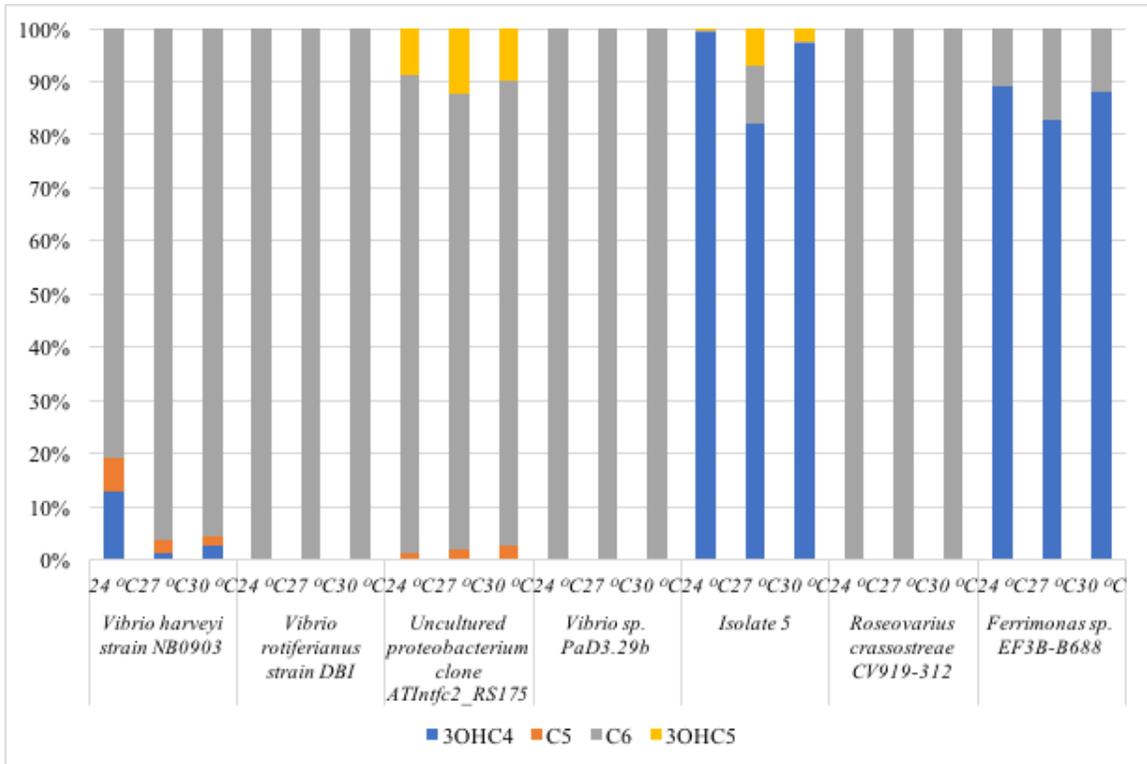


Figure 4-3: Differences in gene sequences of BBD metagenomes as annotated against Subsystems database, under categories of ‘Regulation and cell signaling’, ‘Secondary metabolism’ and Virulence, disease and defense’.

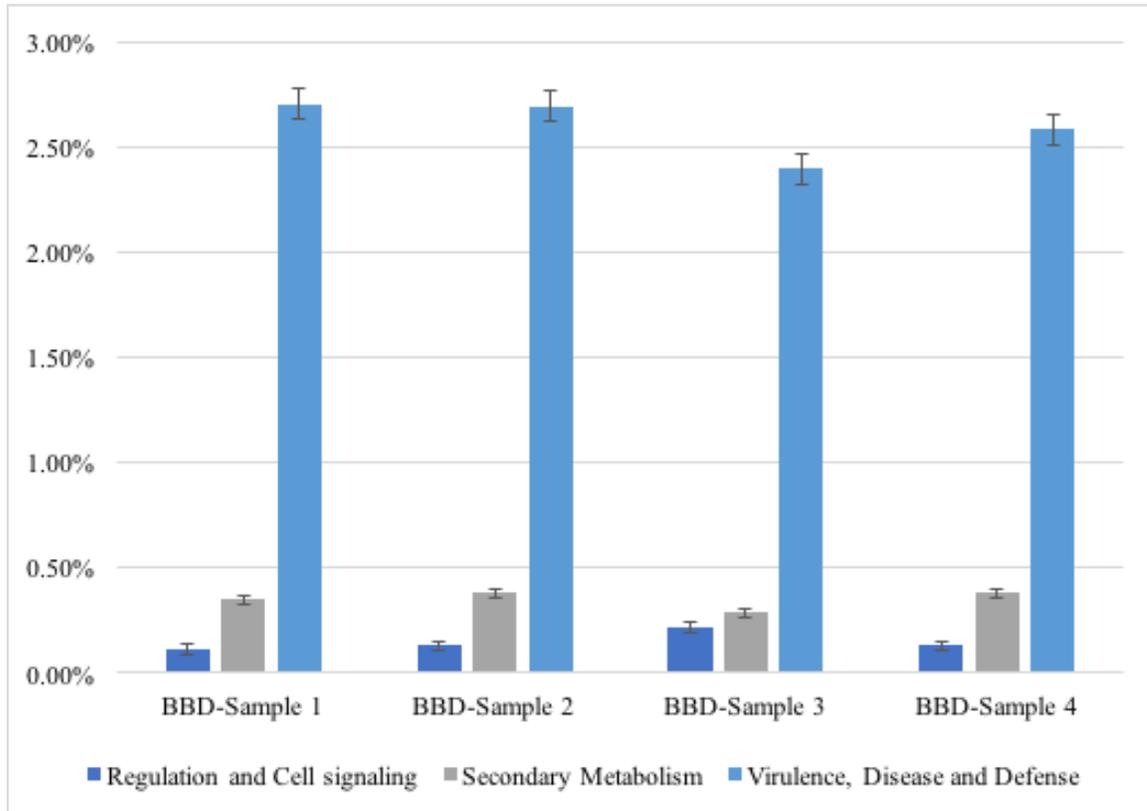


Figure 4-4: Examining secondary metabolites in metagenomes of BBD and comparing with coral atoll (Dinsdale et al. 2008) and apparently healthy coral metagenomes (Littman et al. 2011) with using subsystems database.

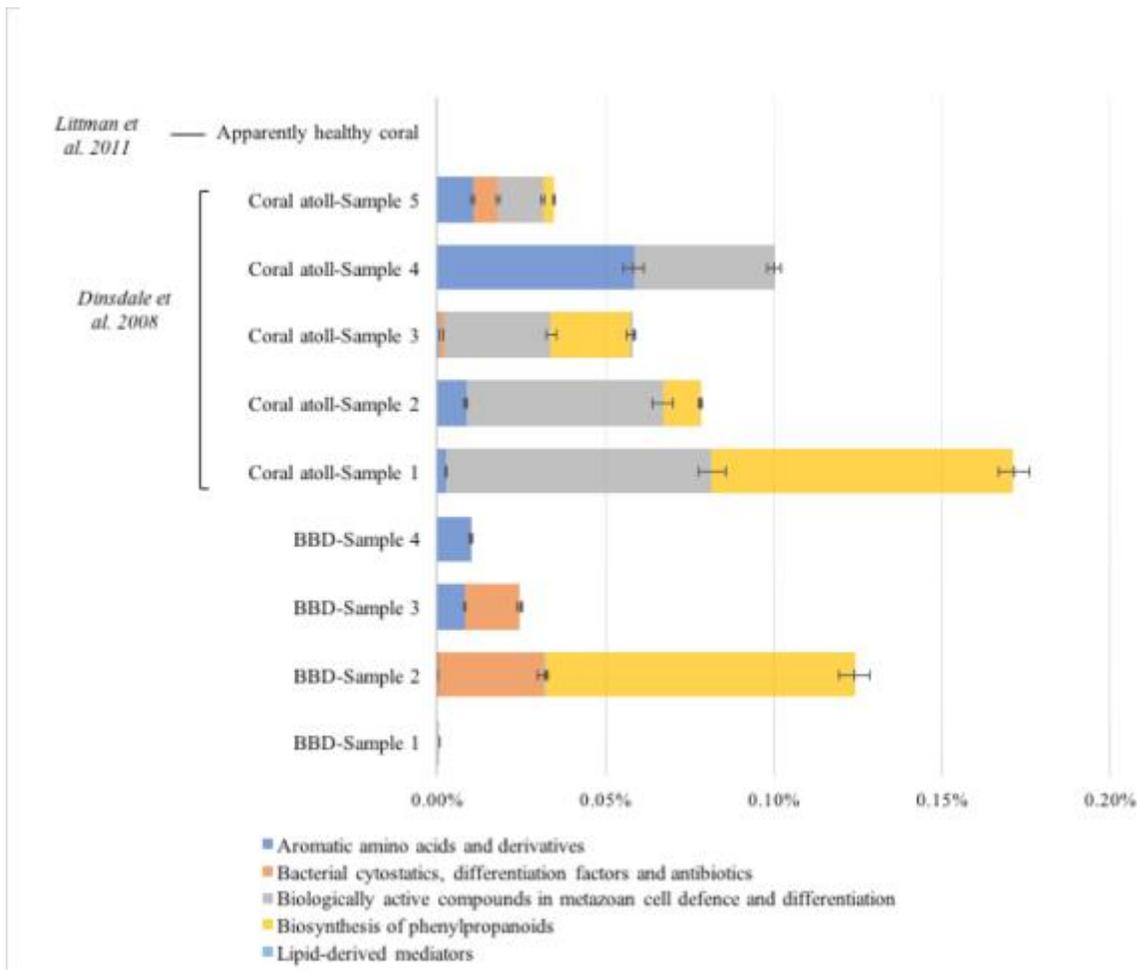


Figure 4-5: Sequences annotated using subsystems database as level 4 functions of ‘QS and biofilm formation’ while comparing BBD metagenomes, coral atoll metagenomes (Dinsdale et al. 2008) and apparently healthy coral metagenome (Littman et al. 2011).

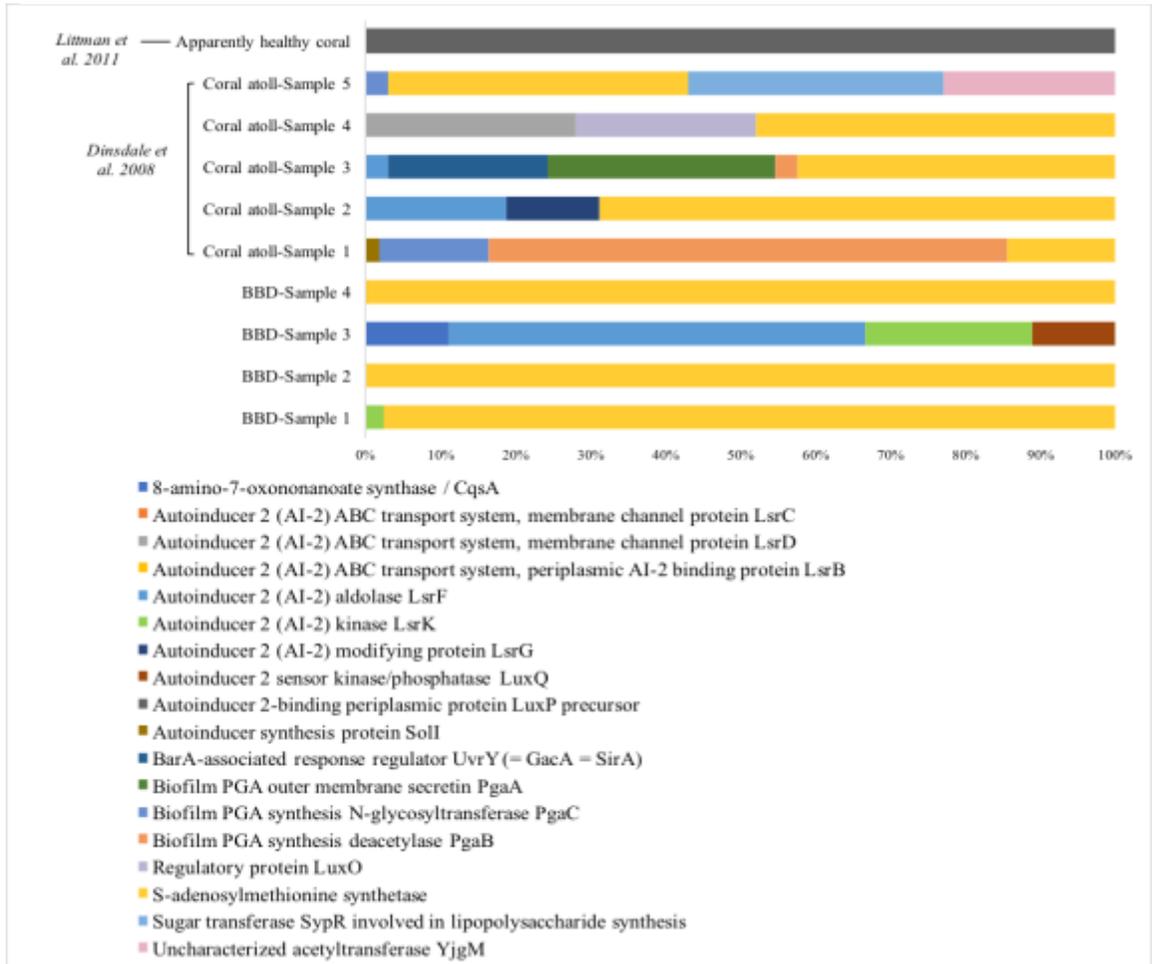


Figure 4-6: Sequences annotated using subsystems database at level 3 under level 2 category of 'Resistance to antibiotics and toxic compounds' comparing BBD, coral atoll (Dinsdale et al. 2008) and apparently healthy coral metagenomes (Littman et al. 2011).

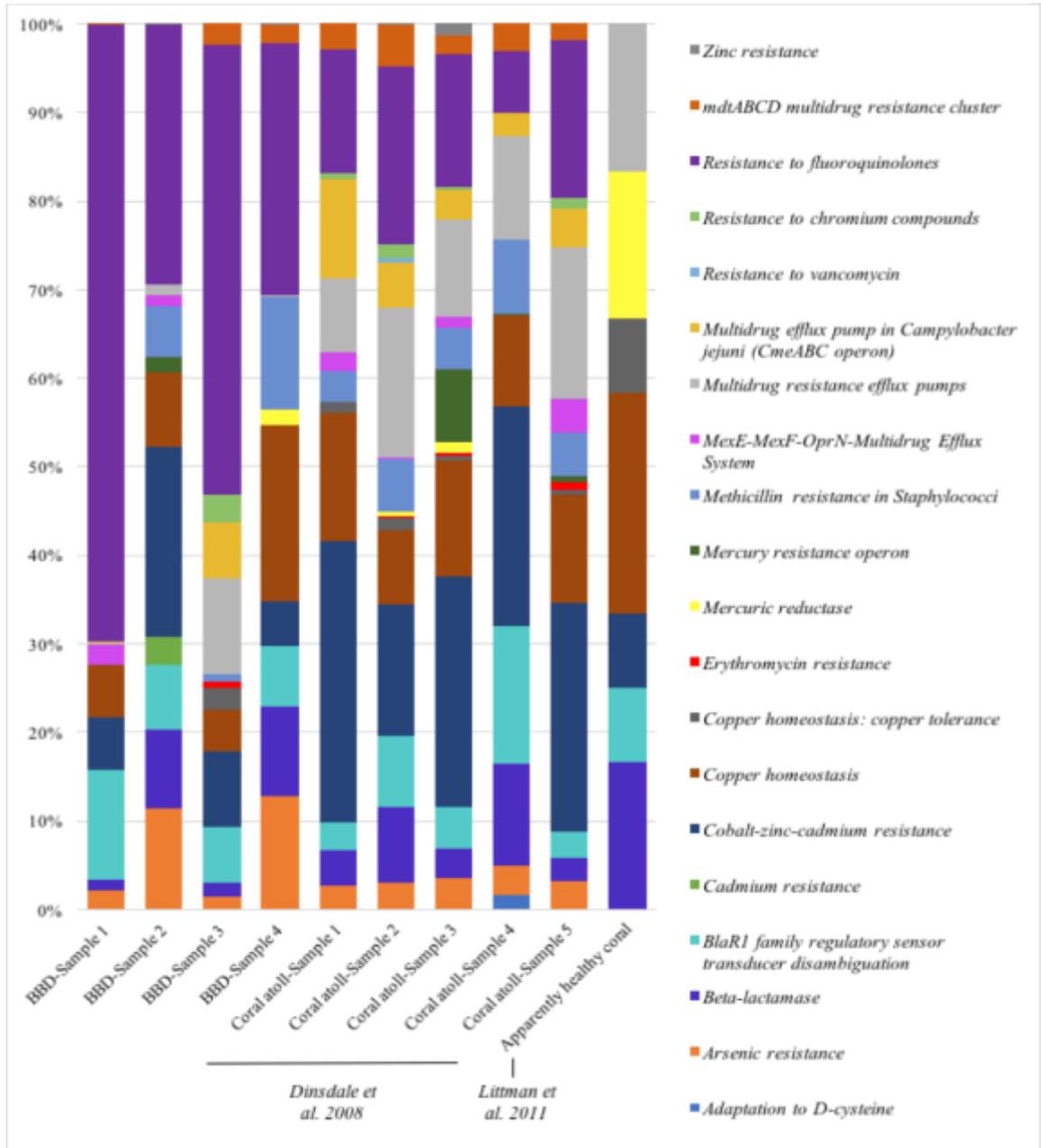
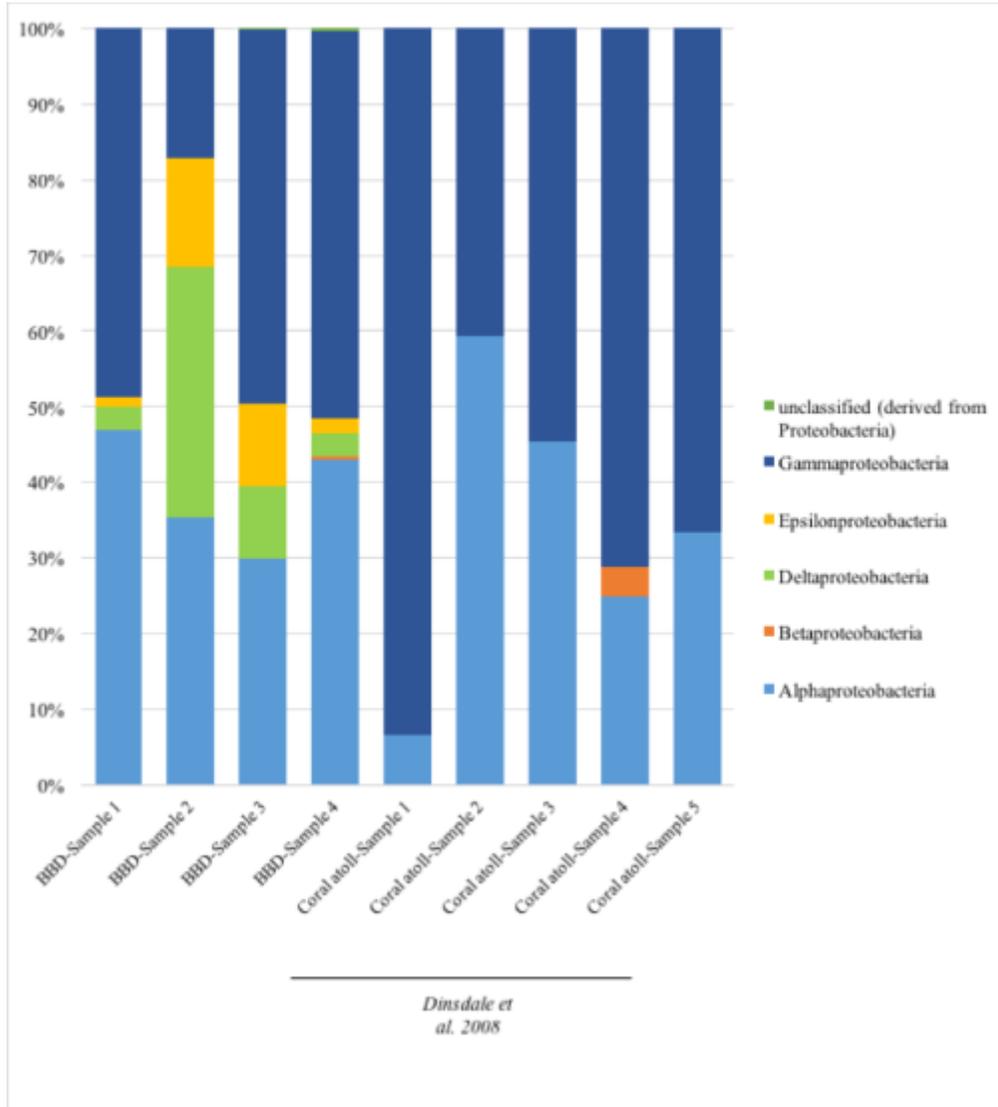


Figure 4-7: Taxonomic affiliations of proteobacteria as compared across metagenomes annotated using RDP database.



CHAPTER 5

CONCLUSIONS

The principal goal of this dissertation was to evaluate the presence of quorum sensing (QS) signals in BBD heterotrophs and assess their potential implication in BBD pathogenicity employing culture dependent and independent methodologies.

The results of Chapter two revealed that DPD, the precursor for the universal signaling molecule AI-2, was produced by nine bacterial isolates from three coral health states. Twelve AHL producing bacteria were also detected and the AHLs produced were identified by LC-MS/MS (Zimmer et al. 2014).

The results of Chapter three revealed a significantly higher number of BBD isolates that produce QS signals (short- to medium chain AHLs) in contrast to isolates from healthy corals and the healthy part of BBD infected corals. Furthermore, these AHLs were identified and it was shown that the production of three of them (3OHC4, 3OHC5, 3OHC6) increased significantly at 30°C, above the observed BBD threshold temperature on the reef. This chapter also exhibited QS related sequences in BBD metagenomes across two geographic locations (Bhedi et al. 2017).

Chapter four examined the antimicrobial production capabilities of isolates across coral health states, tested against two possibly significant BBD isolates, showing the fewest number of isolates inhibiting the two test stains. This chapter also evaluated the AHL production capabilities of seven bacteria (including BBD isolates) with varying temperature, although no significant difference in production was detected across temperatures. The metagenomic aspect of this chapter revealed the presence of QS, antimicrobial synthesis and antimicrobial resistance genes in BBD metagenomes as compared with non-diseased coral metagenomes from publicly available databases

(Dinsdale et al. 2008; Littman et al. 2011). BBD metagenomes were shown to display a large selection of antimicrobial resistance genes. The proteobacterial affiliations of sequences across metagenomes showed the exclusive presence of delta- and epsilonproteobacteria in BBD metagenomes.

Based on the results obtained from this study, and supported by a review of the literature, a model is proposed (Figure 1) for BBD heterotrophic interactions as primarily influenced by QS. In this model, beginning from the top of Figure 1, the bacteria inhabiting the healthy coral SML are exposed to thermal stress due to an increase in surrounding sea-water temperature. This leads to a change in the bacterial community and dynamics, as discussed in (Reshef et al. 2006; Ainsworth and Hoegh-Guldberg 2009; Ainsworth et al. 2010; Krediet et al. 2013). Under these conditions, new pathogens are recruited from the water column near the corals, and existing resident bacteria undergo shifts to adapt to the change in the environmental condition, as per the coral probiotic hypothesis (Reshef et al. 2006). Antimicrobial production by bacteria inhabiting healthy SML then decreases due to the warmer temperatures (Mao-Jones et al. 2010; Rypien et al. 2010; Glasl et al. 2016). Concurrently, specific bacteria increase their abundance (population density) with the increase in temperature, leading to initiation of QS. Quorum sensing then leads to gene expression of virulence factors in opportunistic pathogens like vibrios as well as selection of antimicrobial resistant microbes. QS signals have been known to also function as cues for bacterial recruitment (Joint et al. 2002; Huang et al. 2008), further increasing chances of recruiting pathogens from the water column. Decreased antimicrobial production by SML bacteria allows growth of putative pathogens capable of QS. The collective effect of these exchanges facilitates

establishment of the BBD heterotrophic population, leading to dominance by proteobacteria. QS by vibrios (and other potential QS microbes) within BBD results in production of AHLs (3OHC4, 3OHC5 and 3OHC6) shown to be induced at warmer temperatures (Bhedi et al. 2017). As BBD progresses, the production of these AHLs directly or indirectly influences secretion of proteases (Munn 2015), potentially involved in coral tissue degradation. AHL aided QS additionally enables acquisition and/or expression of antimicrobial resistance genes (Jain et al. 1999). The overall effect of these microbial interactions escalates the virulence of the BBD mat community and encourages maintenance of the community dynamics.

The primary findings from this dissertation provide evidence that specific QS molecules are produced by BBD heterotrophs and that their production is affected by temperature, known to be a controlling factor of coral diseases on the reef. These findings are supported by the presence of virulence genes and their potential expression as revealed by analysis of BBD metagenomes. The results of this dissertation also provide insights into antagonistic interactions within the BBD bacterial population based on antimicrobial production and/or resistance. It is likely that these microbial and environmental (temperature) interactions are involved in the establishment and maintenance of BBD infections. The results from this dissertation offer further information to elucidate the mechanism of one of the most complex, destructive and intricate coral diseases.

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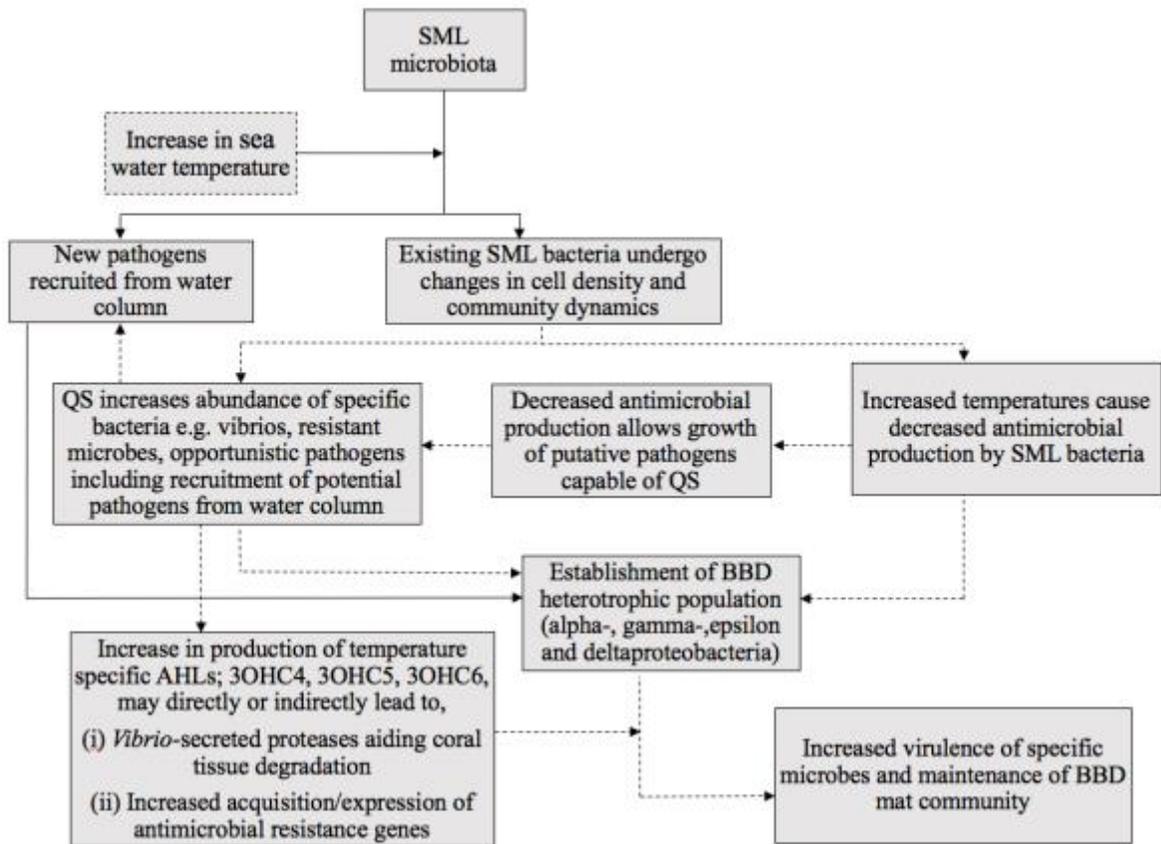
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Figure 5-1: Model of microbial interactions for establishment and maintenance of BBD heterotrophic population, primarily based on quorum sensing.



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PUBLICATIONS AND PRESENTATIONS

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