Phylogeny, Bioactivity, and Physiology of Unique Bacterial Isolates from Hawaiian Marine Sponges from Hawaii Island: Insights into the Bacteria-Sponge Relationship

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ABSTRACT

The goals of this study were to 1) investigate the diversity of cultured bacterial isolates from Hawaiian marine sponges *Haliclona* sp., *Petrosia* sp., and *Iotrochota protea* from Coconut Island and Puhi Bay on the East side of Hawaii Island, 2) identify any unique and/or strong candidates for carrying out further bioactivity and gene screening such as nonribosomal peptide synthetase and polyketide peptide synthase assays and 3) begin to assess their significance to their sponge host. The bacterial isolates contained unique and diverse bacterial isolates according to phylogenetic analysis, nonribosomal peptide synthetase and polyketide peptide synthase screening, Violacein synthetic gene screening, bioactivity assay, and physiological assessments. One isolate, designated PB004-2 (A1), contrasts with its most similar BLAST neighbor, *Brevibacterium frigoritolerans*, in color, form, and media and temperature growth conditions. Another isolate, designated S0022 B001, whose BLAST neighbor is a *Pseudomonas* sp., shows nonmotility, absence of flagella in SEM images, and may possess Violacein-like synthesis genes, indicating S0022 B001 may be a new *Pseudomonas* species. The crystal formation, inconclusively precipitated calcium carbonate, may serve a purpose in the sponge. Findings from this research may indicate that 1) bacteria isolated from the Hawaiian sponges have their unique role in their respective sponge host, 2) may be important to each other's health, and 3) may be important to sponge health.

Key words: bacteria, marine sponges, Hawaii, phylogenetic analysis, physiology, NRPS, PKS, Violacein, *Brevibacterium frigoritolerans*, *Pseudomonas*, bioactivity, antibiotics, sponge health

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CHAPTER ONE

INTRODUCTION

Bacteria residing inside marine sponges from different areas around the world have been observed for well over the past 40 years (Vancelet and Donadey 1977; Hentschel et al. 2002; Sipkema et al. 2015). Recent studies have just begun to reveal the important and sometimes essential role of bacterial colonization within the sponge tissues and cells (Enticknap et al. 2006; Sipkema et al. 2015). Bacteria have consistently and persistently colonized marine sponge tissue both spatially and temporally (Wang et al. 2009). These sponge-associated bacteria have been observed and speculated to contribute to sponge health by way of providing natural defense chemicals for the sponge, for example (Sipkema et al. 2015). Bacteria and the antibiotics that they produce may be so important to the sponge that the absence of bacteria might compromise sponge health (Webster and Blackall 2009). Studying bacteria, the antibiotics that they may produce, and the genes associated with the antibiotics is an area of active research that may provide insight into the bacteria-sponge relationship and possible role of bacteria in their sponge host health (Webster and Blackall 2009).

Bacterial Residential Establishment Inside Host Sponges

The earliest evidence that bacteria resided in marine sponges came in the 1970's with the use of electron microscopy (Vancelet and Donadey 1977; Wilkinson 1978). Images captured bacteria present inside sponge tissue, most notably the mesohyl tissue of the sponge. More recently, techniques incorporating fluorescence in situ hybridization (FISH) have enabled more

specific classification of bacterial species and in other regions, such as the sponge cortex, as well as evidence of vertical transmission of bacteria from one generation of sponges to the next generation as seen by the presence of bacteria in larval cells, sponge nurse cells surrounding oocytes, the periphery of embryos, and oocytes (Enticknap et al. 2006; Manz et al. 2000; Sharp et al. 2007; Schmitt et al. 2008; Sipkema et al. 2015).

Nature of Bacteria-Sponge Relationship

Despite documentation of bacteria and other microbes over the past 40 years, the exact nature of bacteria-sponge relationships has been an area of active research with some questions beginning to be answered. Sponges are sessile animals that can pass up to 24,000 L of seawater per kilogram per day when filter feeding, expelling sterile water in the process (Hentschel et al. 2002). Though the vast majority of microbes and other dissolved nutrients are metabolized by the sponge, accounting for the role as transient food sources, bacteria and other microbes can persist inside the sponge, comprising as much as 60% of total sponge biomass (Hardoim et al. 2009).

In some cases microbes may be pathogenic to the sponge. The presence of bacteria associated with sponge necrotic tissue indicates sponge disease in some cases (Webster 2007; Webster and Taylor 2012). Bacteria, such as *Vibrio* and *Pseudomonas* isolated from sponges contain genes linked with virulence factors such as those involved in quorum sensing (Hentschel et al. 2000; Holden et al. 1999; Wagner-Dobler et al. 2005). Microbes have also been present in healthy sponges (Webster and Hill 2001; Bondarev et al. 2013). Considering that bacteria are present in both healthy and diseased marine sponge tissue, disease and nutrient acquisition

account for only two of the many possible roles bacteria serve in their sponge host (Webster 2007).

Another possibility may be a commensal relationship for either the benefit of the sponge or bacteria. Sponges may provide bacteria with a substrate or shelter to colonize (Bondarev et al. 2013), or bacteria may be able to utilize sponge common metabolic by-products that may be harmful to the sponge if not processed by the bacteria (Hoffmann et al. 2009).

Yet much consideration is placed into the possibility of a symbiotic relationship between bacteria and sponge. Various studies have been conducted with the idea of symbiosis in mind. For example, the most common bacterial isolates *Pseudovibrio* and *Spongiobacter* isolated from the demosponge *Suberites carnosus* have been repeatedly isolated from other demosponges, suggesting a symbiotic relationship; however, the most abundant bacterial isolates from a sponge *Leucosolenia* sp., bacteria *Pseudoalteromonas* and *Vibrio*, though common isolates among other sponge species, are also prolific in seawater, suggesting a less close or even opportunistic establishment with sponges (Kennedy et al. 2008; Flemer et al. 2012; Ozturk et al. 2013). In another case, microbes producing the same bioactive compound but living in different sponge hosts and from different locations offer evidence for symbiotic relationships because the same bioactive compound produced by different species of bacteria may be needed by the sponge host for survival in varied environments (Trindade-Silva et al. 2013).

Though little is known about the dynamics of microbial communities inside sponges and if they seem to aid or facilitate sponge survival in stressed conditions such as processing metabolic waste products, observations of microbes and the characteristics that establish these microbial communities offer many insights into the nature of the bacterial-sponge relationship

(Hoffmann et al. 2009). Furthermore, the dynamics of microbial communities inside sponges may offer support for a symbiotic relationship. Though sponges produce chemical defense compounds and have an immune response, microbes may offer sponges protection from damaged tissues or cells due to predation, opportunistic infections caused by microbes, competitors, and fouling organisms (Imhoff and Stohr 2003; Taylor et al. 2007). Microbes, bacteria in particular, produce biologically active secondary (2°) metabolites in some cases and chemical defense compounds against other microbial growth (Laport et al. 2009). Bacteria, such as from the phyla *Actinobacteria*, *Firmicutes*, and *Proteobacteria*, that are known to produce biologically active 2° metabolites, may supplement the sponge immune system (Hentschel et al. 2001). *Aquamarina, Pseudoalteromonas, Pseudomonas, Pseudovibrio, Spongiobacter,* and *Vibrio* spp. isolated from various marine sponges, have exhibited antibacterial activity (Hentschel et al. 2001; Thiel and Imhoff 2003; Flemer et al. 2011; O'Halloran et al. 2011). Bacteria isolated from marine sponges have also exhibited antimicrobial activity against model lab organisms and pathogenic strains of *Staphylococcus aureus* (SA), *Bacillus cereus* (BC), *Escherichia coli* (EC), *Pseudomonas aeruginosa* (PA), and *Candida albicans* (Santos et al. 2010). Because mounting research suggests bacteria and sponges may be symbionts, identifying cultured bacterial community constituents in a geographic area that has not been researched before, such as Hawaii Island, testing for bioactivity of bacterial isolates against each other, and testing for the presence of genes involved in antibiotic production may provide insight into the bacteria-sponge relationship and indicate symbiosis.

Techniques for Identifying the Bacterial Community inside Marine Sponges

Identifying the exact and complete bacterial community inside marine sponges has been one of the challenges to studying the bacteria-sponge relationship. Techniques used to study bacteria independent of culturing have estimated the culturable bacteria to be less than 1% of the total bacterial population (Hentschel et al. 2012). The use of culture-independent techniques such as denaturing gradient gel electrophoresis (DGGE), clonal libraries, next generation sequencing, and FISH have provided insight into bacterial community structure (Webster et al. 2001; Enticknap et al. 2006; Isaacs et al. 2009; Wang et al. 2009; Jackson et al. 2012; Ozturk et al. 2013).

Molecular approaches, including phylogenetics can provide clues about growing conditions such as genes involved in metabolism and physiological niches that can facilitate or encourage the growth of previously uncultured bacteria (Webster et al. 2001). Molecular approaches can also give a more accurate representation of the true bacterial community or constituents (Hugenholz et al. 1998). Culture-independent methods are useful for providing a better representation of the true bacterial constituents of the community; however, certain downfalls have been observed with some culture-independent methods. For example, Jackson et al. (2012) reported in their culture-independent versus culture-dependent methods that the culture-independent pyrosequencing data contained all cultured Proteobacteria that were identified in the pyrosequencing; however, in the phyla Actinobacteria and Firmicutes, many of the cultured isolates did not appear in the pyrosequencing data. This can be attributed to extremely low abundance of certain bacteria affecting methodology and in turn producing error. Isolated cultured bacteria absent in the culture-independent set have also arisen in other studies but may not be a common occurrence (Zhu et al. 2008).

Culture-dependent methods are useful for assessing phenotypic and genotypic information such as physiology, metabolic profiles, and biosynthetic potential (Xi et al. 2012). Culture-dependent studies are also performed to validate function of genes by *in vivo* and *in vitro* experiments to bacteria's host and to study bioactive efficacy, especially in studies designed with symbiosis and bacteria host interactions (Ozturk et al. 2013). When investigating the bacteria inside CI and PB marine sponges, culture-dependent techniques were employed to be able to see what bacteria could be most easily enumerated, what colony morphology and other colony physical characteristics could be observed, what form the bacteria took on and any distinguishing characteristics associated with the bacteria like flagella under microscopy, test for bioactivity, test for NRPS and PKS genes and any other genes that may be under question due to physical characteristics of the bacteria, and test for any other culture-dependent trait due to identifying the bacterial genera.

Bacterial Identities

Prior to 2000, the phylogenetics and similarity or dissimilarity of bacterial communities from different areas of the world were practically unknown (Hentschel et al. 2002). Hentschel et al. (2002) conducted an extensive diversity survey based on 16s ribosomal RNA (rRNA) sequences obtained from bacteria from 190 sponges from around the world and included their own two distantly related marine sponges *Theonella swinhoei* from the sea near France and *Aplysina aerophoba* from marine waters near Japan, Palau, and the Red Sea (Figure 1.1). Phylogenetics analysis using 16s rRNA data rather than morphological or metabolic data is almost exclusively used to date because 1) comparing molecular sequences has become a commanding tool to determine evolutionary relationships, and 2) the 16s molecule of the small

ribosomal 30s subunit copies itself with great precision but small changes also occur slowly over time, allowing for the detection of relatedness among bacteria (Woese and Fox 1977). The two most dominant groups of the bacteria obtained from this global sample of sponges were from phyla Acidobacteria and Chloroflexi. Other groups present among the sponges were phyla Actinobacteria, Cyanobacteria, Nitrospira, and classes Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria, and the minor phyla, Bacteroidetes and Spirochaetes. Furthermore, bacterial clones from *T. swinhoei* and *A. aerophoba* clustered closely with the bacterial species found among the global sponges.

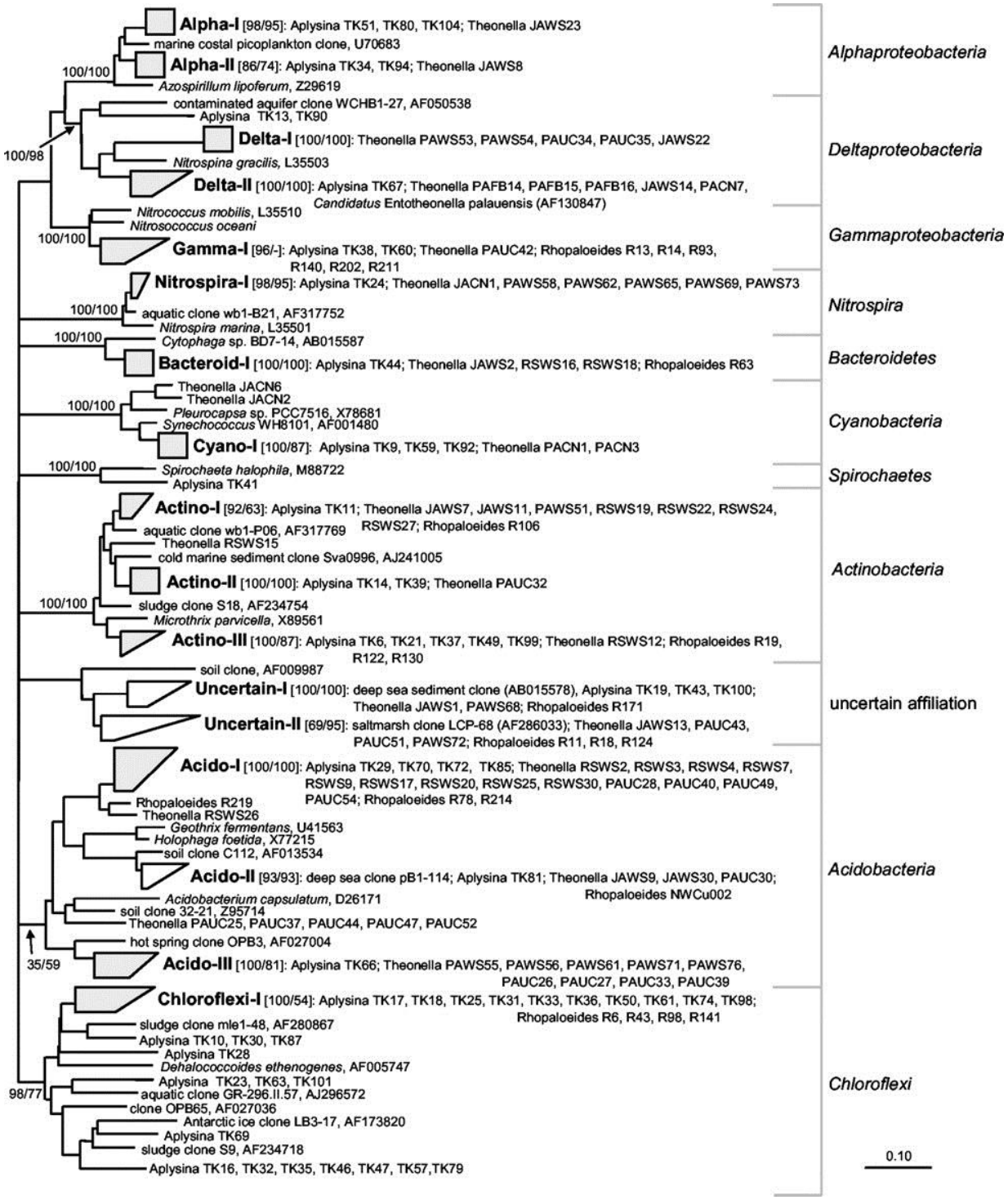

Figure 1.1. Phylogenetic tree calculated with all publicly available 16S rDNA sequences from marine sponges. (Source: Hentschel et al. 2002)
Neighbor-joining method used with 1000 bootstrap replications, scale bar represents number of nucleotide substitutions per base pair.

Since the metagenomic study conducted by Hentschel et al. (2002), many more species of bacteria have been found residing inside a wide variety of marine sponge species worldwide using culture-independent methods, and for the most part, the marine associated bacteria are distinct from those bacteria found in terrestrial environments (Philippot et al. 2010).

a. Survey of Culture-Independent Bacterial Identity Assessment.

Research investigating bacterial communities within sponges from around the world in the last 10 years involving culture–independent methods revealed 14 phyla and 46 genera of bacteria (Li and Liu 2006; Thiel et al. 2007; Kennedy et al. 2008; Mohamed et al. 2008; Hardoim et al. 2008; Isaacs et al. 2009; Kamke et al. 2010; Radwan et al. 2010; Bruck et al. 2012; Jackson et al. 2012; Ozturk et al. 2013) (Table 1.1). Major groups of bacteria varied among sponges and geographic locations. The dominant bacterial groups among the sponges and locations included Proteobacteria from *Chondrella australiensis* near China (Li and Liu 2006), Cyanobacteria from Aplysina *fulva* near Brazil (Hardoim et al. 2008), Delta- and Gammproteobacteria from *Hyrtios erectus* and *Amphimedon* sp. in the Red Sea (Radwan et al. 2010), Proteobacteria from *Raspailia ramosa* and *Stelligera stuposa* near Ireland (Jackson et al. 2012), Actinobacteria from *Arenosclera braziliensis* near Brazil (Trindade-Silva et al. 2013), and Betaproteobacteria from *Crambe crambe* in the Mediterranean (Ozturk et al. 2013). *Spongiobacter* sp. from class Gammaproteobacteria have been found among several sponges including *Chondrilla nucula* from the Mediterranean, *Haliclona simulans* from Irish seas, and *Mycale laxissima* from Key Largo, Florida (Thiel et al. 2007; Kennedy et al. 2008; Mohamed et al. 2008). Many genera occurred multiple times from different sponge hosts and geographic locations such as *Actinomyces and Streptomyces* sp. present in sponges Theonella *swinhoei* from the Atlantic near

France, Aplysina *aerophoba* from the Pacific near Palau, and the *Craniella austrialiensis* in the Pacific near China (Hentschel et al. 2002; Li and Liu 2006).

Table 1.1. Culture-Independent Bacteria from Marine Sponges Around the World. (Sources: Hentschel et al. 2002; Li and Liu 2006; Thiel et al. 2007; Kennedy et al. 2008; Mohamed et al. 2008; Hardoim et al. 2008; Isaacs et al. 2009; Kamke et al. 2010; Radwan et al. 2010; Bruck et al. 2012; Jackson et al. 2012; Ozturk et al. 2013)

PHYLUM	CLASS	GENUS
Acidobacteria		*Geothrix* *Holophaga*
Actinobacteria	Actinobacteria	*Acidomicrobium* *Actinomyces* *Microthrix* *Streptomyces*
Aquificae		
Bacteroidetes		*Aequorivita* *Bacteroides* *Bizionia* *Cytophaga* *Flavobacterium* *Provotella* *Rhodothermus*
Chloroflexi		*Dehalococcoides*
Cyanobacteria		*Pleurocapsa* *Prochlorococcus* *Synechococcus*
Deferribacteres		
Dictyoglomi		
Firmicutes	Bacilli	*Bacillus* *Carnobacterium*
Gemmatimonadetes		
Nitrospira		*Nitrospira* *Leptosprillum* *Thermodesulfovibrio*
Planktomycetes		

Table 1.1. Culture-Independent Bacteria from Marine Sponges Around the World (continued). (Sources: Hentschel et al. 2002; Li and Liu 2006; Thiel et al. 2007; Kennedy et al. 2008; Mohamed et al. 2008; Hardoin et al. 2008; Isaacs et al. 2009; Kamke et al. 2010; Radwan et al. 2010; Bruck et al. 2012; Jackson et al. 2012; Ozturk et al. 2013)

PHYLUM	CLASS	GENUS
Proteobacteria	Alpha- Beta- Delta- Gamma-	(Alpha-) *Achrombacter* *Azospirillium* *Hyphomicrobium* *Rhodobacter* *Rhodospirillum* (Beta-) *Alcaligenes* *Thiobacillus* Delta- *Desulfobacter* *Desulfovibrio* *Paleobacter* *Geobacter* *Bdellovibrio* *Nitrospina* (Gamma-) *Enterobacter* *Halomonas* *Nitrococcus* *Oceanospirillum* *Pseudoalteromonas* *Pseudomonas* *Spongiobacter*

Overall, many more different species of bacteria have been discovered following culture-independent techniques such as next-generation sequencing; however, as noted earlier, culture-independent techniques may not capture the whole bacterial community (Zhu et al. 2008; Jackson et al. 2012). This survey is by no means complete as many more studies have been done but not included in this survey, but it does provide ample insight to bacterial communities inside marine sponges from around the world.

b. Survey of Culture-Dependent Bacterial Identity Assessment.

Culture-dependent studies have also yielded a diverse set of bacteria species from marine sponges. A survey of culture-dependent bacterial identity assessment among numerous studies done since the early 2000s reveals the cultured bacterial community contained diverse species regardless of sponge host and geographic location. Taken together, this survey of culture–dependent studies showed 7 different genera and 53 genera of bacteria (Webster et al. 2001; Webster and Hill 2001; Thakur et al. 2005; Diekmann et al. 2004; Kennedy et al. 2009; Isaacs et al. 2009; Zhang et al. 2009; Flemer et al. 2011; Xi et al. 2012; Bruck et al. 2012; Jackson et al. 2012; Ozturk et al. 2013) (Table 1.2). Major groups of bacteria varied among sponges and geographic locations. The dominant bacteria among the sponges and locations included Alphaproteobacteria from *Rhopaloides odorable* from Australia, Gammaproteobacteria from *Haliclona simulans* from Ireland, (Webster and Hill 2001; Kennedy et al. 2009). The dominant class was Gammaproteobacteria and dominant genus was *Pseudoalteromonas* from *Clathria prolifera* from the Northeast coast of the USA in the Atlantic Ocean (Isaacs et al. 2009). The dominant classes from *Suberites carnosis* and *Lecosolenia* sp. from Ireland were Alpha- and Gammaproteobacteria, respectively, and the dominant genera from *Lecosolenia* sp. was *Pseudoalteromonas* and *Vibrio* sp. from the class Gammaproteobacteria. (Flemer et al. 2011). The dominant class was Gammaproteobacteria from *Raspailia ramosa* and *Stelligera stuposa* from Ireland (Jackson et al. 2012). The most abundant isolates were Pseudovibrio sp. from class Alphaproteobacteria from *Crambe crambe* from Spain (Ozturk et al. 2013). In regards to sponges and geographic location, bacteria cultured are both common as well as unique at the genus level. For example, we see many isolates from the genera *Bacillis, Vibrio, Pseudovibrio,*

and Pseudoalteromonas but some genera are not as common in the cultured group, for example *Spongiobacter*, *Formosa*, and *Colwellia* to name a few. At phylum and class level, we see isolates belonging to a few Phyla or classes, notably Actinobacteria, Bacillus, Proteobacteria, and Bacteroidetes. Between the two studies of bacterial isolates from *Rhopaloides odorabile* sampled at different times but in similar locations from the Great Barrier Reef, both studies cultured *Bacillus* sp. (Webster et al. 2001; Webster and Hill 2001). Isolates were predominately from Alphaproteobacteria in Webster and Hill (2001) but only Gamma-, Delta-, and Betaproteobacteria were cultured in Webster et al. (2001). Also in Webster et al. (2001) Planctomyces, Green Sulfur Bacteria, Green non-Sulfur Bacteria, and Actinobacteria were cultured, whereas in Webster and Hill (2001) Cyanobacteria were cultured.

Table 1.2. Culture-Dependent Bacteria from Marine Sponges Around the World. (Sources: Webster et al. 2001; Webster and Hill 2001; Thakur et al. 2005; Diekmann et al. 2004; Kennedy et al. 2009; Isaacs et al. 2009; Zhang et al. 2009; Flemer et al. 2011; Xi et al. 2012; Bruck et al. 2012; Jackson et al. 2012; Ozrurk et al. 2013)

PHYLUM	CLASS	GENUS
Actinobacteria	Actinobacteria	*Actinobiospora* *Actinomadura* *Arthrobacter* *Blastococcus* *Catenuloplanes* *Frigoribacterium* *Georgenia* *Gordonia* *Kocuria* *Micrococcus* *Micromonospora* *Nonomuraea* *Norcardiopsis* *Pseudonocardia* *Rhodococcus* *Sacchaomonospora* *Salinispora* *Streptomyces* *Verrucosispora*
Bacteroidetes		*Cytophaga* *Formosa* *Maribacter* *Polaribacter* *Salegentibacter*
Cyanobacteria		*Leptolyngbya* *Plectonema*
Firmicutes	Bacilli	*Bacillus* *Marinococcus* *Staphylococcus*
Green Sulfur Bacteria		
Green non-Sulfur Bacteria		
Planktomycetes		

Table 1.2. Culture-Dependent Bacteria from Marine Sponges Around the World (continued). (Sources: Webster et al. 2001; Webster and Hill 2001; Thakur et al. 2005; Diekmann et al. 2004; Kennedy et al. 2009; Isaacs et al. 2009; Zhang et al. 2009; Flemer et al. 2011; Xi et al. 2012; Bruck et al. 2012; Jackson et al. 2012; Ozrurk et al. 2013)

PHYLUM	CLASS	GENUS
Proteobacteria	Alpha- Beta- Delta- Gamma-	(Alpha-) *Erythrobacter* *Paracoccus* *Pseudovibrio* *Rhodobacter* *Rugueria* *Sulfitobacter* *Sphingomonas* *Sphingopyxis* *Roseobacter* (Beta-) *Alcaligenes* (Gamma-) *Aliivibrio* *Alteromonas* *Colwellia* *Halomonas* *Idiomarina* *Marinobacter* *Microbulbifer* *Photobacterium* *Pseudoalteromonas* *Pseudomonas* *Psychrobacter* *Shewanella* *Spongiobacter* *Vibrio*

This survey is by no means complete as many more studies have been done but not included in this survey, but it does provide ample insight to bacterial communities inside marine sponges from around the world. Culture-dependent techniques will be used for studying part of the bacterial community inside Coconut Island (CI) and Puhi Bay (PB) sponges because these manipulations in culture can be used to further assess bacterial significance to the sponge (Xi et

al. 2012). Given that there was much overlap of bacterial genera classified with culture-dependent methods with the culture-independent methods, this investigation predicted that culture-dependent would reveal a sufficient—albeit biased sample because of the culture media used—subset of the total bacterial community.

c. Bacteria from Marine Sponges Found in Hawaii

Though numerous sponges from different geographic locations have been studied to determine what bacteria are residing inside, only two studies have investigated the bacteria that are present inside sponges from Hawaii. The bacteria-sponge studies employed culture-dependent and culture–independent methods and yielded common as well as different phyla.

Culture-independent methods revealed the sponge *Suberites zeteki* from Kaneohe Bay near the coast of Oahu Island, Hawaii harbored phyla Clamydiae, Plantomycetes, Bacteroidetes, Proteobacteria—specifically classes Alpha and Gamma (Zhu et al. 2008). Cultured isolates from *S. zeteki* belonged to the phylum Firmicutes and classes Actinobacteria and Gammaproteobacteria. Overall, the dominant isolates were *Bacillus* species from Firmicutes. The other Firmicutes isolates were from *Peanibacillus*. In Actinobacteria, isolates were from the genera *Streptomyces, Microbacterium*, and *Rhodococcus*. In class Gammaproteobacteria, isolates were from the genera *Vibrio, Acinetobacteria, Pseudomonas,* and *Microbulbifer* and Family Alteromonadaceae. The isolates in phylum Actinobacteria were more closely related to terrestrial bacteria than to other marine environments or sources including sponges. The cultured isolates belonging to the groups Firmicutes, Actinobacteria, and Gammaproteobacteria have also been isolated from the marine sponges Aplysina *aerophoba,* Aplysina *cavernicola, Halidrondra panacea*, and *Rhopaloides odorabile* (Freidrich et al. 2001; Imhoff and Stoehr 2003; Webster and Hill 2001). Low sequence similarity of many Alphaproteobacteria, Planctomycetes, and

Clamydiae individuals suggest these bacteria found in *S. zeteki* may represent new species. Based on phylogenetic values, overall cultured and uncultured bacteria from *S. zeteki* showed both unique species compared to other bacteria from sponges around the world, and common species found in other marine sponges (Webster and Hill 2001; Hentschel et al. 2002; Hentschel et al. 2003; Imhoff and Stoehr 2003; Taylor et al. 2004; Webster et al. 2004; Zhu et al. 2008).

In the other Hawaiian sponge study that looked at bacteria, culture-independent methods revealed the Hawaiian sponges *Mycale armata* and *Dysidea* sp. from Kaneohe Bay off the coast of Oahu Island, Hawaii contained bacteria from the following phyla: Proteobacteria, Actinobacteria, Bacteroidetes, Cyanobacteria, Acidobacteria, Chloroflexi, and Verrucomicrobia (Wang et al. 2009). Most of the bacteria were predominately from class Alphaproteobacteria. Other Proteobacteria represented were Beta-, Delta-, and Gamma-. Alphaproteobacteria in sponges has been reported in other research and is the most dominant class in many sponges including *Halichondria panacea* and *Rhopaloides odorabile* (Webster and Hill 2001; Hentschel et al. 2006; Wichels et al. 2006; Taylor et al. 2007; Thiel et al. 2007; Zhu et al. 2008). Species *Pseudovibrio denitrificans*, an Alphaproteobacteria, found in *Dysidea* sp. was also found in other sponges from different areas of the world (Enticknap et al. 2006; Hentschel et al. 2006; Taylor et al. 2007). *Vibrio* sp. from Gammaproteobacteria were related to the Hawaiian *S. zeteki* as well as *R. odorable* from Australia (Webster et al. 2001; Zhu et al. 2008). Other bacteria from *M. armata* in classes Actinobacteria and Beta- and Deltaproteobacteria were also present in sponges *Tethya aurantium* from the Mediterranean and *Latrunculia apicalis* and *Mycale acerata* from Antarctica (Webster et al. 2004). In DGGE, bacterial bands varied among *M. armata*, *Dysidea* sp., *Gelloides fibrosa*, *Tedania* sp., and *Scopalina* sp., but were similar with the same sponge species collected at different times, indicating the bacterial community is sponge specific,

consistent, and persistent over time. Two bands were common in the sponges *M. armata, Dysidea* sp., *G. fibrosa, Tedania* sp., *Scopalina* sp. and two bands were unique in two samples of *M. armata* collected at different years. The DGGE patterns described above suggest that *M. armata* and other Hawaiian sponges had similar bacterial phylotypes as well as different phylotypes and were consistent throughout time.

This current investigation in CI and PB, using culture-dependent methods, was predicted to yield similar as well as different bacteria phyla and/or genera compared to the culture-independent and culture-dependent Hawaiian studies and other world studies.

2° Metabolite Production May Produce Antibiotic Compounds

a. Bioactivity of Bacteria from Sponges

Among the many roles suggested for bacterial existence inside marine sponges, the role of bacteria producing 2° metabolites with biological activity is of particular interest for understanding bacteria-sponge symbiosis, sponge ecology, bacteria ecology, and biotechnological and pharmaceutical utility. Bacteria found in sponges may assist in host defense, a hypothesis that was proposed because some bacteria, such as *Pseudovibrio* sp., that were found in healthy sponges were absent in diseased sponges (Webster et al. 2008; Penesyan et al. 2011). Isolating 2° metabolites of biological activity from bacteria present inside sponges may indicate that bacteria may benefit sponge health (Webster et al. 2008) such as defending sponges against unhealthy colonization and pathogens (Penesyan et al. 2011).

Many bacteria cultured from marine sponges have inhibited the growth of other marine bacteria (Penesyan et al. 2011). For example, Penesyan et al. (2011) showed *Pseudovibrio denitrificans*, one strain isolated from a sponge, produced topodithietic acid (TDA) which

displayed bioactivity against various marine bacterial species from classes Alpha- and Gammaproteobacteria and phyla Bacteroidetes, Firmicutes, and Actinobacteria using the disc diffusion method (Table 1.3.) (Figure 1.2). Having antibiotic activity present in the sponge may be helpful in keeping 1) the growth of bacteria that are stable residents of sponges under control and 2) transient bacteria from colonizing in the sponge (Penesyan et al. 2011). Bacteria in both cases may be opportunistic pathogens for the sponge, therefore bacteria producing antibiotics such as TDA found in the sponge may be aid in sponge health.

Table 1.3. Inhibitory activity of TDA by *Pseudovibrio denitrificans* strain against various marine bacteria using the disc diffusion method. (Source: Penesyan et al. 2011)

Target Strains	Phylum	Origin	Inhibition by TDA
Alpha-proteobacterium D323 ^	*Alphaproteobacteria*	seaweed *Delisea pulchra*	-
Phaeobacter inhibens T5 ^	*Alphaproteobacteria*	marine sediment	-
Phaeobacter inhibens T5-3	*Alphaproteobacteria*	marine sediment	-
Nautella sp. R11	*Alphaproteobacteria*	seaweed *Delisea pulchra*	+++
Phaeobacter gallaeciensis 2.10 ^	*Alphaproteobacteria*	seaweed *Ulva lactuca*	-
Oceanicola granulosus	*Alphaproteobacteria*	seawater	++
Oceanicola batsensis	*Alphaproteobacteria*	seawater	+++
Roseovarius sp. 2601	*Alphaproteobacteria*	seawater	++
Oceanicaulis alexandrii	*Alphaproteobacteria*	seawater	++
Rhodobacterales bacterium	*Alphaproteobacteria*	seawater	+++
Phaeobacter gallaeciensis BS107 ^	*Alphaproteobacteria*	scallop *Pecten maximus*	-
Vibrio harveyi	*Gammaproteobacteria*	seawater	+++
Pseudoalteromonas tunicata	*Gammaproteobacteria*	tunicate *Ciona intestinalis*	+++
Pseudoalteromonas undina	*Gammaproteobacteria*	seawater	++
Pseudoalteromonas piscicida	*Gammaproteobacteria*	dead fish	+
Pseudoalteromonas citrea	*Gammaproteobacteria*	seawater	++
Pseudoalteromonas haloplanktis	*Gammaproteobacteria*	oyster *Crassostrea gigas*	++
Pseudoalteromonas ulvae	*Gammaproteobacteria*	seaweed *Ulva lactuca*	++
Pseudoalteromonas flavipulchra	*Gammaproteobacteria*	seawater	+
Acinetobacter sp. ESS07	*Gammaproteobacteria*	seaweed *Delisea pulchra*	+++
Marinomonas sp. ND73	*Gammaproteobacteria*	seaweed *Delisea pulchra*	+++
Shewanella sp. ND51	*Gammaproteobacteria*	seaweed *Delisea pulchra*	++
Thalassomonas sp. ND29	*Gammaproteobacteria*	seaweed *Delisea pulchra*	+++
Thalassomonas sp. ND49	*Gammaproteobacteria*	seaweed *Delisea pulchra*	++
Aestuariibacter sp. ND16	*Gammaproteobacteria*	seaweed *Delisea pulchra*	++
Vibrio sp. ND23	*Gammaproteobacteria*	seaweed *Delisea pulchra*	++
Dokdonia sp. ESS16	*Bacteroidetes*	seaweed *Delisea pulchra*	++
Aquimarina sp. ND19	*Bacteroidetes*	seaweed *Delisea pulchra*	+
Tenacibaculum sp. ND71	*Bacteroidetes*	seaweed *Delisea pulchra*	+
Bacillus sp. D203	*Firmicutes*	seaweed *Delisea pulchra*	++
Bacillus sp. ESS03	*Firmicutes*	seaweed *Delisea pulchra*	+++
Micrococcus sp. ESS26	*Actinobacteria*	seaweed *Delisea pulchra*	++
Agrococcus sp. LSS27	*Actinobacteria*	seaweed *Delisea pulchra*	++

+ zone of clearance in disc diffusion up to 3 mm from the edge of the disc; ++ 3–5 mm; +++ more than 5 mm; - no inhibition observed; ^ TDA-producing bacteria.

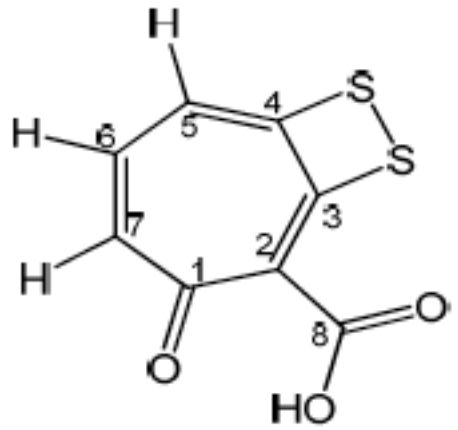

Figure 1.2. Topdithietic Acid. (Source: Penesyan et al. 2011)

In addition to the studies looking into the bioactivity of sponge-associated bacteria against other marine bacteria, numerous studies have tested bacteria isolated from various species of marine sponges from different geographic locations against model lab bacteria and common human pathogens (eg. Thakur et al. 2005; Li and Liu 2006; Santos et al. 2010; Flemer et al. 2011). Some sponge-associated bacteria showing bioactivity include *Spongiobacter, Shewanella, Pseudoalteromonas, Psychrobacter, Strenotrophomas* spp. from class Gammaproteobacteria; *Vigibacillus* and *Staphylococcus* from phylum Firmicutes; and *Formosa* from phylum Planctomycetes (Santos et al. 2010; Flemer et al. 2011); however, a great number of bacteria from marine sponges with bioactivity have been identified as *Bacillus, Peudovibrio,* and *Pseudomonas* sp. and in Phylum Actinobacteria (eg. Thakur et al. 2005; Li and Liu 2006; Muscholl-Silberhorn et al. 2007; Kennedy et al. 2008; Santos et al. 2010; Flemer et al. 2011, O'Halloran et al. 2011). These bacteria in the ladder list are of great interest to ecological studies but more especially to human bacterial pathogens studies, and the investigation in CI and PB had hope to find isolates from the latter group to perform further analysis. Cultured bacteria that are predominant in culture but appear as a minor group or not at all in pyrosequences, for instance, may be prolific due to opportunistic profiles (Zhu et al. 2008; Jackson et al. 2012).

b. Genes Involved in 2° Metabolite Production: Non-Ribosomal Peptide Synthetase and Polyketide Synthase Genes

Non-Ribosomal Peptide Synthetases (NRPS) and Polyketide Synthases (PKS) are enzymatic structures responsible for some 2° metabolite production (Jenke-Kodama et al. 2005; Strieker et al. 2010). PKS and NRPSs structures synthesize products from the successive condensation of ketides (Fishbach and Walsh 2006) and peptides, respectively, to produce structurally and functionally diverse molecules that may have pharmaceutical or industrial applications and ecological implications ((Jenke-Kodama et al. 2005; Strieker et al. 2010). In the past few decades, most of the discovered NRPS 2° compounds produced by bacteria came from classes Actinobacteria and Bacilli from the soil, (Cheng et al. 2002; Horwood et al. 2004); however in recent years, marine microorganisms have also been shown to produce useful NPRS 2° compounds to be applied in pharmaceutics, industry, and ecology (Blunt et al. 2007; Blunt et al. 2008.) Degenerate NRPS PCR primers can be used to amplify NRPS genes of documented or taxon classified Actinobacteria as well as undocumented or unclassified Actinobacteria; for example (Ayuso-Sacido and Genilloud). Likewise PKS genes can also be screened in bacteria to indicate possible useful 2° metabolite production (Kim and Fuerst 2006). The synthesis of 2° metabolites, often produced by NRPS/PKS, by marine bacterial symbionts from sponges may provide chemical defenses against pathogens or predators for both the bacteria and sponge (Schneemann et al. 2010).

Importance of Actinobacteria, Bacillus, Pseudomonas, and Pseudovibrio

As described above, bacteria from the phylum Actinobacteria and genera *Bacillus, Pseudomonas,* and *Pseudovibrio* are of particular interest in bacteria-sponge research because they provide insight to symbiosis in ecosystems and organisms with diverse sets of genes for adaptation to changing environments. Also, their 2° metabolite production can be used for industrial, biotechnological, and pharmaceutical applications such as the case of antibiotics.

Species from the phylum Actinobacteria are found in terrestrial as well as marine environments and are the leading producers of natural biologically active products, accounting for two-thirds out of the thousands discovered (Cheng et al. 2002; Lang et al. 2004; Molinski et al. 2008; Nicolaou et al. 2009; Thomas et al. 2010; Hu et al. 2011; Zhu et al. 2013). Actinobacteria that possess antibiotic activity have come from a plethora of species including *Actinobiospira, Actinomadura, Arthrobacter, Brachybacteria, Gordonia, Janibacter, Knoellia, Kocuria, Microbacterium, Micrococcus, Micromonospora, Norcardia, Pseudonocardia, Rhodococcus, Salinospora,* and *Streptomyces* and sponge species including *Cymbastela concenrica, Halichondria panacea, Ircina fusca, Pseudoceratina clavata, Rhabdastrella globostellata, Rhopaloeides odorabile, Scleritoderma* sp., and *Xetospongia* sp., and the same Actinobacteria have been found among different species of sponges from different geographic locations (Hentschel et al. 2001; Webster et al. 2001; Hentschel et al. 2002; Kim et al. 2005; Lafi et al. 2005; Montalvo et al. 2005; Enticknap et al. 2006; Zhu et al. 2008; Xi et al. 2012). Xi et al. (2012) found their 222 out of 244 Actinobacteria cultured from sponges off the coast of China contained NRPS and/or PKS genes. Li and Liu (2006) showed most Actinobacteria isolated from sponge *C. australiensis* showed close relationships to *Streptomyces* in GenBank but two strains out of 23 were distantly related to *Streptomyces* in GenBank indicating possible new

species. Taken together, Actinobacteria documented so far were considered non-harmful and produced bioactive metabolites that may be beneficial to the sponge, ecosystem, and other areas of interest (Kim et al. 2006; Jiang et al. 2007; Izumikawa et al. 2010; Schneemann et al. 2010; Thomas et al. 2010).

The genus *Bacillus* is also found in diverse niches ranging from terrestrial and aquatic, and many different *Bacillus* species show a wide range of bioactivity (Horwood et al. 2004; Muscholl-Silberhorn et al. 2007; Santos et al. 2010). In particular, they show promise in inhibiting the growth of many human pathogens, and many Bacilli have been isolated from sponges for testing against human pathogens (Santos et al. 2010). They also contain NRPS and PKS genes involved in 2° metabolite production; and *Bacillus* species from sponges have been screened for these NRPS and PKS genes (Siegl and Hentschel 2010).

Pseudomonas sp. live in a broad range of environments, including terrestrial and marine environments, more specifically marine invertebrates, sea sediment, and sea water (Romanenko et al. 2005). *Pseudomonas* sp., including those cultured from marine sponges, have been shown to produce a wide variety and abundance of 2° metabolites including antibiotic activity (Kamei and Isnansetyo 2003; Romanenko et al. 2005; Thakur et al. 2005; Kennedy et al. 2008; Marinho et al. 2009; Santos et al. 2010) found many *Pseudomonas* isolates were from the dominant group, Gammaproteobacteria, and showed the highest level of antibiotic activity in model lab and pathogenic bacteria compared to other genera exhibiting antibiotic activity. Santos et al. also found that *P. fluorescens* and *P. aeruginosa* strains isolated from their sponges exhibited antibiotic activity against both Gram-positive and Gram-negative bacteria (Santos et al. 2010).

Lastly, *Pseudovibrio* has also been a common, and sometimes major, genus among the bacteria discovered in Marine sponges throughout the world and exhibits antibiotic activity

(Enticknap et al. 2006; Kennedy et al. 2008; Santos et al. 2010; O'Halloran et al. 2011; Jackson et al. 2012). Jackson et al. (2012) found in the sponge *S. stuposa*, the Alphaproteobacteria group had isolates most closely related to genera *Roseobacter* and *Ruegeura* sp. while the sponge *R. ramosa* had almost all *Pseudovibrio* sp. *Pseudovibrio* shows promising bioactivity against human bacterial pathogens, with *P. ascidisceicola* and *P. denitrificans* generally more effective against Gram-positive bacteria (Santos et al. 2010). Antimicrobial activity has been shown to be variable among *Pseudovibrio* sp. (Muscholl-Silberhorn et al. 2007; Santos et al. 2010). Recent studies have uncovered the bioactivity and bioactive potential of *Pseudovibrio* through bioactivity assays and NRPS and PKS screening (Kennedy et al. 2008). Though *Pseudovibrio* have been isolated from sea water, the majority of isolates and known presence of *Pseudovibrio* have been from marine invertebrates, suggesting a symbiotic relationship, and often *Pseudovibrio* found in sponges have not been found in the nearby water column (Shieh et al. 2004; Enticknap et al. 2006; Fukanaga et al. 2006; Hosoya and Yokota 2007; O'Halloran et al. 2013).

This current research hoped to culture bacteria from phylum Actinobacteria and genera *Bacillus, Pseudomonas,* and *Pseudovibrio* because isolates belonging to these groups would be strong candidates for bioactivity, NRPS/PKS gene screening, and other physiological assessments.

The aims of this research were to investigate the diversity of cultured bacterial isolates from three Hawaiian marine sponge species, identify any unique or strong candidates for carrying out further bioactivity and NRPS and PKS assays, and to assess the environmental significance of the identified unique candidates.

The specific research goals of this present study were:

1) Isolate and identify sponge bacteria from Coconut Island (CI) and Puhi Bay (PB)

    A) using traditional culture dependent methods.

    B) determining genus identification through phylogenetics

2) Assay the isolated bacteria for bioactivity using

    A) disc diffusion assay against model bacterial pathogens

    B) disc diffusion assay against isolates from its own sponge bacterial community

3) Screen cultured bacteria from CI and PB sponges for PKS and NRPS genes involved in 2° metabolite biosynthesis.

CHAPTER TWO

MATERIALS AND METHODS

Sponge collection

Sponges that were observed to be disease-free were collected off of Coconut Island (CI) and in Puhi Bay (PB), Hawaii Island, on March 7 and June 18, 2013 by SCUBA at a depth of 1 meter by Dr. Anthony Wright and Kehau Hagiwara in collaboration with Dr. Wright, College of Pharmacy, UH Hilo. Sponges were cut with sterile scalpels, immediately transferred to plastic bags containing ambient seawater, kept on ice, and transported to the laboratory within 3 hours. Three individuals of a *Haliclona sp.* from CI, 2 individuals from PB of a *Petrosia sp.*, and 5 individuals of *I. protea* from PB were collected (Table 2.1).

Table 2.1. Sponge Collection, Sample Name, and Location

Coconut Island (CI) ***Haliclona* sp.**	**Puhi Bay (PB)** ***Petrosia* sp.**	**Puhi Bay (PB)** ***Iotrochota protea***
CI 1-1	PB 4-1	PB 5-1
CI 1-2	PB 4-2	PB 5-2
CI 1-3		PB 5-3
		PB 5-4
		S0022 B001

Sponge preparation, Bacterial Isolation on Agar

All cell culture was performed in a biosafety laminar flow hood using aseptic techniques. All media, equipment, and other reagents were sterilized before use. Approximately 5 grams of sponge tissue was surface sterilized with 70% ethanol and air-dried. Each 5 gram sample was sliced to about 1 cm thickness and set on Difco brand marine agar (MA) to incubate for 7 days at ambient lab temperature (22-25°C). In addition, approximately 5 grams of sponge tissue was surface sterilized with 70% ethanol, air-dried, and the sponge surface was spread on agar plates as a negative control for outside bacteria growth. Sponges were observed for different microbial growth—based on color, colony morphology, and other distinguishing characteristics—and

microbial growth was streaked for isolation on MA and Actinomycetes with filtered natural seawater agar and glycerol (ASA) plates.

Documentation of Colony Characteristics

Sponges and microbes were photographed and described by location, texture, color, colony morphology, and other distinguishing characteristics. All isolates were collected as pure cultures until DNA sequencing and stored at -80ºC in 20% glycerol.

PCR and Sequencing of 16S rRNA Region

The total 16S rRNA region is approximately 1500 bp. Colony PCR was carried out using colony as template, 16S rRNA forward primer Integrated DNA Technologies 27F and reverse primer 981R (Table 2.2), nuclease-free water, and Promega Go Taq Green Master Mix to give an amplicon approximately 1200 bp. The Eppendorf Mastercycler Pro with Vapo.protect thermocycler was set to the following cycling condition: 15 min at 94°C for 1 cycle; 1 min at 94°C, 30 sec at 52°C, 30 sec at 72°C for 30 cycles; 10 min at 72°C for 1 cycle; 11 min at 10°C for 1 cycle; and held at 4°C. PCR bacterial products were verified on a 1.5% agarose gel run at 80 volts for approximately 30 min and using Promega 100 bp ladder.

Colonies that failed to yield a band from colony PCR were grown in Difco marine broth (MB) or Difco Actinomycetes with natural sea water broth (ASW) overnight at ambient lab temperature (22-25°C). DNA was extracted from cultures using MoBio Ultraclean Microbial DNA Isolation Kit following manufacturer instructions. DNA extractions were quantified using NanoDrop ND-1000 spectrophotometer. PCR was set to the following parameters: 3 min at 94°C for 1 cycle; 1 min at 94°C, 30 sec at 54°C, 30 sec at 72°C for 30 cycles; 10 min at 72°C for 1 cycle; and held at 4°C.

PCR products were cleaned using Wizard PCR cleanup kit following manufacturer's instructions. Cleaned amplicons were quantified using NanoDrop ND-1000 spectrophotometer. Sanger sequencing reactions were set up for separate forward, using 27F, and reverse, using 981R, and run on Applied Biosystems Hitachi 3500 Genetic Analyzer sequencer. Forward and reverse sequences were corrected and reassembled using Sequencher 6.0.

Isolate S0022 B001

Isolate S0022 B001 1200 bp amplicon was gel purified using Wizard PCR cleanup kit following manufacturer's instructions. The clean product was quantified (NanoDrop ND-1000 spectrophotometer) and cloned into pGEM using Promega pGEM-T easy vector systems following manufacturer's protocol. Ligated products were grown on Luria-Bertani agar with 10 ug per ml ampicillin and incubated at 37ºC for 3 days. One white colony was selected for plasmid prep using Wizard SV mini purification system following manufacturer's instructions. Sequencing was performed as described previously with the exception of using forward primer M13F and reverse primer M13R from Invitrogen (Table 2.2).

Table 2.2. Primers Used in Experiments.

Name	Primer Sequence	Length of target gene fragment (bp)	Description and Purpose	Reference
27 F 981R	5'-AGA GTT TGA TCM TGG CTC AG -3' 5'-GGG TTG CGC TCG TTG CGG G -3'	1200	Universal 16s rRNA	Donachie et al. 2004
M13F M13R	5'-GTA AAA CGA CGG CCA GT -3' 5'-AAC AGC TAT GAC CAT G -3'	1200	Cloning using pGEM	Invitrogen 1999
A3F A7R	5′-GCSTACSYSATSTACACSTCSGG-3′ 5′-SASGTCVCCSGTSCGGTAS-3′	700-800	NRPS	Ayuso-Sacido and Genilloud 2005
K1F M6R	5′-TSAAGTCSAACATCGGBCA-3′ 5′-CGCAGGTTSCSGTACCAGTA-3′	1200-1400	PKS-1	Ayuso-Sacido and Genilloud 2005
KS_{α} KS_{β}	5′-TSGCSTGCTTGGAYGCSATC-3′ 5′-TGG AANCCG CCGAABCCTCT-3′	600	PKS-2	Metsa-Ketela et al. 1999
VPA3 VPA4	5′-CCRCAGCTSCAYCCGCATTTCCAG-3′ 5′-CAGGCYGCCCTCCATCCA GCCRCA-3′	1000	*vioA*	Hakvag et al. 2009
VPB1 VPB2	5′-CTGTTCAATATGTCGACGCCGC-3′ 5′-GCGGATCGCACATCTGCCACATC-3	900	*vioB*	Hakvag et al. 2009
VPB3 VPB4	5′-CCGGCCGGCCGSCTGCTGC-3′ 5′-GSCGCGAGCGSCKSAGGCTGC-3′	1850	*vioB*	Hakvag et al. 2009

NCBI BLAST

Sixty-four bacterial gene sequences were analyzed with Basic Local Alignment Search Tool (BLAST) set to find highly similar sequences on GenBank at National Center for Biotechnology Information (NCBI). The top 100 BLAST highest similarity hits were saved on an Excel file and highest percent identity shown in Table 3.2.

Phylogenetic analysis

Assembled sequences from Sequencher were grouped into genera and imported into MEGA 4.0. Comparable genera from other sponge bacteria papers and/or closest BLAST neighbors as well as the type species were accessed on NCBI Genbank and included in alignment analyses and phylogenetics from each genera group (Table 2.3). Sequences were aligned using ClustalX pairwise and multiple alignments with default settings. Maximum likelihood (ML) phylogenetic analyses were carried out by first testing the following models to find the best fit for nucleotide substitution and distribution under the Bayesian Information Criterion: General Time Reversible, Hasegawa-Kishiro-Yano, Tamura-Nei, and Tamura 3-parameter, Kimura 2-parameter, Jukes-Cantor, uniform rates, gamma distributed, and invariant sites. One-thousand bootstrap replications were used to test tree strength. Default settings were used for ML sequences.

Table 2.3 NCBI GenBank 16s rRNA sequences used in Phylogenetic analysis.

NCBI GenBank Accession #	Description	Reference
DQ091007	*Bacillus*	Yu et al. 2009
DQ180149	*Bacillus*	Li and Liu 2006
AF333558	*Bacillus*	Webster et al. 2001
EU862089	*Bacillus*	Santos et al. 2010
DQ903941	*Bacillus*	Zhu et al. 2008
DQ903942	*Bacillus*	Zhu et al. 2008
DQ903958	*Bacillus*	Zhu et al. 2008
DQ903963	*Bacillus*	Zhu et al. 2008
DQ903970	*Bacillus*	Zhu et al. 2008
DQ874993	*Bacillus*	Wang et al. 2009
DQ874989	*Bacillus*	Wang et al. 2009
DQ874983	*Bacillus*	Wang et al. 2009
DQ874978	*Bacillus*	Wang et al. 2009
DQ874975	*Bacillus*	Wang et al. 2009
EU768819	*Bacillus*	Kennedy et al. 2009
JF820795	*Bacillus*	Jackson et al. 2012
KM888109	*Bacillus* BLAST close neighbor	NA*
KF535117	*Bacillus* BLAST close neighbor	NA*
NR_121761	*Bacillus* BLAST close neighbor	NA*
KC990823	*Bacillus* type species	NA*
LK021111	*Virgibacillus* outgroup to *Bacillus*	NA*
GQ153944	*Brevibacterium*	Kiran et al. 2010a
GQ153943	*Brevibacterium*	Kiran et al. 2010b
GQ163719	*Brevibacterium*	Radwan et al. 2010
GQ504255	*Brevibacterium*	Sun et al. 2010
EU086802	*Brevibacterium*	Selvakuma et al. 2011
EU725777	*Brevibacterium*	Selvakuma et al. 2011
FJ853165	*Brevibacterium*	Selvakuma et al. 2011
KC778370	*Brevibacterium* BLAST close neighbor	NA*
NR026166	*Brevibacterium* type species	Selvakuma et al. 2011
FN673681	*Micrococcus* outgroup to *Brevibacterium*	NA*
AB245514	*Ferrimonas*	Nakagawa et al. 2006
DQ778094	*Ferrimonas*	Campbell et al. 2007
GU391222	*Ferrimonas*	Ji et al. 2013
KC545309	*Ferrimonas* BLAST close neighbor	NA*
NR_027 602	*Ferrimonas* type species	NA*
GU070670	*Paraferrimonas* outgroup to *Ferrimonas*	NA*

*Not Applicable

Table 2.3 NCBI Genbank 16s rRNA sequences used in Phylogenetic analysis (continued).

NCBI GenBank Accession #	Description	Reference
GU929559	*Photobacterium*	Bruck et al. 2012
JX477106	*Photobacterium*	Dupont et al. 2014
AJ842344	*Photobacterium*	Thompson et al. 2005
HQ449970	*Photobacterium* BLAST close neighbor	NA*
KC012646	*Photobacterium* BLAST close neighbor	NA*
AY435156	*Photobacterium* type species	NA*
DQ903940	*Vibrio* outgroup to *Photobacterium*	NA*
EU237124	*Pseudoalteromonas*	Mangango et al. 2009
DQ180160	*Pseudoalteromonas*	Li and Liu 2006
EU768826	*Pseudoalteromonas*	Kennedy et al. 2009
JF820668	*Pseudoalteromonas*	Jackson et al. 2012
JF820733	*Pseudoalteromonas*	Jackson et al. 2012
EF414041	*Pseudoalteromonas*	Isaacs et al. 2009
KJ557112	*Pseudoalteromonas* BLAST close neighbor	NA*
KF359482	*Pseudoalteromonas* BLAST close neighbor	NA*
AB681739	*Pseudoalteromonas* type species	NA*
AY771763	*Algicola* outgroup to *Pseudoalteromonas*	NA*
EU237143	*Pseudomonas*	Mangango et al. 2009
EU862079	*Pseudomonas*	Santos et al. 2010
GU929556	*Pseudomonas*	Bruck et al. 2012
DQ875001	*Pseudomonas*	Wang et al. 2009
DQ875000	*Pseudomonas*	Wang et al. 2009
EU768833	*Pseudomonas*	Kennedy et al. 2009
JF820764	*Pseudomonas*	Jackson et al. 2012
JX477107	*Pseudomonas*	Dupont et al. 2014
KC108954	*Pseudomonas* BLAST close neighbor	NA*
AM934696	*Pseudomonas* BLAST close neighbor	NA*
AM685632	*Pseudomonas* BLAST close neighbor	NA*
FJ972538	*Pseudomonas* type species	NA*
JQ922425	*Cellvibrio* outgroup to *Pseudomonas*	NA*
EU862088	*Pseudovibrio*	Santos et al. 2010
DQ874974	*Pseudovibrio*	Wang et al. 2009
JF820700	*Pseudovibrio*	Kennedy et al. 2009
JF820777	*Pseudovibrio*	Kennedy et al. 2009
JX477103	*Pseudovibrio*	Dupont et al. 2014
EF414057	*Pseudovibrio*	Isaacs et al. 2009

*Not Applicable

Table 2.3 NCBI Genbank 16s rRNA sequences used in Phylogenetic analysis (continued).

NCBI GenBank Accession #	Description	Reference
AB681198	*Pseudovibrio* BLAST close neighbor	NA*
NR_029112	*Pseudovibrio* BLAST close neighbor; type species	NA*
HE584768	*Pseudovibrio* BLAST close neighbor; type species	NA*
AJ550939	*Oceanibulbus* outgroup to *Pseudovibrio*	NA*
EU237120	*Shewanella*	Mangango et al. 2009
JF820681	*Shewanella*	Jackson et al. 2012
JF820760	*Shewanella*	Jackson et al. 2012
JX477104	*Shewanella*	Dupont et al. 2014
GU289647	*Shewanella* BLAST close neighbor	NA*
DQ307731	*Shewanella* type species	NA*
AY682202	*Alteromonas* outgroup to *Shewanella*	NA*
AF358664	*Thalassospira*	Lopez-Lopez et al. 2002
AB786710	*Thalassospira*	Tsubouchi et al. 2014
AB786711	*Thalassospira*	Tsubouchi et al. 2014
AB548215	*Thalassospira*	Nogi et al. 2014
AY186195	*Thalassospira*	Liu et al. 2007
AY189753	*Thalassospira*	Liu et al. 2007
AB265822	*Thalassospira*	Kodama et al. 2008
EU017546	*Thalassospira*	Zhao et al. 2010
KC534432	*Thalassospira* BLAST close neighbor	NA*
FJ860275	*Thalassospira* type species	NA*
JN6255553	*Thalassobaculum* outgroup to *Thalassospira*	NA*
DQ903940	*Vibrio*	Zhu et al. 2008
DQ903944	*Vibrio*	Zhu et al. 2008
DQ903947	*Vibrio*	Zhu et al. 2008
DQ903951	*Vibrio*	Zhu et al. 2008
DQ903952	*Vibrio*	Zhu et al. 2008
JF820685	*Vibrio*	Jackson et al. 2012
JF820750	*Vibrio*	Jackson et al. 2012
JX477102	*Vibrio*	Dupont et al. 2014
FJ154796	*Vibrio* BLAST close neighbor	NA*
HQ161740	*Vibrio* BLAST close neighbor	NA*
KC884660	*Vibrio* BLAST close neighbor	NA*
HG315011	*Vibrio* BLAST close neighbor	NA*
NR_115936	*Vibrio* type species	NA*
HQ449970	*Photobacterium* outgroup to *Vibrio*	NA*

*Not Applicable

ISOLATES PB 4-2 (A1) and S0022 B001

Isolates PB 4-2 (A1) and S0022 B001 were chosen for further analyses because BLAST revealed their closest neighbor to be a *Brevibacterium* and *Pseudomonas* sp., respectively, making isolates PB 4-2 (A1) and S0022 B001 good candidates for bioactivity, NRPS and PKS assays, and environmental and biochemical analyses. Isolate S0022 B001 was chosen also because of its deep purple color and crystal formation on agar plates.

Growth with Various Media

For PB 4-2 (A1), cells were inoculated in vials containing ASW, MB, sterile cotton submerged in MB, nutrient broth (NB), or Mueller-Hinton broth (MHB) and streaked on Actinomycetes agar (AA), ASA+glycerol, ASA+glycerol+peptone, MA, nutrient agar (NA), and Mueller-Hinton agar (MHA). For S0022 B001, cells were inoculated in vials containing MB, NB, MHB and streaked on ASA, ASA+glycerol, MA, NA, and MHA. All cells were incubated at ambient lab temperature 21ºC for 7 days. Plates and broths were checked for red or purple growth, unusual formations, and agar color change.

Temperature Growth range

For PB 4-2 (A1), cells were streaked on ASA and incubated at the following temperatures: 15, 18, 21, 24, 27, 28, and 37ºC. For S0022 B001, cells were streaked on MA and incubated at the following temperatures: 21, 24 and 37ºC. All plates were incubated for 7 days and checked for red or purple growth.

Light Microscopy/Gram Stain

PB 4-2 (A1) cells grown on ASA and S0022 B001 cells grown on MA were transferred to slides and resuspended with a loop of water, heat fixed, and Gram stained. Cells were viewed under a compound telescope with the highest magnification under oil immersion (1000x total).

Electron Microscopy

Cell and crystal visualization. All preparation of cells was done in a chemical flow hood using aseptic technique. PB 4-2 (A1) cells grown on ASA and S0022 B001 cells grown on MA were resuspended in sterile autoclaved Millipore water, centrifuged, and water decanted 3 times. Cells were fixed with 4% glutaraldehyde in 0.1 M sodium cacodylate with 0.35 M sucrose, pH 7.6, for 1 hour. The cell mixture was loaded onto a 0.2 um filter and the liquid was filtered through. Cells were washed in 0.1 M sodium cacodylate with 0.44 M sucrose, 2 times, 20 minutes total. Cells were then dehydrated in a graded ethanol series of 30, 50, 70, 85, and 95%, 2 times for 3 minutes at each dilution. Cells were dried using 100% ethanol, 3 times for 10 minutes each time. Cells were critical point dried with hexamethyldisilazane. The filter containing the prepared cells was mounted on conductive carbon tape that was mounted on an aluminum disc. Silver polish was dotted from the edge of the disk to the edge of the filter. The cells were gold sputter coated for 60 sec in a vacuum chamber. Cells were visualized using Hitachi S-3400N Variable Pressure Scanning Electron Microscope. For crystal visualization, another sample of S0022 B001 was taken directly from plates and fixed in the same procedure as described above.

NRPS, PKS I, PKS II Genes Screening of Coconut Island and Puhi Bay Isolates and Violacein Genes Screening of S0022 B001

All isolates were screened for amplification of parts of the adenylation domains of NRPS, amplification of parts of ketosynthase and acyltransferase domains of PKS I, and amplification of parts of ketosynthase of PKS II genes using degenerate primers A3F, A7R, K1F, M6R, $KS_{\alpha,}$ and KS_{β} (Table 2.2). Colonies were grown in MB overnight or taken from MA or ASA plates at ambient lab temperature (22-25°C). DNA was extracted from cultures using MoBio Ultraclean Microbial DNA Isolation Kit following manufacturer instructions. DNA extractions were quantified using NanoDrop ND-1000 spectrophotometer. PCR was set to the following parameters for touchdown: 1 cycle at 95°C for 5 min; 10 cycles at 95°C for 30 sec, 60°C for 30 sec with 2º decrease each cycle, 72°C for 90 sec; 30 cycles at 95°C for 30 sec, 40°C for 30 sec; 1 cycle at 72°C for 7 min; and held at 4°C (Kennedy et al. 2008). PCR bacterial products were verified on a 1.5% agarose gel run at 80 volts for approximately 30-60 min and using Promega 100 bp ladder. *Pseudomonas aeruginosa* was used as a positive control. *Escherichia coli* and nuclease-free water were used as negative controls.

S0022 B001 was screened for parts of the Violacein synthesis structures using degenerate primer sets VPA3 and VPA4 which amplifies the flavoenzyme part of VioA, VPB1 and VPB2 which amplifies the heme protein part of VioB, VPB3 and VPB4 which also amplifies the heme protein part of VioB (Hakvag et al. 2009) (Table 2.2). PCR was set to the following parameters: initial denaturation at 96 °C for 5 minutes, followed by 35 cycles at 95 °C for 1 minute, 60 °C for 1 minute, and 72 °C for 2 minutes. Final extension was at 72 °C for 10 minutes. The amplicons were cloned as described for the S0022 B001 16S rRNA. PCR bacterial products

were verified on a 1% agarose gel run at 80 volts for approximately 30-60 min and using Promega 1 kb ladder. *Escherichia coli* and nuclease-free water were used as negative controls.

S0022 B001 Motility Assay

S0022 B001 was spotted in the center of 0.5% and 1.0% MA plates. As controls S0022 B001 was spotted on the center of 1.5% and *P. aeruginosa* was spotted in the center of 0.5%, 1.0%, and 1.5% MA plates. All plates were incubated at ambient lab temp 22ºC for 7 days.

CHAPTER THREE

RESULTS

Isolation of Sponge-Associated Bacteria

After 7 days of incubation on complex media agar plates, bacteria were transferred to MA and ASA plates and streaked for isolation. Each colony was considered an isolate due to a variety of colony characteristics including different colors, colony sizes, and colony (Table 3.1, Figure 3.1). Some colonies exhibited changes in color over time. After two consecutive rounds of re-isolation of single colonies, 68 culture isolates were considered pure cultures until 16s DNA analysis was performed. The negative control MA and ASA agar plates, ones that were spread with the outside surface of sponges that had been surfaced sterilized with ethanol, showed no growth.

Table 3.1. Bacterial isolate location, sponge origin, and colony characteristics.

Cultured Isolate ID	Location & Sponge	Colony Characteristics
CI 1-1 (A1)	Coconut Island, *Haliclona* sp.	Cream; shiny; smooth-edged; small-sized
CI 1-1 (B1)	Coconut Island, *Haliclona* sp.	Cream/light brown; shiny smooth-edged; small-sized
CI 1-1 (B2)	Coconut Island, *Haliclona* sp.	Cream; shiny; smooth-edged; small-sized
CI 1-1 (C1)	Coconut Island, *Haliclona* sp.	Cream, light brown, light orange; shiny; smooth-edged; small-sized
CI 1-1 (D1)	Coconut Island, *Haliclona* sp.	Light brown with brown ring; shiny; smooth-edged; small-sized
CI 1-1 (D2)	Coconut Island, *Haliclona* sp.	Cream; shiny; smooth-edged; small-sized
CI 1-2 (A1)	Coconut Island, *Haliclona* sp.	Brown; gooey; shiny; smooth-edged; small-sized
CI 1-2 (B1)	Coconut Island, *Haliclona* sp.	Off-white; shiny; smooth edged; small-sized
CI 1-2 (B2)	Coconut Island, *Haliclona* sp.	Pale apricot; shiny; smooth-edged; small-sized

Table 3.1. Bacterial isolate location, sponge origin, and colony characteristics (continued).

Cultured Isolate ID	Location & Sponge	Colony Characteristics
CI 1-2 (B3)	Coconut Island, *Haliclona* sp.	Cream; shiny; smooth-edged; small-sized
CI 1-2 (C1)	Coconut Island, *Haliclona* sp.	Brown; shiny; smooth-edged; medium-sized
CI 1-2 (C2)	Coconut Island, *Haliclona* sp.	Cream; shiny; smooth-edged; small-sized
CI 1-2 (D1)	Coconut Island, *Haliclona* sp.	Orange; shiny; smooth-edged; medium-sized
CI 1-2 (E1)	Coconut Island, *Haliclona* sp.	Brown; gooey; shiny; smooth-edged; small-medium-sized
CI 1-2 (E2)	Coconut Island, *Haliclona* sp.	Cream/slightly orange; shiny; smooth-edged; small-sized
CI 1-2 (F1)	Coconut Island, *Haliclona* sp.	Cream; shiny; smooth-edged; medium-sized
CI 1-2 (G1)	Coconut Island, *Haliclona* sp.	Cream; shiny; smooth-edged; small-sized
CI 1-3 (A1)	Coconut Island, *Haliclona* sp.	White; shiny; smooth-edged; small-sized
CI 1-3 (A2)	Coconut Island, *Haliclona* sp.	Off-white; shiny; smooth-edged; small-sized
CI 1-3 (B1)	Coconut Island, *Haliclona* sp.	Brown; gooey; shiny; smooth-edged; small-medium-sized
CI 1-3 (C1)	Coconut Island, *Haliclona* sp.	Cream/transparent; shiny; smooth-edged; small-medium-sized
CI 1-3 (D1)	Coconut Island, *Haliclona* sp.	Light brown/transparent; shiny; smooth-edged; medium-sized
CI 1-3 (E1)	Coconut Island, *Haliclona* sp.	Light brown/transparent; shiny; smooth-edged; medium-sized
PB 4-1 (A1)	Puhi Bay, *Petrosia* sp.	Light brown/transparent; shiny; irregular-edged; large-sized
PB 4-1 (B1)	Puhi Bay, *Petrosia* sp.	Gray/light-brown; shiny; smooth-edged; medium-sized
PB 4-1 (B2)	Puhi Bay, *Petrosia* sp.	Cream; shiny; smooth-edged; small-sized
PB 4-2 (A1)	Puhi Bay, *Petrosia* sp.	Red; shiny; smooth-edged; medium-sized
PB 4-2 (A2)	Puhi Bay, *Petrosia* sp.	Light gray/cream; shiny; smooth-edged; small-sized
PB 4-2 (A3)	Puhi Bay, *Petrosia* sp.	Cream; shiny; smooth-edged; small-sized

Table 3.1. Bacterial isolate location, sponge origin, and colony characteristics (continued).

Cultured Isolate ID	Location & Sponge	Colony Characteristics
PB 4-2 (B1)	Puhi Bay, *Petrosia* sp.	Cream; shiny; smooth-edged; medium-sized
PB 4-2 (C1)	Puhi Bay, *Petrosia* sp.	Gray brown; shiny; smooth-edged; small-sized
PB 4-2 (C2)	Puhi Bay, *Petrosia* sp.	Light brown/transparent; shiny; smooth-edged, irregular when big; medium-sized
PB 5-1 (A1)	Puhi Bay, *Iotrochota protea*	Peach orange; shiny; smooth-edged; medium-sized
PB 5-1 (A2)	Puhi Bay, *Iotrochota protea*	Cream white; shiny; smooth-edged; small-sized
PB 5-1 (A3)	Puhi Bay, *Iotrochota protea*	Gray brown; shiny; smooth, gray edge and cream opaque center; small-sized
PB 5-1 (B1)	Puhi Bay, *Iotrochota protea*	Gray cream brown; shiny; smooth-edged; small-sized
PB 5-1 (C1)	Puhi Bay, *Iotrochota protea*	Cream; shiny; smooth-edged; small-sized
PB 5-1 (D1)	Puhi Bay, *Iotrochota protea*	Gray brown; shiny; smooth-edged, irregular when big; medium-sized
PB 5-2 (A1)	Puhi Bay, *Iotrochota protea*	Cream light orange; shiny; smooth-edged; medium-sized
PB 5-2 (B1)	Puhi Bay, *Iotrochota protea*	Cream; shiny; smooth-edged; medium - sized
PB 5-2 (C1)	Puhi Bay, *Iotrochota protea*	Cream; shiny; smooth-edged; medium - sized
PB 5-2 (D1)	Puhi Bay, *Iotrochota protea*	Cream; shiny; smooth-edged; small-sized
PB 5-2 (D2)	Puhi Bay, *Iotrochota protea*	Cream; shiny; smooth-edged; small-sized
PB 5-2 (E1)	Puhi Bay, *Iotrochota protea*	Cream brown; shiny; smooth-edged; small-sized
PB 5-2 (F1)	Puhi Bay, *Iotrochota protea*	Brown; gooey; shiny; smooth-edged; small-sized
PB 5-3 (A1)	Puhi Bay, *Iotrochota protea*	Cream gray brown; shiny; smooth-edged; small-sized
PB 5-3 (A2)	Puhi Bay, *Iotrochota protea*	Cream; shiny; smooth-edged; small-sized
PB 5-3 (B1)	Puhi Bay, *Iotrochota protea*	Gray brown; shiny; smooth, gray edge and cream opaque center; small-sized

Table 3.1. Bacterial isolate location, sponge origin, and colony characteristics (continued).

Cultured Isolate ID	Location & Sponge	Colony Characteristics
PB 5-3 (C1)	Puhi Bay, *Iotrochota protea*	Cream beige; shiny; smooth-edged; large-sized
PB 5-3 (C2)	Puhi Bay, *Iotrochota protea*	White cream; shiny; smooth-edged; small-sized
PB 5-3 (D1)	Puhi Bay, *Iotrochota protea*	Cream; shiny; smooth-edged; large-sized
PB 5-3 (D2)	Puhi Bay, *Iotrochota protea*	Cream gray; shiny; smooth-edged; large-sized
PB 5-3 (E1)	Puhi Bay, *Iotrochota protea*	Cream gray; shiny; irregular-edged; large-sized
PB 5-4 (A1)	Puhi Bay, *Iotrochota protea*	Red; shiny; smooth-edged; medium-sized
PB 5-4 (A2)	Puhi Bay, *Iotrochota protea*	White; shiny; smooth-edged; large-sized
PB 5-4 (A3)	Puhi Bay, *Iotrochota protea*	Cream white/green gray; shiny; smooth-edged; small-sized
PB 5-4 (A4)	Puhi Bay, *Iotrochota protea*	Cream; shiny; smooth-edged; small-sized
PB 5-4 (B1)	Puhi Bay, *Iotrochota protea*	Gray green/brown; shiny; smooth-edged; small-sized
PB 5-4 (B2)	Puhi Bay, *Iotrochota protea*	Cream yellow; shiny; smooth-edged; small-sized
PB 5-4 (B3)	Puhi Bay, *Iotrochota protea*	Cream yellow; shiny; smooth-edged; medium-sized
PB 5-4 (C1)	Puhi Bay, *Iotrochota protea*	Cream yellow; gooey; shiny; smooth-edged; small-sized
PB 5-4 (C2)	Puhi Bay, *Iotrochota protea*	Gray cream; shiny; smooth-edged; small-sized
PB 5-4 (D1)	Puhi Bay, *Iotrochota protea*	Cream; shiny; smooth-edged; small-sized
S0022 B001	Puhi Bay, *Iotrochota protea*	Purple pigment; shiny; smooth edge; medium-sized; crystal formation

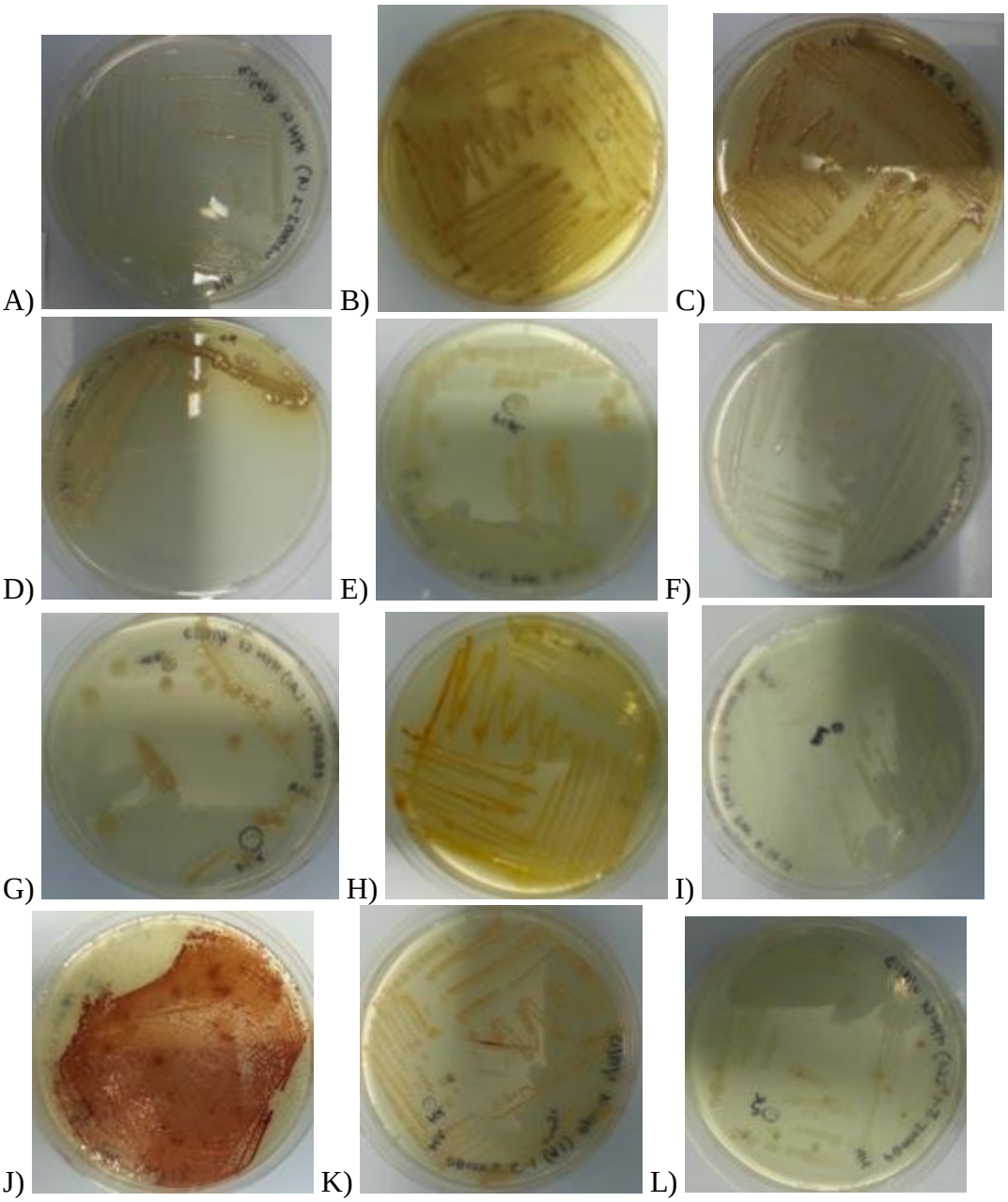

Figure 3.1. Photos of Representative Cultures on Marine Agar.
Isolate ID is followed by closest BLAST neighbor description. A) CI 1-1 (A1) *Pseudovibrio denitrificans.* B) CI 1-2 (C1) *Pseudovibrio denitrificans.* C) CI 1-2 (A1) *Pseudovibrio denitrificans.* D) CI 1-1 (B1) *Vibrio harveyi.* E) PB 5-2 (A1) *Vibrio harveyi.* F) CI 1-2 (B3) *Photobacterium rosenbergii.* G) PB 4-1 (A1) *Shewanella sp.* H) CI 1-2 (D1) *Pseudoalteromonas piscida.* I) PB 5-4 (B2) *Ferrimonas kyonanensis.* J) PB 5-4 (A1) *Thalassospira xianhensis.* K) PB 5-1 (A1) *Bacillus hwajinpoensis.* L) PB 5-1 (C1) *Pseudomonas fragi.*

NCBI BLAST

The cultured bacterial isolates of CI and PB showed the closest relationships to bacteria found in marine environments (seawater, sea sediment, marine sponges and tunicates, marine fish, marine algae, marine plants) according to BLAST results in Genbank (Table 3.2). The major species and phylum or class that CI and PB isolates were most similar to included *Bacillus* sp. from class Bacilli, *Brevibacterium* sp. from phylum Actinobacteria, *Ferrimonas* sp. from class Gammaproteobacteria, *Photobacterium* sp. from class Gammaproteobacteria, *Pseudoalteromonas* sp. from class Gammaproteobacteria, *Pseudomonas* sp. from class Gammaproteobacteria, *Shewanella* sp. from class Gammaproteobacteria, *Thalassospira* sp. from class Alphaproteobacteria, *Pseudovibrio* from class Alphaproteobacteria, and *Vibrio* sp. from class Gammaproteobacteria. Most CI and PB isolates had percent identities above 97% with their closest neighbors (56/64=87.5%). Exceptions include CI 1-1 (B1) whose closest BLAST neighbor was *Vibrio harveyi* at 94%, CI 1-1 (B2) whose closest BLAST neighbor was *Pseudovibrio denitrificans* at 83%, CI 1-2 (B2) whose closest BLAST neighbor was *Pseudomonas fragi* at 96%, PB 5-2 (D1) whose closest BLAST neighbor was *Vibrio rotiferianus* at 91%, PB 5-3 (C2) whose closest BLAST neighbor was *Photobacterium rosenbergii* at 91%, PB 5-4 (A2) whose closest BLAST neighbor was *Thalassospira permensis* at 84%, PB 5-4 (A4) whose closest BLAST neighbor was *Vibrio parahaemolyticus* at 87%, PB 5-4 (B2) whose closest BLAST neighbor was *Ferrimonas kyonanensis* at 96%.

Table 3.2. Bacterial Isolate closest NCBI GenBank BLAST neighbor and closest neighbor accession number.

Cultured Isolate ID	NCBI BLAST Closest Neighbor Description	Percent Identification	Closest Neighbor Accession #
CI 1-1 (A1)	*Pseudovibrio denitrificans*	99	AB681263
CI 1-1 (B1)	*Vibrio harveyi*	94	KC884656
CI 1-1 (B2)	*Pseudovibrio denitrificans*	83	KC751084
CI 1-1 (C1)	*Vibrio alginolyticus*	99	JF700503
CI 1-1 (D1)	*Pseudovibrio denitrificans*	99	AB681263
CI 1-1 (D2)	*Vibrio parahaemolyticus*	100	KC210810
CI 1-2 (A1)	*Pseudovibrio denitrificans*	100	AB681263
CI 1-2 (B1)	*Vibrio harveyi*	99	HM008704
CI 1-2 (B2)	*Pseudomonas fragi*	96	AM933514
CI 1-2 (B3)	*Photobacterium rosenbergii*	99	HQ449970
CI 1-2 (C1)	*Pseudovibrio denitrificans*	99	NR_029112
CI 1-2 (C2)	*Photobacterium rosenbergii*	99	HQ449970
CI 1-2 (D1)	*Pseudoalteromonas piscicida*	99	KC59328
CI 1-2 (E1)	*Pseudovibrio denitrificans*	99	JN128253
CI 1-2 (E2)	*Vibrio parahaemolyticus*	99	JN188415
CI 1-2 (F1)	*Vibrio rotiferianus*	99	KC756840
CI 1-2 (G1)	*Vibrio alginolyticus*	100	KC884660
CI 1-3 (A1)	*Bacillus cereus*	100	KF278103
CI 1-3 (A2)	*Bacillus thuringiensis*	99	KF010790
CI 1-3 (B1)	*Pseudovibrio denitrificans*	100	JN128253
CI 1-3 (C1)	*Vibrio communis*	99	JF836185
CI 1-3 (D1)	*Vibrio harveyi*	99	FJ161347
CI 1-3 (E1)	*Vibrio fischeri*	99	DQ530290
PB 4-1 (A1)	*Shewanella sp.*	97	GU289647
PB 4-1 (B1)	*Pseudovibrio denitrificans*	99	HE584768
PB 4-1 (B2)	*Photobacterium rosenbergii*	97	HQ449969
PB 4-2 (A1)	*Brevibacterium frigoritolerans*	99	KC778370
PB 4-2 (A2)	*Pseudovibrio denitrificans*	100	AB681263
PB 4-2 (A3)	*Vibrio maritimus*	98	GU929925
PB 4-2 (B1)	*Vibrio harveyi*	99	JF412246
PB 4-2 (C1)	*Pseudovibrio denitrificans*	99	HE584768
PB 4-2 (C2)	*Pseudoalteromonas arabiensis*	100	AB576636
PB 5-1 (A1)	*Bacillus hwajinpoensis*	98	GQ903399
PB 5-1 (A2)	*Bacillus stratosphericus*	98	KF535117
PB 5-1 (A3)	*Pseudovibrio denitrificans*	99	HE584768
PB 5-1 (B1)	*Pseudovibrio denitrificans*	99	NR_029112
PB 5-1 (C1)	*Pseudomonas fragi*	99	AB685632
PB 5-1 (D1)	*Pseudovibrio denitrificans*	100	HE584768

Table 3.2. Bacterial Isolate closest NCBI BLAST neighbor and closest neighbor accession number (continued).

Cultured Isolate ID	NCBI BLAST Closest Neighbor Description	Percent Identification	Closest Neighbor Accession #
PB 5-2 (A1)	*Vibrio harveyi*	98	HQ161740
PB 5-2 (B1)	*Vibrio aestuarianus*	99	DQ985231
PB 5-2 (C1)	*Vibrio alginolyticus*	100	KC884660
PB 5-2 (D1)	*Vibrio rotiferianus*	91	KC756840
PB 5-2 (D2)	*Vibrio rotiferianus*	99	HQ890462
PB 5-2 (E1)	*Vibrio owensii*	99	HG315011
PB 5-2 (F1)	*Pseudovibrio ascidiaceicola*	99	AB681198
PB 5-3 (A1)	*Pseudovibrio denitrificans*	100	HE584768
PB 5-3 (A2)	*Vibrio harveyi*	100	KF193917
PB 5-3 (B1)	*Pseudovibrio denitrificans*	99	AB681263
PB 5-3 (C1)	*Pseudovibrio denitrificans*	99	AB681263
PB 5-3 (C2)	*Photobacterium rosenbergii*	92	HQ439539
PB 5-3 (D1)	*Vibrio harveyi*	98	FJ161347
PB 5-3 (D2)	*Pseudovibrio denitrificans*	99	HE584768
PB 5-3 (E1)	*Vibrio natriegens*	99	KF245479
PB 5-4 (A1)	*Thalassospira xianhensis*	99	KC534432
PB 5-4 (A2)	*Thalassospira permensis*	84	FJ860275
PB 5-4 (A3)	*Vibrio natriegens*	99	KF245479
PB 5-4 (A4)	*Vibrio parahaemolyticus*	87	KF142388
PB 5-4 (B1)	*Pseudovibrio denitrificans*	99	HQ908691
PB 5-4 (B2)	*Ferrimonas kyonanensis*	96	NR_041387
PB 5-4 (B3)	*Vibrio harveyi*	99	FJ154796
PB 5-4 (C1)	*Vibrio harveyi*	99	HM008704
PB 5-4 (C2)	*Pseudovibrio denitrificans*	100	AB681263
PB 5-4 (D1)	*Vibrio azureus*	99	KC210817
S0022 B001	*Pseudomonas* sp.	99	AM934696

The dominant isolates belonged the *Vibrio* (n=27, 42.19%) and *Pseudovibrio* groups (n=20, 31.25%) (Table 3.3, Figure 3.2). The remaining groups that were isolated are *Bacillus* (n=4, 6.25%), *Photobacterium* (n=4, 6.25%), *Pseudoalteromonas* (n=2, 3.13%), *Pseudomonas* (n=2, 3.13%), *Thallasospira* (n=2, 3.13%), *Brevibacterium* (n=1, 1.56%), *Ferrimonas* (n=1, 1.56%), and *Shewanella* (n=1, 1.56%).

Table 3.3 Coconut Island &Puhi Bay Bacteria Count

Bacteria Genus	Count	Percent
Bacillus	4	6.25%
Brevibacterium	1	1.56%
Ferrimonas	1	1.56%
Photobacterium	4	6.25%
Pseudoalteromonas	2	3.13%
Pseudomonas	2	3.13%
Pseudovibrio	20	31.25%
Shewanella	1	1.56%
Thallasospira	2	3.13%
Vibrio	27	42.19%
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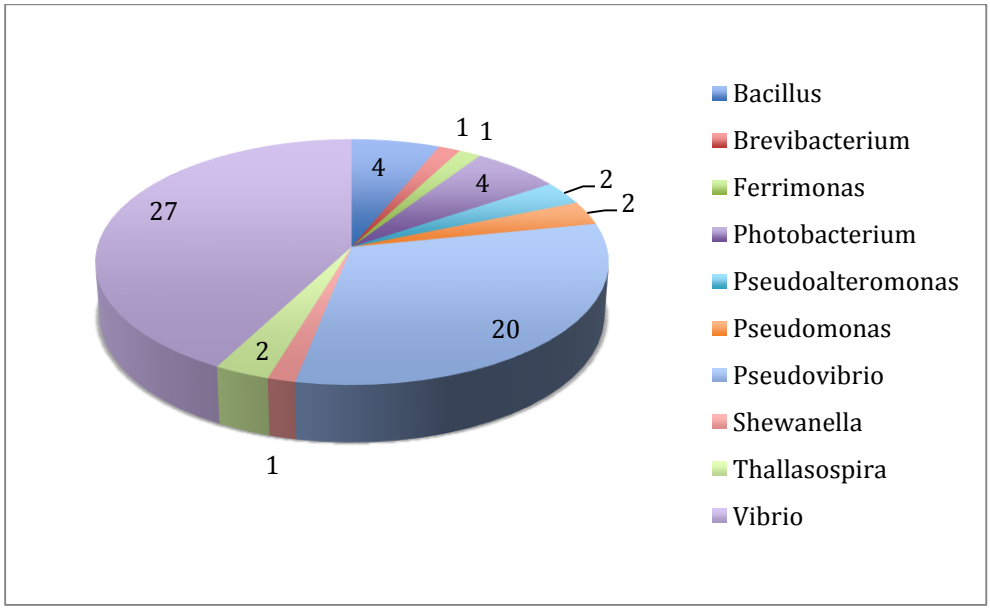


Figure 3.2 Coconut Island & Puhi Bay Bacteria Count
4 isolates are *Bacillus* sp., 1 isolate is a *Brevibacterium* sp., 1 isolate is a *Ferrimonas* sp., 4 isolates are *Photobacterium* sp., 2 isolates are *Pseudoalteromonas* sp., 2 isolates are *Pseudomonas* sp., 20 isolates are *Pseudovibrio* sp., 1 isolate is a *Shewanella* sp., 2 isolates are *Thalassospira* sp., and 27 isolates are *Vibrio* sp.

The genera *Photobacterium*, *Pseudovibrio*, and *Vibrio* occur among CI (*Haliclona* sp.), PB 4 (*Petrosia* sp.), and PB 5 (*I. protea*) groups (Figure 3.3). The genera *Brevibacterium* and *Shewanella* occur only in the PB 4 group. The genera *Ferrimonas* and *Thalassospira* occur only in the PB 5 group. The genus *Pseudoalteromonas* occurs between the CI and PB 4 groups. The genera *Bacillus* and *Pseudomonas* occur between the CI and PB 5 groups.

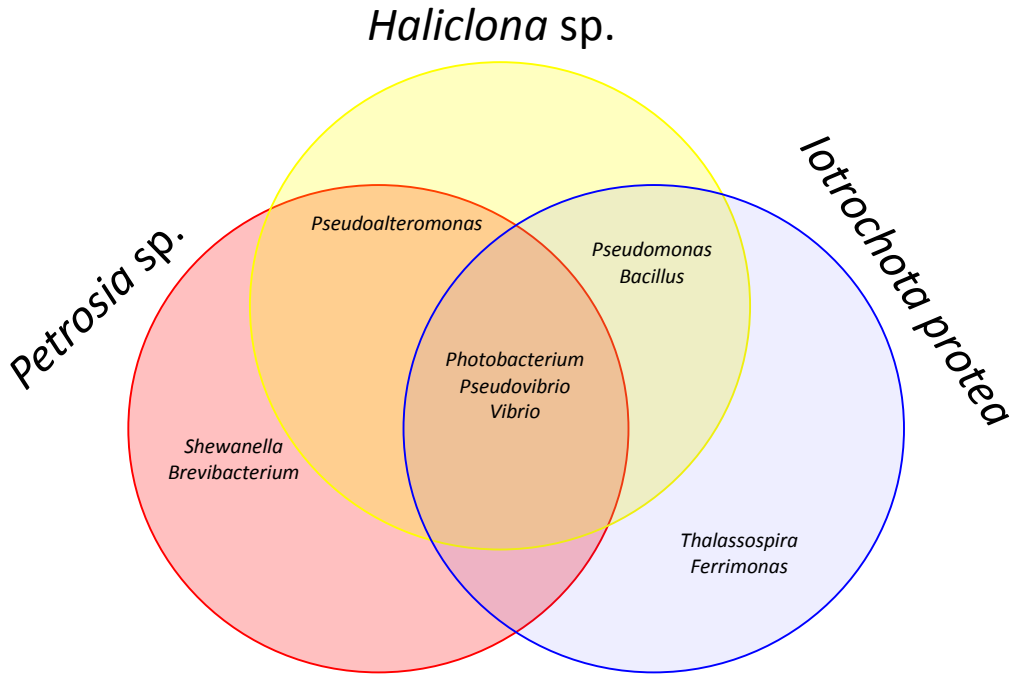


Figure 3.3. Sponges *Haliclona* sp, *Petrosia* sp., and *Iotrochota protea* show common and distinct cultured bacterial genera.
The sponge *Petrosia* sp. is represented as the red circle. The sponge *Haliclona* sp. is represented as the yellow circle. The sponge *Iotrochota protea* is represented as the blue circle. PB 4 isolates BLAST closest genera occur 1) in only *Petrosia* sp. (red), 2) in both *Petrosia* sp. and *Haliclona* sp. (the intersection of red and yellow), or 3) among all *Haliclona* sp., *Petrosia* sp., and *Iotrochota protea* (the intersection of red, yellow, and blue); CI isolates BLAST closest genera occur 4) in both *Petrosia* sp. and *Haliclona* sp. (the intersection of red and yellow), 5) in both *Haliclona* sp.and *Iotrochota protea* (the intersection of yellow and blue) or 6) among all *Haliclona* sp., *Petrosia* sp., and *Iotrochota protea* (the intersection of red, yellow, and blue); PB 5 isolates BLAST closest genera occur 7) in only *Iotrochota protea* (blue), 8) in both *Iotrochota protea* and *Haliclona* sp. (the intersection of blue and yellow), or 9) among all *Haliclona* sp., *Petrosia* sp., and *Iotrochota protea* (the intersection of red, yellow, and blue). S0022 B001, closest BLAST genera is *Pseudomonas* sp., is from *Iotrochota protea.*

Phylogenetics

Bootstrap consensus values 90-100% are considered strong, 70-89% moderate, and 0-69% weak for the Maximum Likelihood analysis. Bootstrap values ≥70% are shown at the nodes except in the cases where values <70% are at nodes connected to CI or PB isolates.

Bacillus Group. Bootstrap values generally show moderate support for this tree being a close representation of the true evolutionary relationship among the bacteria (Figure 3.4A). The Bacillus CI and PB isolates clustered in separate branches away from each other. The isolates clustered with their respective closest neighbor but had weak bootstrap values. Isolate CI 1-3 (A2) also clustered with a *Bacillus* from Oahu but had a weak bootstrap value. PB 5-1 (A2) clustered most closely with a *Bacillus* from Brazil but with a weak bootstrap value. PB 5-1 (A1) clustered most closely with a *Bacillus* from Oahu but had a weak bootstrap value. The isolates clustered away from the type species but PB 5-1 (A1) was closest to the type species.

Brevibacterium Group. Bootstrap values generally show weak support for this tree being a close representation of the true evolutionary relationship among the bacteria (Figure 3.4B). Isolate PB 4-2 (A1) clustered most closely with known *B. frigoritolerans* including its closest BLAST neighbor and had a moderate bootstrap value. The isolate clustered separately from known *Brevibacterium* from world sponges. The isolate clustered away from the type species.

Ferrimonas Group. Bootstrap values show strong support for this tree being a close representation of the true evolutionary relationship among the bacteria (Figure 3.4C). The isolates clustered together on a separate branch away from other marine *Ferrimomas* sp. The isolates clustered with their closest BLAST neighbor with moderate to strong bootstrap values. The isolates also clustered away from the type species.

***Photobacterium Group*.** Bootstrap values generally show moderate support for this tree being a close representation of the true evolutionary relationship among the bacteria (Figure 3.4D). Isolates PB 4-1 (B2) and PB 5-3 (C2) clustered together with a moderate bootstrap value. Isolate CI 1-2 (B3) clustered in a separate branch with its closest BLAST neighbor and had a strong bootstrap value. The closest BLAST neighbor to isolates PB 4-1 (B2) and PB 5-3 (C2) clustered more closely with bacteria from Australia and Brazil with a strong bootstrap value. The isolates did not cluster closely with any bacteria from world sponges. The isolates also did not cluster with the type species.

***Pseudoalteromonas Group*.** Bootstrap values generally show moderate support for this tree being a close representation of the true evolutionary relationship among the bacteria (Figure 3.4E). The isolates clustered on separate branches away from each other. The isolates clustered most closely with their respective BLAST closest neighbor with moderate to strong bootstrap values. Isolate CI 1-2 (D1) clustered closely with bacteria from China and the Northeast USA but with a weak bootstrap value. Isolate PB 4-2 (C2) did not closely cluster with any bacteria from world sponges. The isolates did not cluster with the type species.

***Pseudomonas Group*.** Bootstrap values generally show weak support for this tree being a close representation of the true evolutionary relationship among the bacteria (Figure 3.4F). The isolates clustered separately on different branches. Isolate PB 5-1 (C1) clustered with its closest BLAST neighbor. S0022 B001 clustered most closely with a bacterium from Antarctica but had a weak bootstrap value. The isolates clustered away from the type species.

***Pseudovibrio Group*.** The preliminary phylogenetic tree that included all CI and PB isolates along with closest BLAST neighbors and world isolates in the *Pseudovibrio* group showed very weak support with the highest bootstrap value being 78 and the rest being 34 and below (data not

shown), though 19 of the 20 CI and PB isolates had closest BLAST identities 97% and above (Table 3.2). The tree in Figure 3.4G includes the sequences that had the strongest bootstrap value, and the tree is generally moderately supported. PB 5-1 (B1) and PB 4-1 (B1) clustered together but with weak bootstrap values. Isolate PB 5-2 (F1) clustered most closely with a *Pseudovibrio* sp. from France but had a weak bootstrap value. PB 5-1 (B1) and PB 4-1 (B1) clustered with their closest BLAST neighbor which was also their type species. The isolates clustered closely with a *Pseudovibrio* from Oahu but had a weak bootstrap value.

***Shewanella Group*.** Bootstrap values generally show weak support for this tree being a close representation of the true evolutionary relationship among the bacteria (Figure 3.4H). The PB isolate clustered most closely with its closest BLAST neighbor but had a weak bootstrap value. The isolate did not cluster with bacteria from world sponges. The isolate also did not cluster with the type species.

***Thalassospira Group*.** Bootstrap values generally show weak support for this tree being a close representation of the true evolutionary relationship among the bacteria (Figure 3.4I). The two isolates clustered together but with weak bootstrap values. The isolates clustered with their closest BLAST neighbors but with weak bootstrap values. The isolates did not cluster closely with bacteria from world sponges. The isolate did not cluster with the type species.

***Vibrio Group*.** The preliminary phylogenetic tree that included all CI and PB isolates along with closest BLAST neighbors and world isolates in the *Vibrio* group showed very weak support with the one-third of bootstrap values between 50-89 and the rest being below 50 (data not shown), though 24 of the 26 CI and PB isolates had closest BLAST identities 97% and above (Table 3.2). The tree in Figure 3.4 J includes the sequences that had the strongest bootstrap value, and the tree is generally moderately supported. Isolates PB 5-2 (C1) and PB 5-2 (E1) clustered together

but with weak bootstrap values. The isolates PB 5-2 (C1) and PB 5-2 (E1) clustered with their closest BLAST neighbor but with weak bootstrap values. The remaining isolates did not cluster with their closest BLAST neighbors. The isolates clustered away from bacteria from world sponges. The isolates also did not cluster with the type species.

A)

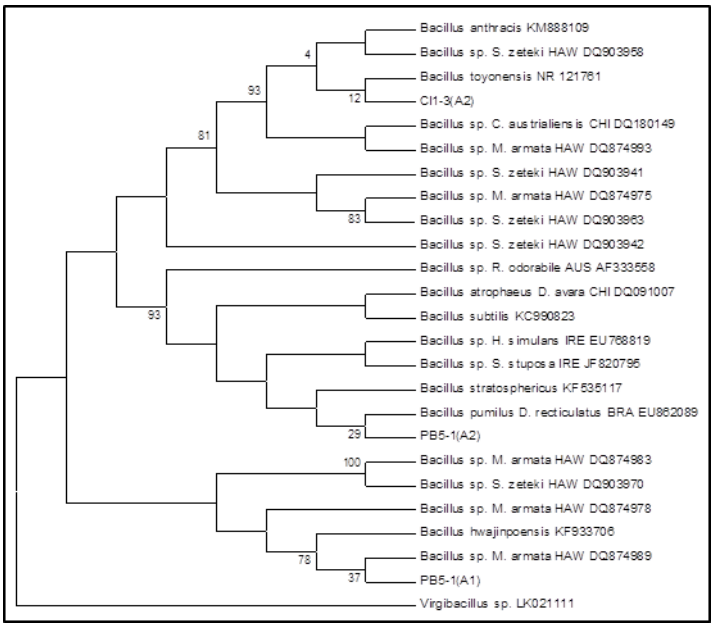
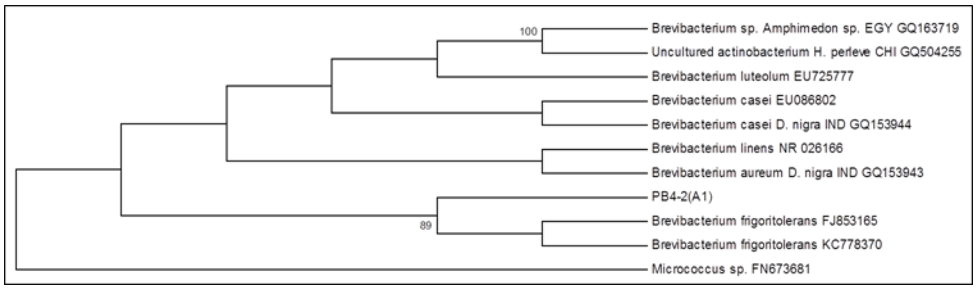


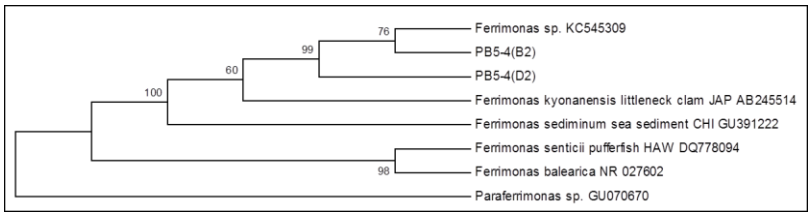
Figure 3.4. Maximum likelihood trees of CI and PB isolates, closest NCBI BLAST neighbors, type species, and corresponding genera of bacteria from world sponges or other sources.

Trees were computed using 1000 bootstrap replicates; all bootstrap values that are 70% and greater are shown at nodes. Values below 70% are not shown except in the cases where values below 70% are at nodes connected to CI or PB isolates. Isolates are given according to their designated ID. For closest neighbors, the bacteria's scientific name is given followed by the GenBank accession number. For bacteria belonging to sponges, bacteria's scientific name is given followed by either the sponge it belongs to or other origin, followed by the geographic location of the sponge, followed by the accession number. Sponge species are *Amphimedon* sp., *Anoxycalyx joubini, Clathria prolifera, Craniella austrialiensis, Mycale armata, Dendrilla nigra, Discodermia* sp., *Dragmacidon reticulatus, Dysidia avara, Phorbas tenacio, Haliclona simulans, Hymeniacidon perleve, Lissodendoryx nobilis, Raspailia ramosa, Rhopaloeides odorabile, Stelligera stuposa,* and *Suberites zeteki.* Geographic locations are ANT—Antarctica, AUS—Austrailia, BRA—Brazil, CAR—Caribbean, CHI—China, EGY—EGY, FRA—France, HAW—Oahu Hawaii, IND—India, IRE—Ireland, JAP—Japan, NEU—Northeast USA, SPA—Spain. A) *Bacillus* sp. group: Kimura 2-parameter model, invariant rates, type species *B. subtilis*, outgroup *Virgibacillus.*

B)



C)



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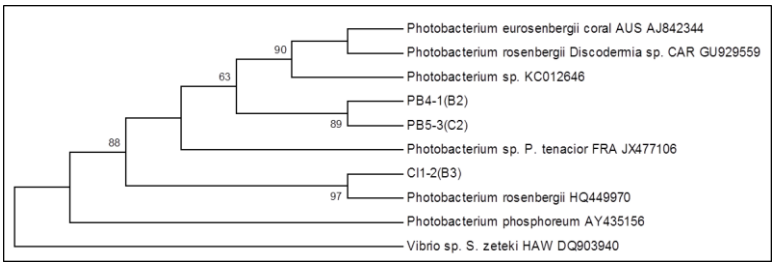


E)

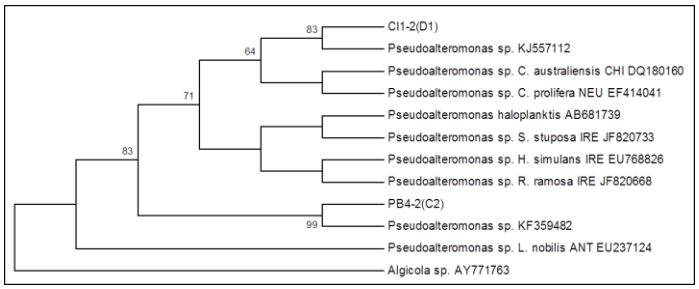
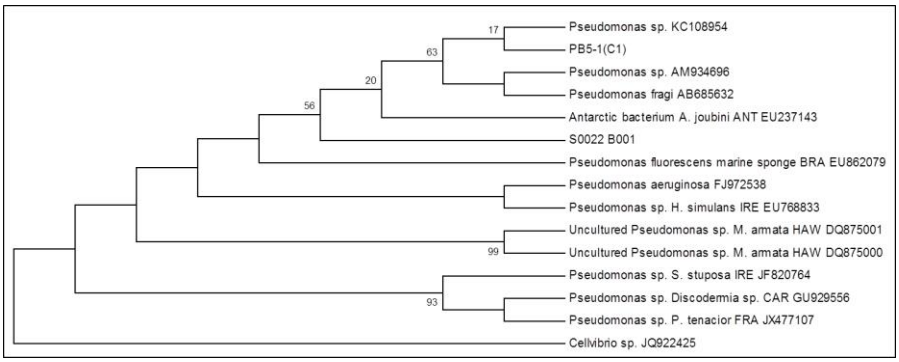


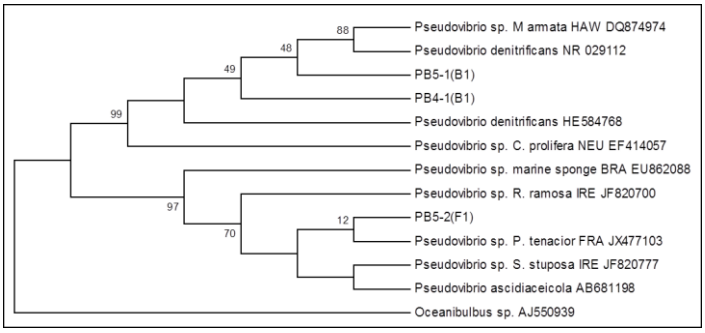
Figure 3.4. Maximum likelihood trees of CI and PB isolates, closest NCBI BLAST neighbors, type species, and corresponding genera of bacteria from sponges or other sources around the world (continued).

B) *Brevibacterium* sp. group: Tamura-Nei model, gamma distribution, type species *B. linens*, outgroup *Micrococcus*. C) *Ferrimonas* sp. group: Tamura-Nei model, gamma distribution, type species *F. balearica*, outgroup *Paraferrimonas*. D) *Photobacterium* sp. group: Kimura 2-parameter model, invariant rates, type species *P. phosphoreum*, outgroup *Vibrio*. E) *Pseudoalteromas* sp. group: Kimura 2-parameter model, invariant rates, type species *P. haloplanktis*, outgroup *Algicola*.

F)



G)



H)

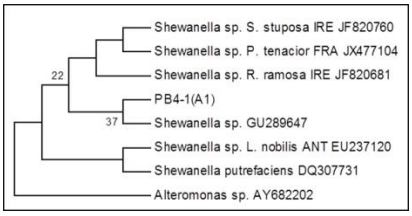
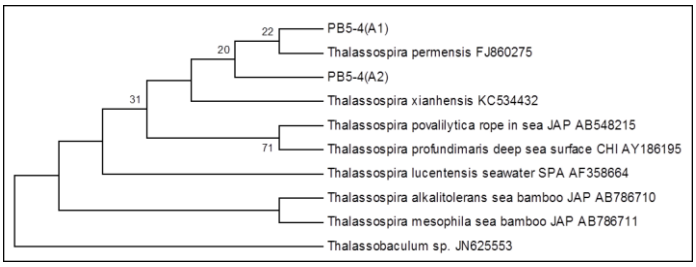


Figure 3.4. Maximum likelihood trees of CI and PB isolates, closest NCBI BLAST neighbors, type species, and corresponding genera of bacteria from sponges or other sources around the world (continued).

F) *Pseudomonas* sp. group: Kimura 2-parameter model, uniform rates, type species *P. aeruginosa*, outgroup *Cellvibrio*. G) *Pseudovibrio* sp. group: Kimura 2-parameter model, uniform rates, type species *P. dentrificans*, outgroup *Oceanibulbus*. H) *Shewanella* sp. group: Kimura 2-parameter model, gamma distribution and invariant rates, type species *S. putrefaciens*, outgroup *Alteromonas*.

I) 

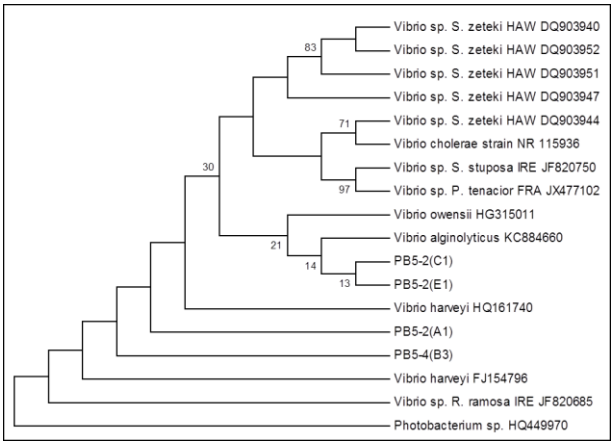
J) 

Figure 3.4. Maximum likelihood trees of CI and PB isolates, closest NCBI BLAST neighbors, type species, and corresponding genera of bacteria from sponges or other sources around the world (continued).
I) *Thalassospira* sp. group: Kimura 2-parameter model, gamma distributed, type species *T. lucentensis*, outgroup *Thalassospira*. J) *Vibrio* sp. group: Kimura 2-paramter model, gamma distribution and invariant rates, type species *V. cholera*, outgroup *Photobacterium.*

Isolate PB 4-2 (A1)

Isolate PB 4-2 (A1) culture growth characteristics, Gram Stain, and microscopy.

Isolate PB 4-2 (A1) displayed a bright red to deep red color on agar plates and broth (Table 3.4 and Figure 3.5A). Growth on MA and ASA start out without pigment then the red pigment develops as the colony gets bigger. The bacterium is able to extract or breakdown precipitated salts/minerals or biomolecules in agar. The bacterium turns the marine agar orange to light brown. The bacteria grow best on ASA+glycerol+peptone and MA. The addition of peptone makes the bacterium produce a deeper red color. The bacterium can grow in a small

volume of ASW and MB, about 5 mL of broth, but needs a substrate like cotton in the MB to grow at larger volumes. The bacterium does not grow in AA, NB, NA, MHB, nor MHA. The bacterium grows from 18-27º C but not at 15º C, 28º C, nor 37º C.

Cells Gram stain positive (purple) and appear coccoid when cultured on agar (Figure 3.5 B). Electron microscopy showed lumpy coccoid forms and well as a few short rods characteristic of *Brevibacterium* (Figure 3.6).

Table 3.4. PB 4-2 (A1) Culture medium characteristics.

Physical Characteristics	
Color: red Color Progression: starts out colorless then develops color as colony gets bigger Color variation: bright red to purple red Agar change: able to extract or breakdown precipitated salts/minerals or biomolecules in agar; turns agar orange light brown	
Growth with Various Media	
Actinomycetes + filtered seawater broth	Growth up to 5 ml, No growth in 100 ml
Actinomycetes + filtered seawater + glycerol agar	Growth
Actinomycetes + filtered seawater + glycerol + peptone agar	Growth, deeper red
Marine broth	Growth up to 5 ml, No growth in 100 ml
Marine broth + cotton	Growth
Marine agar	Growth
Actinomycetes agar	No growth
Nutrient broth	No growth
Nutrient agar	No growth
Mueller-Hinton broth	No growth
Mueller-Hinton agar	No growth
Temperature Growth range	
Temperature (º C)	Growth (yes/no)
15	no
18	yes
21	yes
24	yes
27	yes
28	no
37	no

A) 

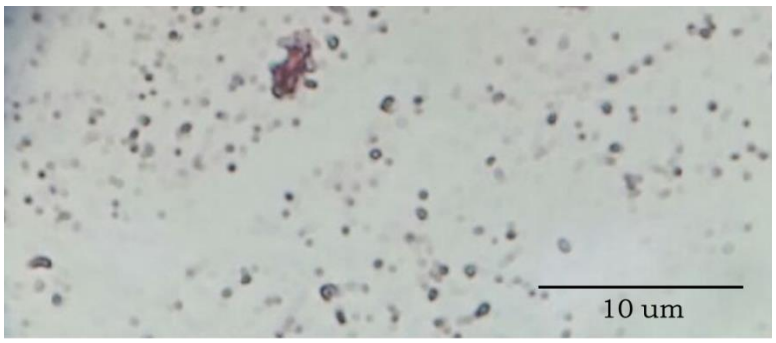
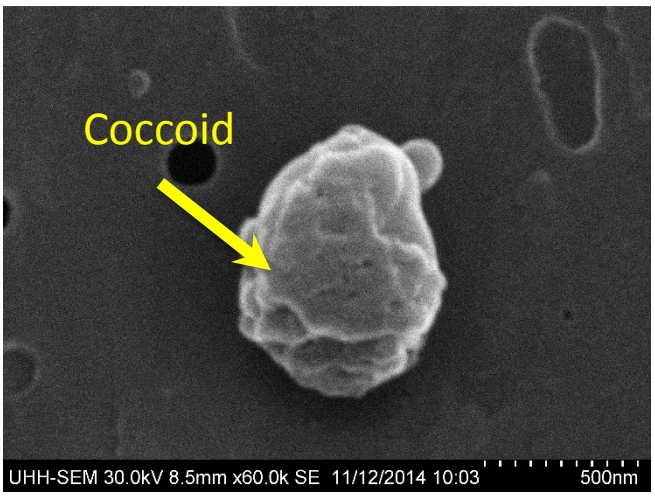
B) 

Figure 3.5. PB 4-2 (A1) Culture, Light Microscopy, and Gram Stain.
A) Cultured isolate PB 4-2 (A1) grown on MA. B) Light Microscopy at 100x magnification; cells stain Gram positive.

A) 

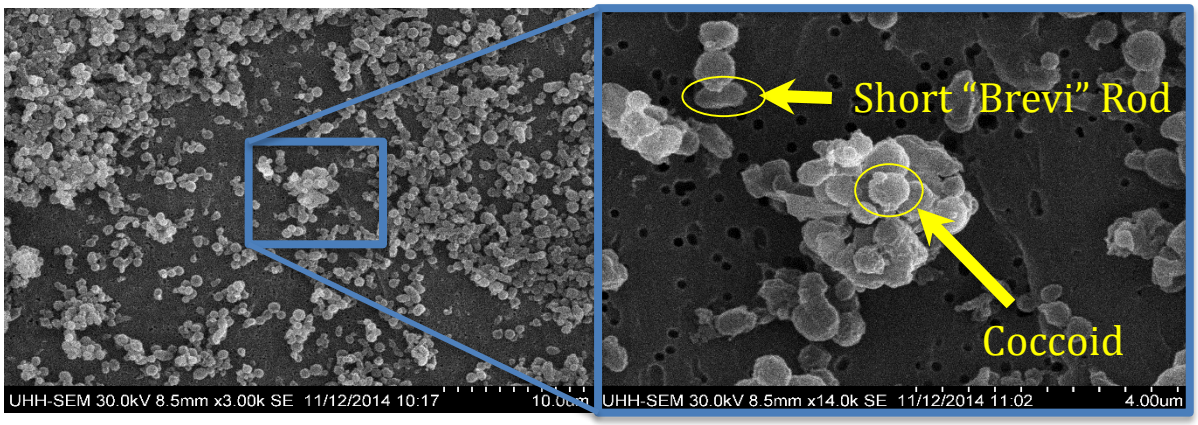
B) 

Figure 3.6. PB 4-2 (A1) Scanning Electron Microscopy.
Scale bars are at bottom right. A) One bacterium as coccoid, 0.65 um in width and 0.85 um in length. B) At left: field of bacteria and at right: close up of cluster of bacteria as coccoid and sort rod forms.

Disc Diffusion Assay of PB 4-2 (A1) against model laboratory organisms and cultured bacteria from Coconut Island and Puhi Bay

Isolate PB 4-2 (A1) inhibits the growth of Gram positive organisms *Staphylococcus aureus* and *Bacillus cereus* but not against Gram negative *Escherichia coli* (Table 3.5 and Figure 3.7). Zones of growth inhibition ranged from 3-8 mm for SA and 3-5 mm for BC. Negative control MB was negative, and positive controls ampicillin (A) and kanamycin (K) were positive.

Table 3.5. Disc Diffusion Assay of PB 4-2 (A1) against model laboratory organisms.

Model Laboratory Organisms	Growth Inhibition Zone Activity				
	Neg control MB	A 10 mg/mL	K 10 mg/mL	PB 4-2 (A1) in mm	+/-
Staphylococcus aureus	-	+	+	5	+
Staphylococcus aureus	-	+	+	4	+
Staphylococcus aureus	-	+	+	3	+
Staphylococcus aureus	-	+	+	7	+
Staphylococcus aureus	-	+	+	8	+
Bacillus cereus	-	+	+	4	+
Bacillus cereus	-	+	+	5	+
Bacillus cereus	-	+	+	4	+
Bacillus cereus	-	+	+	4	+
Bacillus cereus	-	+	+	3	+
Escherichia coli	-	+	+	0	-
Escherichia coli	-	+	+	0	-
Escherichia coli	-	+	+	0	-
Escherichia coli	-	+	+	0	-
Escherichia coli	-	+	+	0	-

A)  B) 

Figure 3.7. Disc Diffusion Plates of Model Lab Bacteria Growth Inhibition with PB 4-2 (A1).
Discs are 6 mm in diameter. A) *Staphylococcus aureus* zone of growth inhibition 8 mm. B) *Bacillus cereus* zone of growth inhibition 3 millimeters.

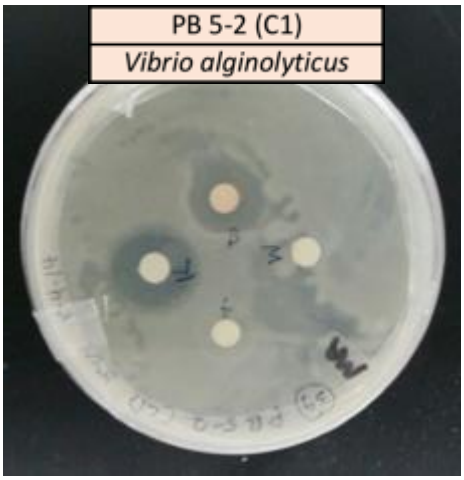
Isolate PB 4-2 (A1) inhibits the growth of most of the cultured bacteria from Coconut Island and Puhi Bay (Table 3.6). Forty-nine out of 59 CI and PB tested positive for growth inhibition, about 75% of the isolates tested. The isolates that tested positive included the following groups (according to BLAST closest neighbors): *Bacillus, Ferrimonas, Photobacterium, Pseudoalteromonas, Pseudovibrio,* and *Vibrio* (Figure 3.8). Zones of inhibition ranged from 0.5-3.5 mm. Isolate PB 4-2 (A1) did not inhibit the growth of 15 out of 59 CI and PB isolates, about 25% of the isolates tested. The isolates that tested negative included the following groups (according to BLAST closest neighbors): *Pseudomonas, Pseudovibrio, Thalassospira,* and *Vibrio.* Negative control MB was negative, and positive controls A and K were positive. Isolate PB 4-2 (A1) tested negative against itself.

Table 3.6. Disc Diffusion Assay of PB 4-2 (A1) against cultured bacteria from Coconut Island and Puhi Bay.

Cultured Isolate ID or control	NCBI BLAST Closest Neighbor Description	Growth Inhibition Zone Activity				
		MB	A 10 mg/mL	K 10 mg/mL	PB 4-2 (A1) in mm	+/-
CI 1-1 (A1)	*Pseudovibrio denitrificans*	-	+	+	0.5	+
CI 1-1 (B1)	*Vibrio harveyi*	-	-	+	0.5	+
CI 1-1 (B2)	*Pseudovibrio denitrificans*	-	-	+	1	+
CI 1-1 (C1)	*Vibrio alginolyticus*	-	-	+	1	+
CI 1-1 (D1)	*Pseudovibrio denitrificans*	-	-	+	3.5	+
CI 1-1 (D2)	*Vibrio parahaemolyticus*	-	-	+	1	+
CI 1-2 (A1)	*Pseudovibrio denitrificans*	-	-	+	2	+
CI 1-2 (B1)	*Vibrio harveyi*	-	-	+	1	+
CI 1-2 (B2)	*Pseudomonas fragi*	-	-	+	0	-
CI 1-2 (B3)	*Photobacterium rosenbergii*	-	+	+	2	+
CI 1-2 (C1)	*Pseudovibrio denitrificans*	-	+	+	2	+
CI 1-2 (C2)	*Photobacterium rosenbergii*	-	+	+	1	+
CI 1-2 (D1)	*Pseudoalteromonas piscicida*	-	+	+	0.5	+
CI 1-2 (E1)	*Pseudovibrio denitrificans*	-	-	+	2	+
CI 1-2 (E2)	*Vibrio parahaemolyticus*	-	-	+	1	+
CI 1-2 (F1)	*Vibrio rotiferianus*	-	-	+	1	+
CI 1-2 (G1)	*Vibrio alginolyticus*	-	-	+	3	+
CI 1-3 (A1)	*Bacillus cereus*	-	+	+	1	+
CI 1-3 (A2)	*Bacillus thuringiensis*	-	+	+	1	+
CI 1-3 (B1)	*Pseudovibrio denitrificans*	-	-	+	2	+
CI 1-3 (C1)	*Vibrio communis*	-	-	+	1	+
CI 1-3 (D1)	*Vibrio harveyi*	-	-	+	1	+
CI 1-3 (E1)	*Vibrio fischeri*	-	-	+	1	+
PB 4-1 (B1)	*Pseudovibrio denitrificans*	-	-	+	2	+
PB 4-1 (B2)	*Photobacterium rosenbergii*	-	+	+	1	+
PB 4-2 (A1)	*Brevibacterium frigoritolerans*	-	-	+	0	-
PB 4-2 (A2)	*Pseudovibrio denitrificans*	-	-	+	0	-
PB 4-2 (B1)	*Vibrio harveyi*	-	-	+	1	+
PB 4-2 (C1)	*Pseudovibrio denitrificans*	-	-	+	2	+
PB 4-2 (C2)	*Pseudoalteromonas arabiensis*	-	+	+	1	+
PB 5-1 (A1)	*Bacillus hwajinpoensis*	-	+	+	1	+
PB 5-1 (A2)	*Bacillus stratosphericus*	-	+	+	1	+
PB 5-1 (A3)	*Pseudovibrio denitrificans*	-	-	+	2	+
PB 5-1 (B1)	*Pseudovibrio denitrificans*	-	+	+	0	-
PB 5-1 (C1)	*Pseudomonas fragi*	-	-	+	0	-
PB 5-1 (D1)	*Pseudovibrio denitrificans*	-	-	+	0	-

Table 3.6. Disc Diffusion Assay of PB 4-2 (A1) against cultured bacteria from Coconut Island and Puhi Bay (continued).

Cultured Isolate ID or control	NCBI BLAST Closest Neighbor Description	Growth Inhibition Zone Activity				
		MB	A 10 mg/mL	K 10 mg/mL	PB 4-2 (A1) in mm	+/-
PB 5-2 (A1)	*Vibrio harveyi*	-	-	+	1	+
PB 5-2 (B1)	*Vibrio aestuarianus*	-	+	+	0.5	+
PB 5-2 (C1)	*Vibrio alginolyticus*	-	-	+	3	+
PB 5-2 (D1)	*Vibrio rotiferianus*	-	-	+	0	-
PB 5-2 (E1)	*Vibrio owensii*	-	-	+	0	-
PB 5-2 (F1)	*Pseudovibrio ascidiaceicola*	-	-	+	0	-
PB 5-3 (A1)	*Pseudovibrio denitrificans*	-	-	+	0	-
PB 5-3 (A2)	*Vibrio harveyi*	-	-	+	1	+
PB 5-3 (B1)	*Pseudovibrio denitrificans*	-	-	+	0.5	+
PB 5-3 (C1)	*Pseudovibrio denitrificans*	-	+	+	0.5	+
PB 5-3 (C2)	*Photobacterium rosenbergii*	-	-	+	0.5	+
PB 5-3 (D1)	*Vibrio harveyi*	-	-	+	0	-
PB 5-3 (D2)	*Pseudovibrio denitrificans*	-	-	+	2	+
PB 5-3 (E1)	*Vibrio natriegens*	-	-	+	0	-
PB 5-4 (A1)	*Thalassospira xianhensis*	-	-	+	0	-
PB 5-4 (A2)	*Thalassospira permensis*	-	+	+	0	-
PB 5-4 (A3)	*Vibrio natriegens*	-	-	+	0	-
PB 5-4 (A4)	*Vibrio parahaemolyticus*	-	-	+	0.5	+
PB 5-4 (B1)	*Pseudovibrio denitrificans*	-	-	+	0	-
PB 5-4 (B2)	*Ferrimonas kyonanensis*	-	+	+	1	+
PB 5-4 (B3)	*Vibrio harveyi*	-	-	+	0.5	+
PB 5-4 (C1)	*Vibrio harveyi*	-	+	+	1	+
PB 5-4 (C2)	*Pseudovibrio denitrificans*	-	-	+	3	+
S0022 B001	*Pseudomonas sp.*	-	+	+	0	-

A) 

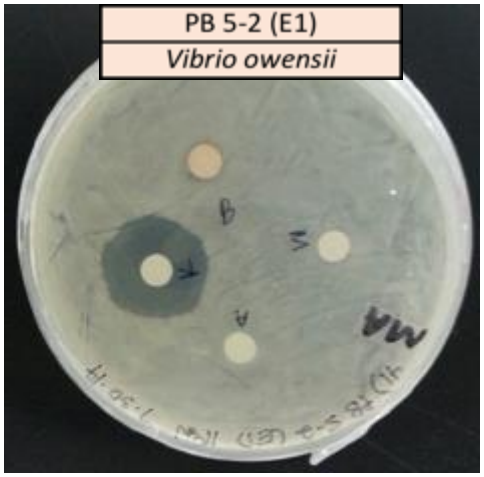
B) 

Figure 3.8. Representative Groups of Disc Diffusion Growth Inhibition with PB 4-2 (A1). Discs are 6 mm in diameter. A) Isolate PB 4-2 (A1) top disc labeled B inhibits the growth of isolate PB 5-2 (C1), BLAST closest neighbor *Vibrio alginolyticus*; zone of inhibition is 3 mm. B) Isolate PB 4-2 (A1) top disc labeled B does not inhibit the growth of isolate PB 5-2 (E1), BLAST closest neighbor *Vibrio owensii*; zone of inhibition is 0 mm.

Isolate S0022 B001

Isolate S0022 B001 culture growth characteristics and microscopy.

Isolate S0022 B001 displayed a deep purple color on agar plates and broth (Table 3.7 and Figure 3.9). Growth on agar plates start out without pigment then the purple pigment develops as the colony gets bigger. The bacterium is able to extract or breakdown precipitated salts/minerals or biomolecules in agar as shown by the clearance of white matter in the agar. The bacterium grows best in MB and MA. The bacteria can grow in a small volume of MB, about 200 mL of broth. The bacterium does not grow in AB, AA, NB, NA, MHB, nor MHA. The bacterium grows from 21-24º C but not at 37º C.

The Gram stain is non-conclusive because the cells are intrinsically purple. A crystal precipitate forms when S0022 B001 is grown on agar (Figures 3.9 and 3.10 B and C). Preliminary elemental analysis shows the crystals are made of Calcium, Carbon, and Oxygen

(graph not shown). Electron microscopy shows a rod-shaped, non-flagellated cell (Figure 3.10 A).

Table 3.7. S0022 B001 Culture Medium Characteristics.

Physical Characteristics	
Color: purple Color variation: pale purple to deep purple; sometimes will lose color on ASA Color Progression: starts out colorless then develops color as colony gets bigger Agar change: able to extract or breakdown precipitated salts/minerals or biomolecules in agar, crystal precipitate will form on agar growth	
Growth with Various Media	
Actinomycetes + filtered seawater + glycerol agar	Growth; color loss
Marine broth	Growth up to about 200 ml, No growth in larger volumes
Marine agar	Growth
Actinomycetes + filtered seawater broth	No growth
Nutrient broth	No growth
Nutrient agar	No growth
Mueller-Hinton broth	No growth
Mueller-Hinton agar	No growth
Temperature Growth range	
Temperature (º C)	Growth (yes/no)
21	yes
24	yes
37	no

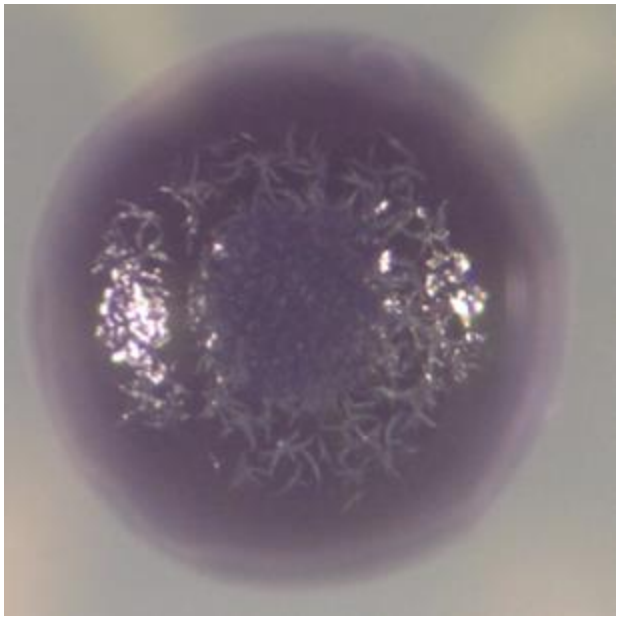
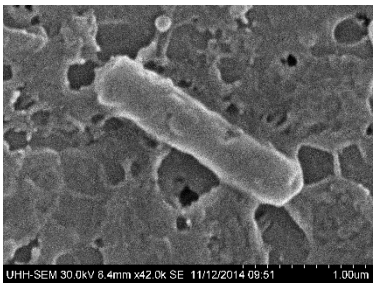
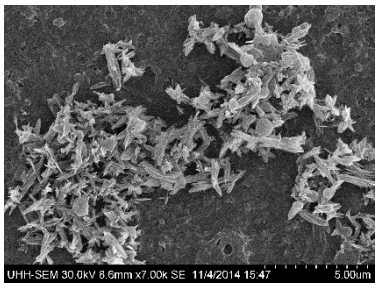


Figure 3.9. S0022 B001 Cultured Plate with Crystal Precipitate.
S0022 B001 colony on MA with crystal formation on top.

A) 

B) 

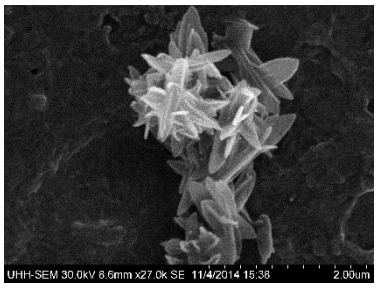
C) 

Figure 3.10. S0022 B001 Scanning Electron Microscopy.
Scale bars are at bottom right. A) One rod-shaped purple bacterium, 0.5 um in width and 2 um in length, embedded in mucus (possibly EPS). B) Field of crystals formed on marine agar culture. C) Close-up of crystals.

Disc Diffusion Assay of S0022 B001 against model laboratory organisms and cultured bacteria from Coconut Island and Puhi Bay

Isolate S0022 B001 inhibits the growth of Gram positive organisms *Staphylococcus aureus* and *Bacillus cereus* but not against Gram negative *Escherichia coli* (Table 3.8). Zones of growth inhibition ranged from 7-9 mm for SA and are 3 mm for BC. Negative control MB was negative, and positive controls A and K were positive.

Table 3.8. Disc Diffusion Assay of S0022 B001 Against Model Laboratory Organisms.

Model Laboratory Organisms	Growth Inhibition Zone Activity				
	MB	A 10 mg/mL	K 10 mg/mL	S0022 B001 in mm	+/-
Staphylococcus aureus	-	+	+	7	+
Staphylococcus aureus	-	+	+	8	+
Staphylococcus aureus	-	+	+	9	+
Bacillus cereus	-	+	+	3	+
Bacillus cereus	-	+	+	3	+
Bacillus cereus	-	+	+	3	+
Escherichia coli	-	+	+	0	-
Escherichia coli	-	+	+	0	-
Escherichia coli	-	+	+	0	-

Isolate S0022 B001 inhibits the growth of some of the cultured bacteria from Coconut Island and Puhi Bay (Table 3.9). Eleven out of 53 CI and PB tested positive for growth inhibition, about 21% of the isolates tested. The isolates that tested positive included the following groups (according to BLAST closest neighbors): *Bacillus, Pseudovibrio, Thalassospira,* and *Vibrio*. Zones of inhibition ranged from 7-10 mm. Isolate S0022 B001 did not inhibit the growth of 42 out of 53 CI and PB isolates, about 79% of the isolates tested. The isolates that tested negative included the following groups (according to BLAST closest neighbors): *Bacillus, Brevibacterium, Ferrimonas, Photobacterium, Pseudoalteromonas, Pseudomonas, Pseudovibrio, Thalassospira,* and *Vibrio.* Negative control MB was negative, and positive controls A and K were positive. Isolate PB 4-2 (A1) tested negative against itself.

Table 3.9. Disc Diffusion Assay of S0022 B001 Against Cultured Bacteria from Coconut Island and Puhi Bay

Cultured Isolate ID or control	NCBI BLAST Closest Neighbor Description	Growth Inhibition Zone Activity			
		MB	K 10 mg/mL	S0022 B001 in mm	+/-
CI 1-1 (A1)	*Pseudovibrio denitrificans*	-	+	7	+
CI 1-1 (B1)	*Vibrio harveyi*	-	+	0	-
CI 1-1 (B2)	*Pseudovibrio denitrificans*	-	+	0	-
CI 1-1 (C1)	*Vibrio alginolyticus*	-	+	0	-
CI 1-1 (D1)	*Pseudovibrio denitrificans*	-	+	8	+
CI 1-1 (D2)	*Vibrio parahaemolyticus*	-	+	0	-
CI 1-2 (A1)	*Pseudovibrio denitrificans*	-	+	0	-
CI 1-2 (B1)	*Vibrio harveyi*	-	+	10	+
CI 1-2 (B2)	*Pseudomonas fragi*	-	+	0	-
CI 1-2 (B3)	*Photobacterium rosenbergii*	-	+	0	-
CI 1-2 (C1)	*Pseudovibrio denitrificans*	-	+	0	-
CI 1-2 (C2)	*Photobacterium rosenbergii*	-	+	0	-
CI 1-2 (D1)	*Pseudoalteromonas piscicida*	-	+	0	-
CI 1-2 (E1)	*Pseudovibrio denitrificans*	-	+	10	+
CI 1-2 (E2)	*Vibrio parahaemolyticus*	-	+	0	-
CI 1-2 (F1)	*Vibrio rotiferianus*	-	+	8	+
CI 1-2 (G1)	*Vibrio alginolyticus*	-	+	10	+
CI 1-3 (A1)	*Bacillus cereus*	-	+	0	-
CI 1-3 (A2)	*Bacillus thuringiensis*	-	+	9	+
CI 1-3 (B1)	*Pseudovibrio denitrificans*	-	+	0	-
CI 1-3 (C1)	*Vibrio communis*	-	+	0	-
CI 1-3 (D1)	*Vibrio harveyi*	-	+	0	-
CI 1-3 (E1)	*Vibrio fischeri*	-	+	0	-
PB 4-1 (B1)	*Pseudovibrio denitrificans*	-	+	0	-
PB 4-1 (B2)	*Photobacterium rosenbergii*	-	+	0	-
PB 4-2 (A1)	*Brevibacterium frigoritolerans*	-	+	0	-
PB 4-2 (A2)	*Pseudovibrio denitrificans*	-	+	0	-
PB 4-2 (B1)	*Vibrio harveyi*	-	+	0	-
PB 4-2 (C1)	*Pseudovibrio denitrificans*	-	+	0	-
PB 4-2 (C2)	*Pseudoalteromonas arabiensis*	-	+	0	-
PB 5-1 (A1)	*Bacillus hwajinpoensis*	-	+	9	+
PB 5-2 (B1)	*Vibrio aestuarianus*	-	+	7	+
PB 5-2 (C1)	*Vibrio alginolyticus*	-	+	10	+
PB 5-2 (D1)	*Vibrio rotiferianus*	-	+	0	-
PB 5-2 (E1)	*Vibrio owensii*	-	+	9	+
PB 5-2 (F1)	*Pseudovibrio ascidiaceicola*	-	+	0	-

Table 3.9. Disc Diffusion Assay of S0022 B001 Against Cultured Bacteria from Coconut Island and Puhi Bay (continued).

Cultured Isolate ID or control	NCBI BLAST Closest Neighbor Description	Growth Inhibition Zone Activity			
		MB	K 10 mg/mL	S0022 B001 in mm	+/-
PB 5-3 (A1)	*Pseudovibrio denitrificans*	-	+	0	-
PB 5-3 (A2)	*Vibrio harveyi*	-	+	0	-
PB 5-3 (B1)	*Pseudovibrio denitrificans*	-	+	0	-
PB 5-3 (C1)	*Pseudovibrio denitrificans*	-	+	0	-
PB 5-3 (C2)	*Photobacterium rosenbergii*	-	+	0	-
PB 5-3 (D1)	*Vibrio harveyi*	-	+	0	-
PB 5-3 (D2)	*Pseudovibrio denitrificans*	-	+	7	+
PB 5-3 (E1)	*Vibrio natriegens*	-	+	0	-
PB 5-4 (A1)	*Thalassospira xianhensis*	-	+	0	-
PB 5-4 (A2)	*Thalassospira permensis*	-	+	10	+
PB 5-4 (A3)	*Vibrio natriegens*	-	+	0	-
PB 5-4 (A4)	*Vibrio parahaemolyticus*	-	+	0	-
PB 5-4 (B1)	*Pseudovibrio denitrificans*	-	+	0	-
PB 5-4 (B2)	*Ferrimonas kyonanensis*	-	+	0	-
PB 5-4 (B3)	*Vibrio harveyi*	-	+	0	-
PB 5-4 (C1)	*Vibrio harveyi*	-	+	0	-
PB 5-4 (C2)	*Pseudovibrio denitrificans*	-	+	0	-

Coconut Island and Puhi Bay NRPS and PKS Genes Screening

Most of the CI and PB isolates tested positive for the amplification of parts of the adenylation domains of NRPS and parts of ketosynthase and acyltransferase domains of PKS genes (Table 3.10 and Figure 3.11). Overall 52 out of 60 isolates, approximately 87%, were positive for both NRPS and PKS or one of the genes. Isolates that tested positive for NRPS genes were in the following groups (according to BLAST closest neighbor results): *Brevibacterium, Photobacterium, Pseudomonas, Pseudovibrio,* and *Vibrio*. Isolates that tested positive for PKS included the following groups (according to BLAST closest neighbor results): *Brevibacterium, Ferrimonas, Photobacterium, Pseudoalteromonas, Pseudomonas, Pseudovibrio, Thallasopira,* and *Vibrio*. Isolates that tested negative for NRPS were in the following groups

(according to BLAST closest neighbor results): *Bacillus, Ferrimonas, Photobacterium, Pseudoalteromonas, Pseudomonas, Pseudovibrio, Thallasopira*, and *Vibrio*. Isolates that tested negative for PKS included the following groups (according to BLAST closest neighbor results): *Bacillus, Pseudoalteromonas,* and *Vibrio*. To highlight, all of the isolates in the Bacillus group were negative for NRPS and PKS genes; all of the *Pseudoalteromonas* and *Thalassospira* were negative for NRPS; all of the *Photobacterium, Pseudomonas, Thalassopira* were positive for PKS; *Brevibacterium* was positive for both NRPS and PKS; some *Photobacterium, Pseudomonas, Pseudovibrio,* and *Vibrio* were positive for NRPS; some *Photobacterium, Pseudomonas, Pseudovibrio,* and *Vibrio* were negative for NRPS; some *Pseuodvibrio* and *Vibrio* were positive for NRPS; and some *Pseudoalteromonas* and *Vibrio* were negative for PKS. Negative controls nuclease-free water (NF water) and *E. coli* were negative. Positive control *P. aeruginosa* was positive.

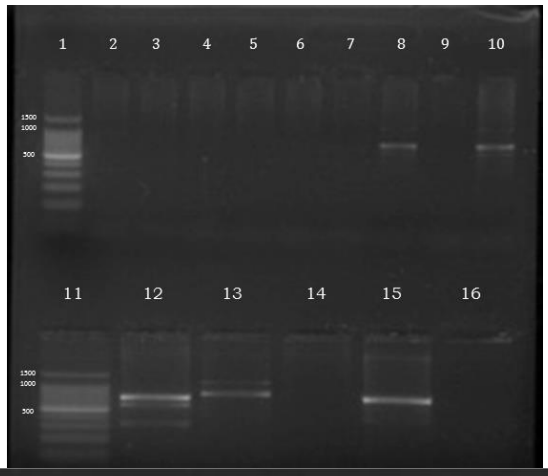
Table 3.10. Coconut Island and Puhi Bay NRPS and PKS Screening

Cultured Isolate ID or control	NCBI BLAST Closest Neighbor Description	NRPS	PKS
CI 1-1 (A1)	*Pseudovibrio denitrificans*	+	+
CI 1-1 (B1)	*Vibrio harveyi*	+	+
CI 1-1 (B2)	*Pseudovibrio denitrificans*	+	+
CI 1-1 (C1)	*Vibrio alginolyticus*	+	+
CI 1-1 (D1)	*Pseudovibrio denitrificans*	+	+
CI 1-1 (D2)	*Vibrio parahaemolyticus*	+	+
CI 1-2 (A1)	*Pseudovibrio denitrificans*	+	+
CI 1-2 (B1)	*Vibrio harveyi*	+	+
CI 1-2 (B2)	*Pseudomonas fragi*	+	+
CI 1-2 (B3)	*Photobacterium rosenbergii*	+	+
CI 1-2 (C1)	*Pseudovibrio denitrificans*	+	+
CI 1-2 (C2)	*Photobacterium rosenbergii*	+	+
CI 1-2 (D1)	*Pseudoalteromonas piscicida*	-	-
CI 1-2 (E1)	*Pseudovibrio denitrificans*	-	+
CI 1-2 (E2)	*Vibrio parahaemolyticus*	-	-
CI 1-2 (F1)	*Vibrio rotiferianus*	-	-
CI 1-2 (G1)	*Vibrio alginolyticus*	-	+
CI 1-3 (A1)	*Bacillus cereus*	-	-
CI 1-3 (A2)	*Bacillus thuringiensis*	-	-
CI 1-3 (B1)	*Pseudovibrio denitrificans*	+	-
CI 1-3 (C1)	*Vibrio communis*	-	+
CI 1-3 (D1)	*Vibrio harveyi*	-	+
CI 1-3 (E1)	*Vibrio fischeri*	-	+
PB 4-1 (B1)	*Pseudovibrio denitrificans*	-	+
PB 4-1 (B2)	*Photobacterium rosenbergii*	-	+
PB 4-2 (A1)	*Brevibacterium frigoritolerans*	+	+
PB 4-2 (A2)	*Pseudovibrio denitrificans*	+	+
PB 4-2 (B1)	*Vibrio harveyi*	-	+
PB 4-2 (C1)	*Pseudovibrio denitrificans*	+	+
PB 4-2 (C2)	*Pseudoalteromonas arabiensis*	-	+
PB 5-1 (A1)	*Bacillus hwajinpoensis*	-	-
PB 5-1 (A2)	*Bacillus stratosphericus*	-	-
PB 5-1 (A3)	*Pseudovibrio denitrificans*	+	+
PB 5-1 (B1)	*Pseudovibrio denitrificans*	-	+
PB 5-1 (C1)	*Pseudomonas fragi*	+	+
PB 5-1 (D1)	*Pseudovibrio denitrificans*	+	+

Table 3.10. Coconut Island and Puhi Bay NRPS and PKS Screening (continued)

Cultured Isolate ID or control	NCBI BLAST Closest Neighbor Description	NRPS	PKS
PB 5-2 (A1)	*Vibrio harveyi*	-	+
CI 1-2 (G1)	*Vibrio alginolyticus*	-	+
PB 5-2 (B1)	*Vibrio aestuarianus*	-	+
PB 5-2 (C1)	*Vibrio alginolyticus*	-	+
PB 5-2 (D1)	*Vibrio rotiferianus*	-	+
PB 5-2 (E1)	*Vibrio owensii*	-	+
PB 5-2 (F1)	*Pseudovibrio ascidiaceicola*	-	+
PB 5-3 (A1)	*Pseudovibrio denitrificans*	+	+
PB 5-3 (A2)	*Vibrio harveyi*	-	-
PB 5-3 (B1)	*Pseudovibrio denitrificans*	+	+
PB 5-3 (C1)	*Pseudovibrio denitrificans*	+	+
PB 5-3 (C2)	*Photobacterium rosenbergii*	+	+
PB 5-3 (D1)	*Vibrio harveyi*	-	+
PB 5-3 (D2)	*Pseudovibrio denitrificans*	+	+
PB 5-3 (E1)	*Vibrio natriegens*	-	+
PB 5-4 (A1)	*Thalassospira xianhensis*	-	+
PB 5-4 (A2)	*Thalassospira permensis*	-	+
PB 5-4 (A3)	*Vibrio natriegens*	-	+
PB 5-4 (A4)	*Vibrio parahaemolyticus*	-	+
PB 5-4 (B1)	*Pseudovibrio denitrificans*	+	+
PB 5-4 (B2)	*Ferrimonas kyonanensis*	-	+
PB 5-4 (B3)	*Vibrio harveyi*	-	+
PB 5-4 (C1)	*Vibrio harveyi*	-	+
PB 5-4 (C2)	*Pseudovibrio denitrificans*	+	+
S0022 B001	*Pseudomonas* sp.	-	+
P. aeruginosa	positive control	+	+
E. coli k12	negative control	-	-
NF water	negative control	-	-

A)



B)

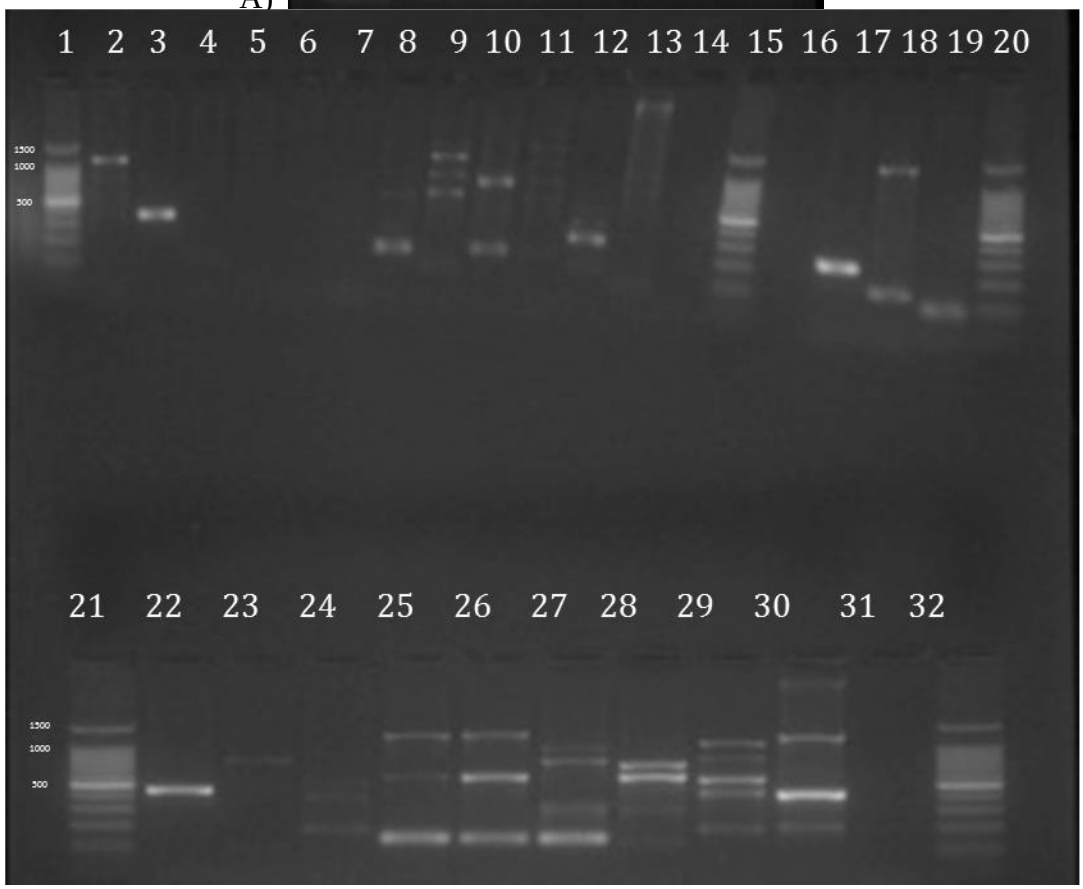


Figure 3.11. PB 4-2 (A1) NRPS and PKS Gene Screen Gel.

A) NRPS gene screen expected product is between 700-800 bp: lanes 1 and 11—ladder, lanes 2 through 10 and 12 through 14—isolates from CI and PB, lane 15—positive control *P. aeruginosa*, lane 16—negative control water. B) PKS I gene screen expected product is between 1200-1400 bp: lanes 1 and 20—ladder, lanes 2 through 13—isolates from CI, lane 14—positive control *P. aeruginosa*, lane 15—negative control water; PKS II gene screen expected product is 600 bp: lanes 16-19—isolates from CI, lanes 21 and 32—ladder, lanes 22 through 29—isolates from CI, lane 30—positive control *P. aeruginosa*, lane 31—negative control water.

Violacein Gene Screening of S0022 B001

For the gene screening, S0022 B001 was positive for the flavoenzyme part of VPA3/VPA4 and VPB3/VPB4 and negative for another part of flavoenzyme VPB1/VPB2 (Figure 3.12). VioA, VPB1 and VPB2 which amplifies the heme protein part of Negative controls were negative.

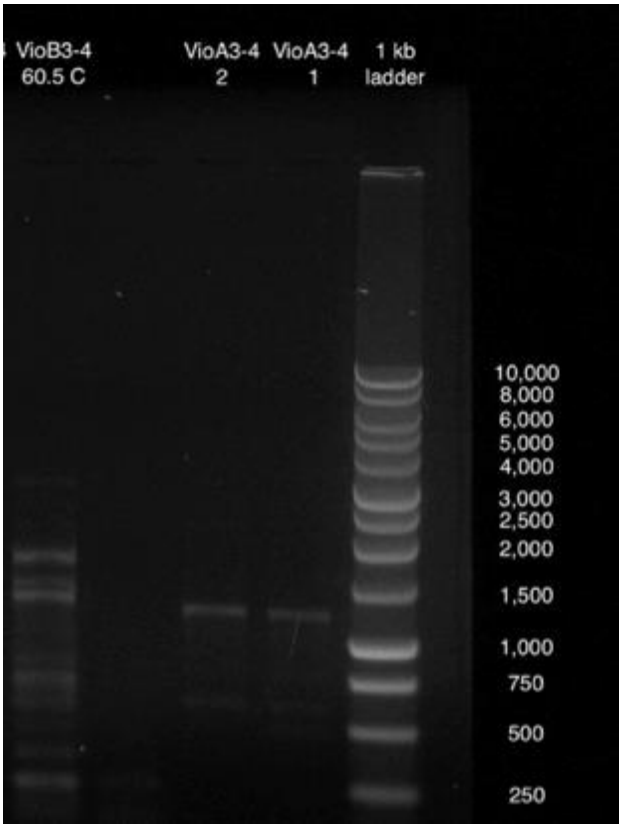
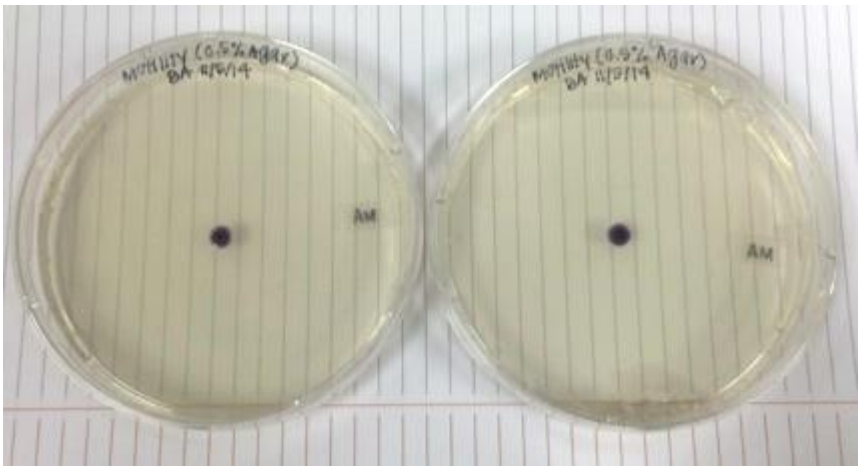


Figure 3.12. Violacein Gene Screening Gel
VioA3-4 expected product is 1000 bp. VioB3-4 expected product is 1850 bp.

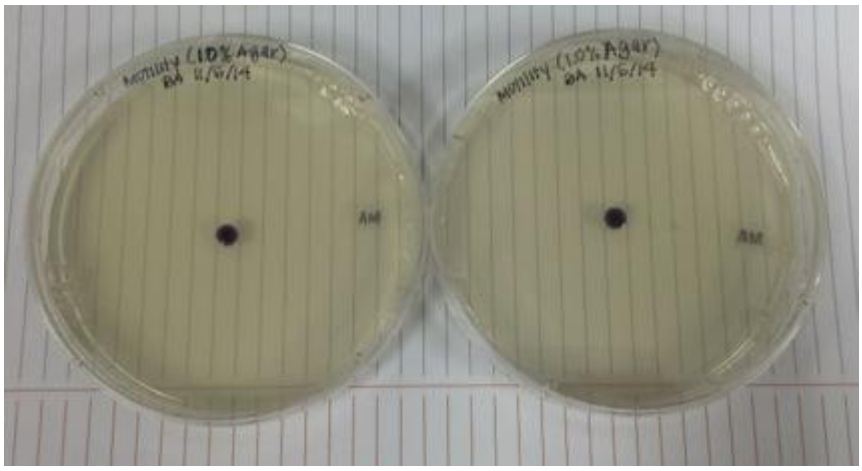
S0022 B001 Motility Assay

S0022 B001 did not show any motility on 0.5% MA, 1.0% MA, and 1.5% MA (Figure 3.13). Positive control *P. aeruginosa* showed motility at each concentration.

A)



B)



C)

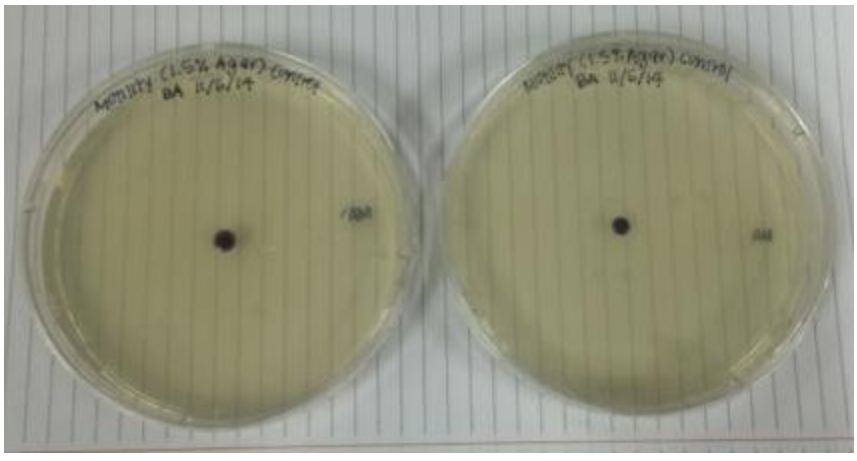


Figure 3.13. S0022 B001 Motility Assay.
A) no motility on 0.5% agar, B) no motility on 1.0% agar, C) no motility on 1.5% control agar.

CHAPTER FOUR

GENERAL DISCUSSION

The goals of this study were to investigate the diversity of cultured bacterial isolates from three Hawaiian marine sponge species, identify any unique and/or strong candidates for carrying out further bioactivity and NRPS and PKS assays as well as begin to assess their significance to their host sponge. *Haliclona* sp., *Petrosia* sp., and *I. protea* sponges were chosen for collection because they are abundant not only in Hawaii but also abundant in other distant geographic locations and were not endangered species nor invasive species in Hawaii to the best of our knowledge (Diekmann et al. 2004; Zhu et al. 2008; Kennedy et al. 2009; Wang et al. 2009; Smith et al. 2009; Glasby et al. 2012; Knapp et al. 2013; Burgsdorf et al. 2014). The bacterial isolates from sponges *Haliclona* sp. off the coast of CI, and *Petrosia* sp. and *I. protea* in PB overall contained unique and diverse bacterial isolates according to phylogenetic analysis, bioactivity assays, and physiological assessments.

Diversity of Coconut Island and Puhi Bay Isolates

Of the 90 or so genera of cultured and uncultured bacteria discovered from marine sponges from other studies, seven of the same genera were also discovered in CI and PB sponges, namely *Bacillus, Photobacterium, Pseudovibrio, Pseudoalteromonas, Pseudomonas, Shewanella*, and *Vibrio* (Figure 4.1). The isolates from CI and PB came from the documented classes Actinobacteria, Bacilli, Alphaproteobacteria, and Gammaproteobacteria. No new phyla or classes were new in the CI and PB samples; however, some new genera were isolated, namely *Brevibacterium* from phylum Actinobacteria, *Thalassospira* from class Alphaproteobacteria, and *Ferrimonas* from class Gammaproteobacteria.

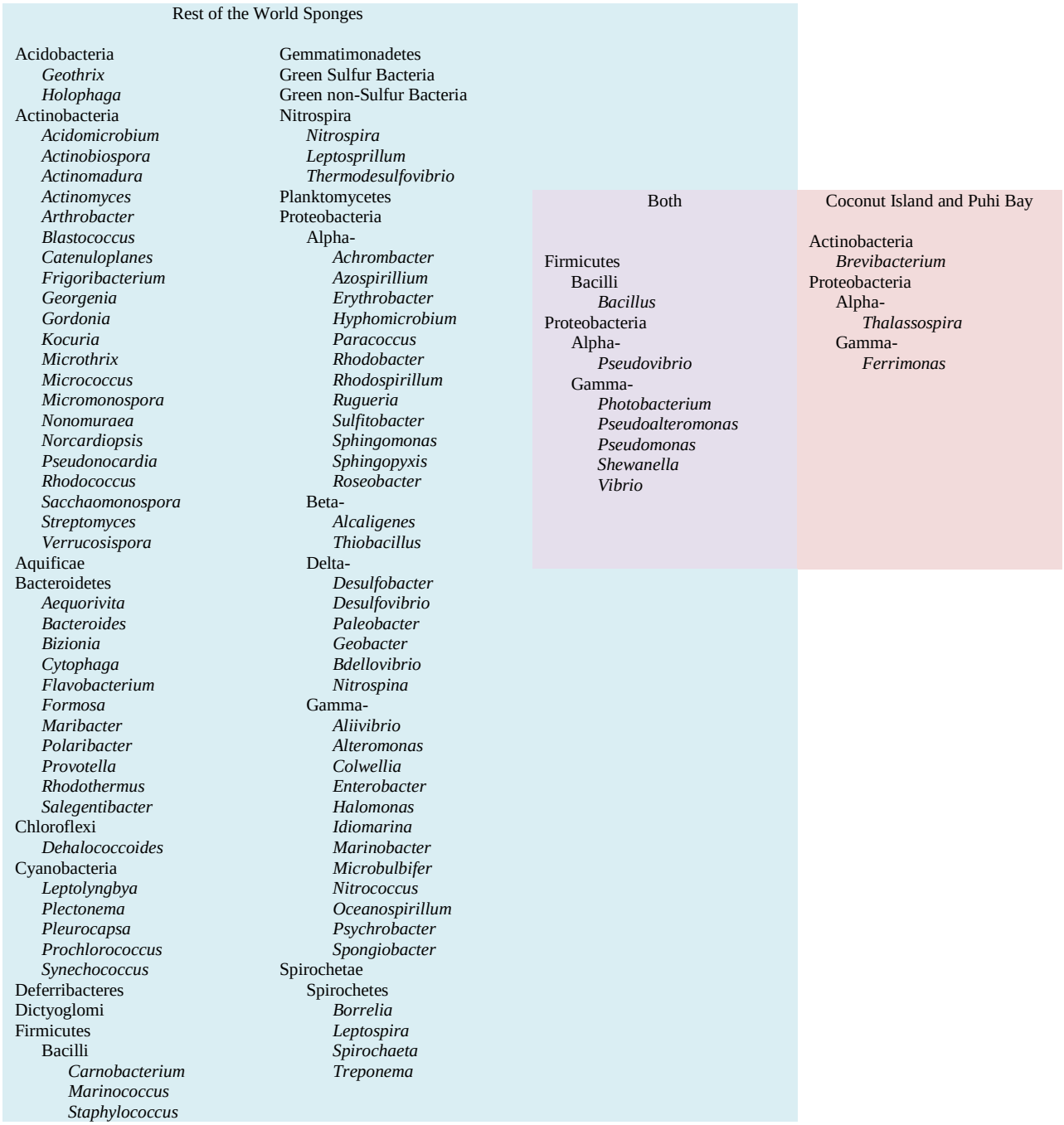


Figure 4.1. Venn Diagram of Coconut Island and Puhi Bay Bacteria with World Bacteria. Phlya are at far left in each column and not italicized, Classes are indented and not italicized, and Genera are italicized. Bacterial Genera found in world sponges are in blue. Newly found bacterial Genera of Coconut Island and Puhi Bay isolates are in red area. Coconut Island and Puhi Bay bacterial Genera also found in world sponges are in purple area. (Sources: See Tables 1.1 and 1.2)

Among the 64 bacteria that were isolated among the three Hawaiian marine sponges, the most dominant isolates belonged to the *Vibrio* and *Pseudovibrio* groups, which are in line with the bacteria cultured from other sponges and areas around the world; however, phylogenetic analyses revealed that many of the CI and PB isolates from the *Vibrio* and *Pseudovibrio* groups did not closely cluster with the other world isolates (Zhu et al. 2007; Isaacs et al. 2009; Kennedy et al. 2009; Santos et al. 2010; Jackson et al. 2012; Dupont et al. 2014). Instances in which a PB isolate from the *Pseudovibrio* group did cluster with the other world isolates, the bootstrap consensus value was well below 70%, suggesting these isolates near Hawaii Island are reasonably unique (Wang et al. 2009). *Vibrio* and *Pseudovibrio* along with *Photobacterium* were also present among all three CI and PB sponges, implying that these particular species are readily grown given the media conditions conducted in this study. Although these isolates were cultured in the laboratory, these bacterial species may not represent the most abundant species within the marine sponge's bacterial community.

The remaining identified CI and PB isolates also did not cluster with the other world isolates (bootstrap consensus <70%) suggesting these isolates near Hawaii Island are unique species. Bacterial isolates characterized from Oahu's marine sponges generally did not cluster or had low bootstrap consensus values as well (Zhu et al. 2007; Wang et al. 2009). All CI and PB isolates clustered away from the respective type species in their group except in the *Pseudovibrio* group (Figure 3.4G). This may be because most of the type species were found in terrestrial sources (Philippot et al. 2010).

Brevibacterium, *Ferrimonas*, and *Thalassospira* spp. have not been reported from culture-independent nor culture-dependent samples from other current research; hence the above named genera represent newly detected isolates from sponges. One interesting note is that the

two most dominant classes of the bacteria obtained from global samples of sponges were from phyla Acidobacteria and Chloroflexi; however no CI and PB isolates were identified from those classes (Hentschel et al. 2002; Hardoim et al. 2008; Kamke et al. 2010; Radwan et al. 2010).

Hawaii Island Sponge Bacteria are Not Closely Related to Oahu Sponge Bacteria Community.

Although similar genera of bacteria were isolated from Hawaii Island and Oahu sponges, *Bacillus* sp. from sponges *S. zeteki* and *M. armata* from Oahu did not cluster with CI and PB *Bacillus* isolates found in this study. The Oahu *Bacillus* sp. isolates were more related to samples from Australia and China (Webster et al. 2001; Li and Liu 2006) and uncultured *Pseudomonas* sp. from *M. armata* from Oahu were more related to species from Ireland, Caribbean, and France than to CI and PB *Pseudomonas* isolates (Bruck et al. 2012; Jackson et al. 2012; Dupont et al. 2014).

87.5% (56/64) of the CI and PB bacterial isolates had 16s rRNA sequence identities matching above 97% of known sequences in Genbank. Less than 12% (8/64) of the CI and PB bacterial isolates had 16s rRNA sequence identities matching below 95% and may represent new species. However, phylogenetic analyses that include other genes such as housekeeping genes *gyrase B, atpD, recA, rpoB, and/or trypB* along with 16s rRNA in the phylogenetic analysis will provide a better assessment of bacterial diversity and relationships with these CI and PB isolates (Xi et al. 2012). Most of the cultured CI and PB bacteria are Gram-negative, which is consistent with the findings of most of the other research; however, Webster et al. (2001) found more of their clones to be Gram-positive than Gram-negative. This leads to the possibility that Gram-positive bacteria residing in marine sponges may be often underestimated, and the bacterial communities inside CI and PB sponges in this present study may indeed have more Gram-

positive bacteria than indicated in our cultured samples or even farther, that the majority of the community may be Gram-positive than what is indicated in this cultured sample.

Isolate PB 4-2 (A1)

Isolate PB 4-2 (A1) was chosen for further analysis because the closest neighbor in BLAST to this isolate was *Brevibacterium frigoritolerans* (99% identical), an Actinobacterium (Table 3.2). Some species of *Brevibacterium* have been known to have bioactivity (Lau et al. 2002; Wietz and Gram 2011). Cultures growing in broth and on agar plates appear red in color (Fig 3.4) and the pigment is retained in the viable cells. PB 4-2 (A1) was often difficult to grow in liquid culture and is able to grow only on AA supplemented with filtered seawater or in MB in a small volume (5mL), alluding to the bacterium needing a suitable substrate such as that provided by *Petrosia* sp. Further evidence lies in the observation that isolate grew on cotton submerged into MB. It was not able to grow in AB, NB, NB supplemented with NaCl, NA, MHB, nor MHA indicating that PB 4-2 (A1) is a strict marine microbe (Table 3.4). Isolate PB 4-2 (A1) may be classified as nonplanktonic and grows within the 18-27ºC range and not at 37ºC, and hence be considered an environmental microorganism (Table 3.4).

NRPS and PKS screening show that PB 4-2 (A1) has both NRPS and PKS genes (Table 3.10). This finding is coupled with the finding that it produces antibiotic activity against both Gram-positive and -negative bacteria and may contain several antibiotic biosynthetic pathways. Isolate PB 4-2 (A1) inhibited the growth of Gram-positive model lab organisms SA and BC but not Gram-negative EC (Table 3.5). PB 4-2 (A1) also inhibited the growth of other bacteria from CI and PB sponges (Table 3.6). About 75% of the CI and PB tested positive for growth inhibition by PB 4-2 (A1) and included a wide range of bacterial genera: *Bacillus, Ferrimonas,*

Photobacterium, *Pseudoalteromonas*, *Pseudovibrio*, and *Vibrio*. *Bacillus* sp. are Gram-positive whereas the rest are Gram-negative; therefore the antibiotic or antibiotics produced by PB 4-2 (A1) may be considered broad spectrum.

In many cases pigment production in bacteria indicate possible natural product formation and also bioactivity (Eagan et al. 2002). The red pigment produced by PB 4-2 (A1) may be involved in the growth inhibition bioactivity. Antimicrobial activity observed from *Brevibacterium* isolate PB 4-2 (A1) may be due to a 2° metabolite, such as heptylprodigiosin, a red-pigmented compound effective against *S. aureus* and produced by *Pseudovibrio denitrificans* (Figure 4.2).

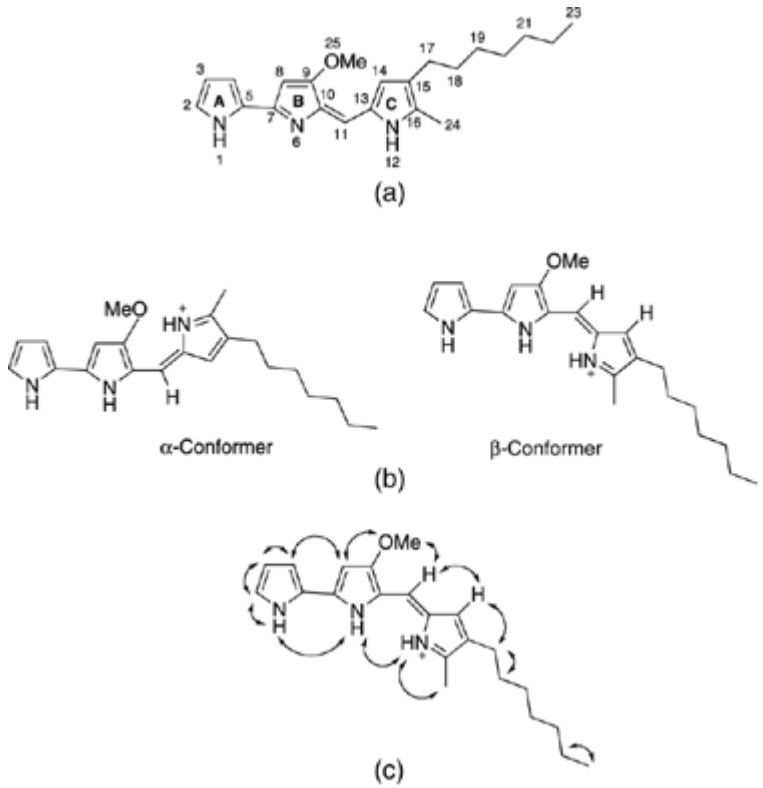


Figure 4.2. Bioactive Compound Heptylprodigiosin Isolated from *Pseudovibrio denitrificans*. (Source: Guzman et al. 2007)
(a) Structure of heptylprodigiosin, (b) Structures of possible conformers of heptylprodigiosin in solution (adopted from Rizzo et al. 1999 and Melvin et al. 1999) and (c) Key NOESY correlations observed for the β-conformer of heptylprodigiosin in $CDCl_3$.

S0022 B001

Isolate S0022 B001 was chosen for further analysis because BLAST analysis revealed it was a *Pseudomonas* species, it produced a deep purple pigment, was isolated from the native Hawaiian sponge *Iotrochota protea* in Puhi Bay, and a white crystal formed on colonies grown on agar (de Laubenfels 1950) (Table 3.2, Figure 3.7). Isolate S0022 B001 displayed a deep purple color on agar plates and broth. Culture conditions such as ability to grow only in MA and MB in a small to moderate volume and inability to grow in AB, NB, NA with NaCl, NA, MHB, nor MHA indicate that S0022 B001 is a strict marine microbe. Growth on agar plates start out without pigment then the purple pigment develops as the colony gets bigger. The purple pigment may be a 2° metabolite synthesized during late stationary phase of growth (Steinbuchel and Schmack 1995).

NRPS and PKS screening show that S0022 B001 has both NRPS and PKS genes. This finding is coupled with the finding that it produces antibiotic activity against both Gram-positive and -negative bacteria. Isolate S0022 B001 inhibited the growth of Gram-positive model lab organisms SA and BC but not Gram-negative EC (Table 3.8). S0022 B001 also inhibited the growth of other bacteria from CI and PB sponges (Table 3.9). About 21% of the CI and PB tested positive for growth inhibition by S0022 B001and included a wide range of bacterial genera: *Bacillus, Pseudovibrio, Thalassospira,* and *Vibrio. Bacillus* sp. are Gram-positive whereas the rest are Gram-negative; therefore the antibiotic or antibiotics produced by S0022 B001 may be considered broad spectrum.

Antibiotic activity observed from *Pseudomonas* isolate S0022 B001 may also be due to a Violacein-like compound, a purple pigment. Violacein has been known to have antibacterial activity since the 1940s (Figure 4.3) (Lichstein and van De Sand 1946). S0022 B001 may

possess Violacein-like genes; consequently, this would be the first documented marine *Pseudomonas* to produce Violacein or a Violacein-like compound (Figure 3.12).

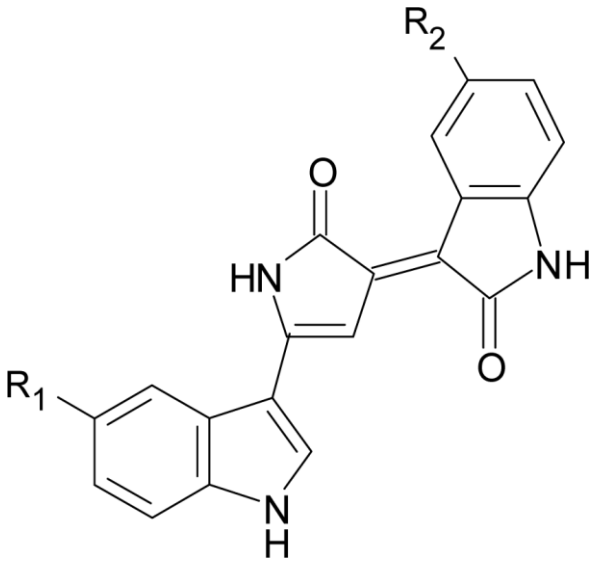


Figure 4.3. Chemical structures of Violacein and Deoxyviolacein (Source: Hakvag et al. 2009).
A) Violacein: R1 = OH, R2 = H. B) Deoxyviolacein: R1 = R2 = H

Screening of CI and PB isolates show that the majority, 52/60, of the isolates were positive for both NRPS and PKS or one of the genes (Table 3.10), indicating that they potentially can produce antibiotic activity and may do so inside their sponge host. Because PB 4-2 (A1) and S0022 B001 show bioactivity against a wide range of bacteria including lab model bacteria and marine bacteria, possess genes involves in 2° metabolite production. These two bacterial isolates may be important to their respective sponge host by synthesizing antibiotics that 1) may defend the host against the other bacteria that may be producing their own antibiotics and 2) may defend the host against pathogens (Zhang et al. 2009; Erken et al. 2013). Though one cannot rule out the possibilities that bacteria present inside CI and PB sponges may be nutritional, pathogenic, or

commensal to the sponge, this current research in CI and PB may support the role of symbiosis (Bondarev et al. 2013; Flores et al. 2013; Bal et al. 2015). Overall, the bacterial community may be producing antibiotics that keep each other's growth under control, may protect the sponge from bacterial and other organism infection, and in turn contribute to sponge health (Webster and Blackall 2009).

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APPENDIX

A.MM1 Spectrophotometry

PB 4-2 (A1) cells grown on ASA and S0022 B001 cells grown on MA were transferred to Promega RNA lysis buffer and incubated at 22ºC for 3 hrs. Cells were pelleted and resuspended in Methanol. The mixture was filtered through a 0.2 um membrane syringe filter. The pigment absorbance was measured from wavelengths 380-900 nm for PB 4-2 (A1) and 400-840 nm for S0022 B001 on a Thermoscientific Genesys 20 Spectrophotometer. Methanol was used as a blank.

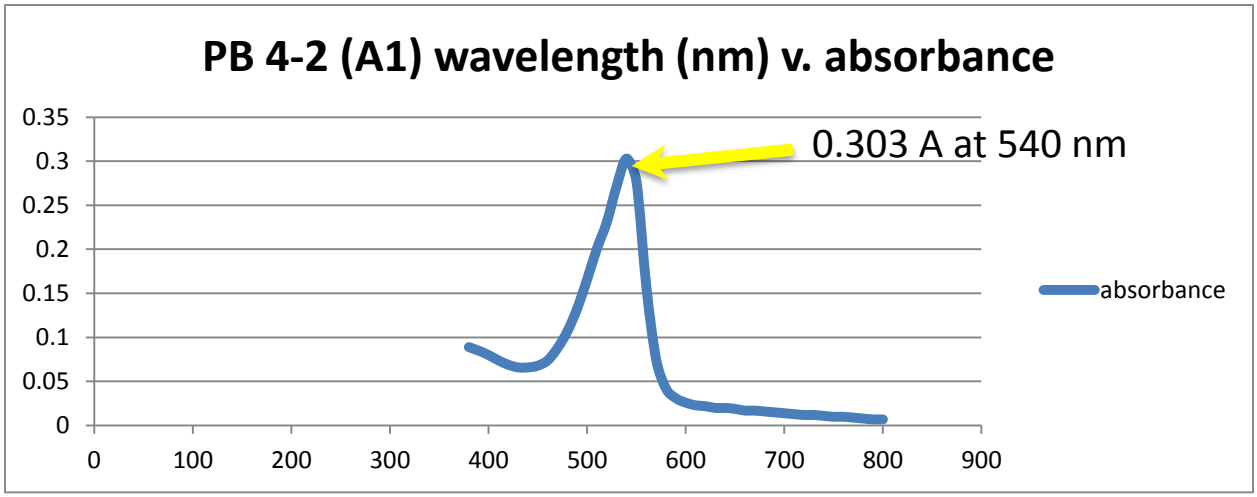

Figure A1. PB 4-2 (A1) Spectrophotometry of red pigment

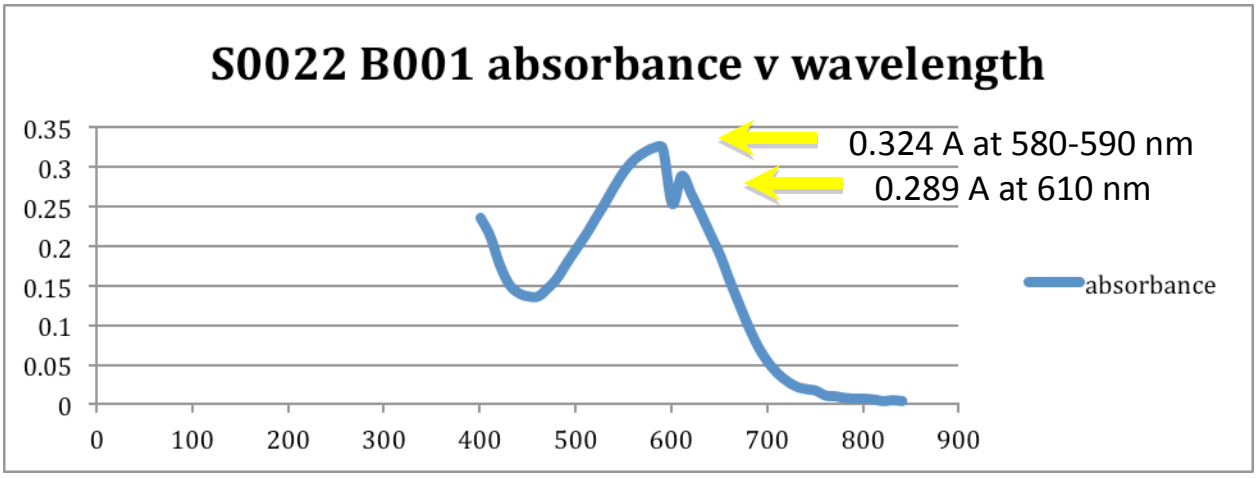

Figure A3. Spectrophotometry of purple pigment.

A.MM2 Pigment Change in the Presence of Acid and Base

PB 4-2 (A1) cells grown on ASA and S0022 B001 cells grown on MA were transferred to sterile autoclaved Millipore water. One ml aliquots were transferred to glass tubes. One set of tubes received HCl and the other set received NaOH. Tubes were given varying amounts of HCl and NaOH and water to bring the final acid or base to final concentrations of 0.08 M, 0.4 M, 0.8 M, and 1.6 M and final volume to 4 ml. A 4 ml volume of cells and Millipore water was used as a pigment comparison.

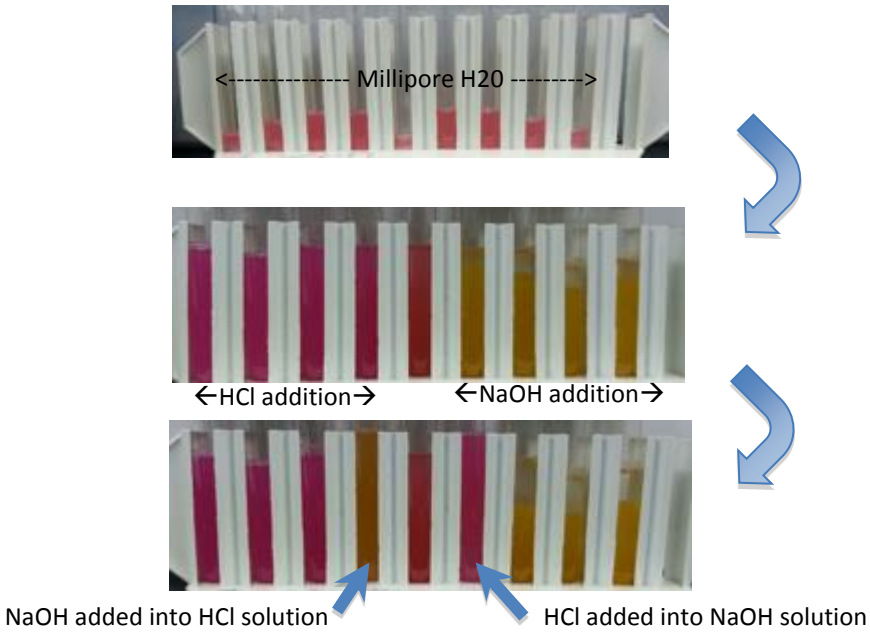

Figure A2. PB 4-2 (A1) Acid (HCl) and Base (NaOH) Red Pigment Change.
Top photo: before acid and base addition. Middle photo: after acid and base addition. Bottom photo: after base added to acid solution, left arrow, and after acid added to base solution, right arrow.

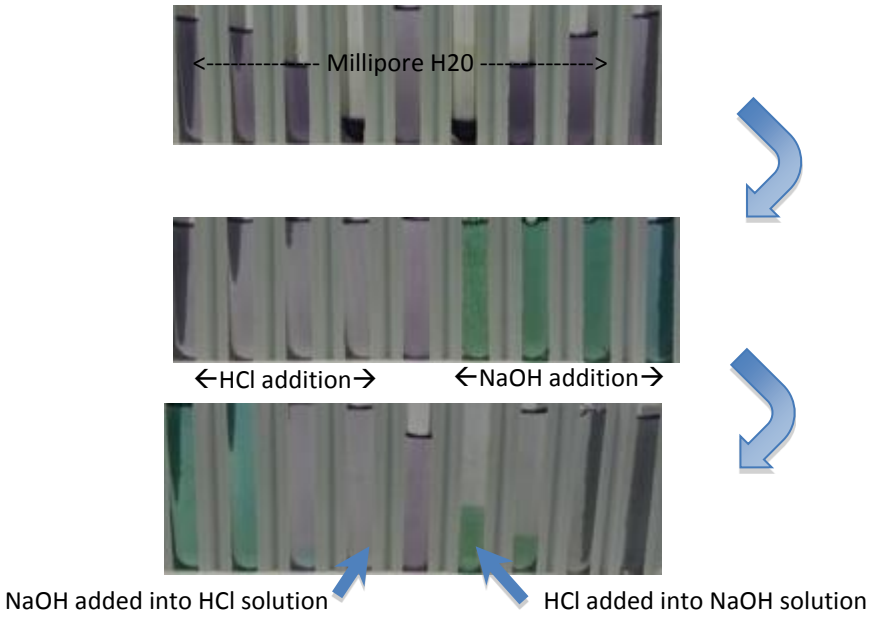

Figure A4. S0022 B001 Acid and Base Purple Pigment Change.
Top photo: before acid and base addition. Middle photo: after acid and base addition. Bottom photo: after base added to acid solution, 4 tubes from the left arrow, and after acid added to base solution, 4 tubes from the right arrow.