THE UNIVERSITY OF CALIFORNIA, SAN DIEGO

A Novel Heterotrophic Bacterial Associate of the Filamentous Cyanobacterium *Moorea producens* JHB

A Thesis submitted in partial satisfaction of the requirements for the degree

Master of Science

in

Biology

by

Susan L. Cummings

Committee in charge:

William H. Gerwick, Chair
Eric Allen, Co-Chair
Lena Gerwick
Milton H. Saier

2015

The Thesis of Susan L. Cummings is approved and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California, San Diego

2015

TABLE OF CONTENTS

Signature Page…… iii

Table of Contents…… iv

List of Figures and Tables……. v

Acknowledgements……. vi

Abstract of Thesis…… vii

Chapter 1: Introduction…… 1
1.1: Theoretical Background…….. 1
References for Theoretical Background……... 7
1.2: Previous Research on the Unknown Heterotrophic Bacterium. 11
References for Previous Research…… 17

Chapter 2: Co-Culturing *Moorea producens* JHB with Other Cyanobacteria: How Specific Is the Association of the Unknown Bacterium with *Moorea producens*?…….. 19
2.1: Methods…….. 20
2.2: Results and Discussion…… 24
References…… 28

Chapter 3: Analysis of the Unknown Bacterium and Proposed Further Experiments…… 29
3.1 Genomic Information……. 29
3.2 Cultivation-Dependent Information…….. 32
References…… 36

Chapter 4: Conclusion…….. 39

Full Reference List……. 41

LIST OF FIGURES AND TABLES

FIGURE 1. Phylogenetic tree comparing the 16S rRNA sequence of Mor1 to those of other bacteria..... 16

TABLE 1. Matrix of co-culturing experiments involving *M. producens* JHB and various other filamentous tropical marine cyanobacteria..... 23

TABLE 2. Primer sequences used in the co-culturing experiment..... 23

FIGURE 2. 16S rRNA PCR using DNA from the co-culturing of *Moorea producens* JHB with other cyanobacteria..... 26

FIGURE 3. *selA* PCR using DNA from the co-culturing of *Moorea producens* JHB with other cyanobacteria..... 27

ACKNOWLEDGEMENTS

All sections of Chapters 1 and 2 are adaptations of material currently being prepared for submission for publication: Cummings SL, Barbé D, Leao TF, Korobeynikov A, Engene N, Glukhov E, Gerwick WH, Gerwick L. “A Novel Uncultured Heterotrophic Bacterial Associate of the Cyanobacterium *Moorea producens* JHB.” The thesis author is the primary author of this paper.

ABSTRACT OF THE THESIS

A Novel Heterotrophic Bacterial Associate of the Filamentous Cyanobacterium *Moorea producens* JHB

by

Susan L. Cummings

Master of Science in Biology

University of California, San Diego, 2015

William H. Gerwick, Chair
Eric Allen, Co-Chair

Filamentous tropical cyanobacteria such as *Moorea producens* live surrounded by a community of heterotrophic bacteria. However, these heterotrophic bacteria and their interactions with the cyanobacteria have not been extensively studied. During efforts to sequence the genome of a culture of *M. producens* strain JHB, the 5.99 Mb genome of an unknown associated bacterium was discovered. This bacterium was found to belong to phylum

Acidobacteria, subgroup 22, and is further referred to in this thesis as Mor1. Mor1 was found to predominantly exist on the outside surfaces of *M. producens* JHB sheaths. Currently, Mor1 is unable to be cultured separately from *M. producens* JHB, and was found to be present only in laboratory cultures of *M. producens* strains. A co-culturing experiment between *M. producens* JHB with Mor1 and other cyanobacterial strains indicated that Mor1 failed to transfer to other cyanobacteria. These data support a specific relationship between Mor1 and *M. producens*, although further experiments would be necessary to confirm a symbiotic relationship between the two organisms. Overall, the study of this previously unknown *Acidobacteria* strain associated with *M. producens* JHB can provide information about an understudied phylum of heterotrophic bacteria as well as a potential novel symbiosis.

CHAPTER 1: INTRODUCTION

1.1: THEORETICAL BACKGROUND

Cyanobacteria, a diverse group of photosynthetic bacteria, play important roles as primary producers in aquatic ecosystems, especially in the ocean. Cyanobacteria interact with other organisms in a variety of different ways, perhaps most distinctively in symbioses with eukaryotic organisms. For example, the organisms commonly referred to as lichens consist of a symbiotic relationship between an algae [either a cyanobacterium (= blue-green alga) or a eukaryotic alga] and a fungus. The most common cyanobacterial partners within lichen consist of the genus *Nostoc*, and these cyanobacteria provide fixed carbon through photosynthesis while the fungal filaments provide structure and gather moisture and nutrients from the environment [1][2]. Cyanobacteria of the nitrogen-fixing genera *Nostoc* and *Anabaena* have also been known to form extremely productive symbioses with aquatic and terrestrial plants such as *Azolla* and *Gunnera*, with the cyanobacteria providing fixed nitrogen for the plant in exchange for carbon [3][4]. Nitrogen-fixing cyanobacteria have further been discovered in symbioses with diatoms, and actually live within the eukaryotic cell. Short filaments of *Richelia* and *Calothrix* (cyanobacteria) reside within the silica frustules of some species of the diatoms *Rhizosolenia*, *Chaetoceros*, or *Hemiaulus*, providing a large amount of fixed nitrogen for their diatom hosts [5][6]. Additionally, it was recently discovered that the globally widespread but

uncultured unicellular marine cyanobacterium known as UCYN-A exists in a symbiotic relationship with a unicellular prymnesiophyte. UCYN-A fixes nitrogen and relies obligately on the prymnesiophyte for carbon fixation, and has an unusually reduced genome [7]. All these relationships contain distinct exchanges of nutrients, especially with the cyanobacterium providing fixed nitrogen for its partner.

Cyanobacteria also interact extensively with heterotrophic bacteria in the environment, often in more complex and less well-characterized ways than the aforementioned eukaryotic symbioses. Diverse communities of heterotrophic bacteria commonly surround filamentous cyanobacteria. The heterotrophic bacteria likely consume nutrients released from the cyanobacteria, but may also produce necessary vitamins and chelators for the cyanobacteria, assist in cycling of CO_2 and phosphate, or help to lower O_2 levels for oxygen-sensitive processes such as nitrogen fixation [8][9]. Various studies have classified what taxa of heterotrophic bacteria live in close proximity to cyanobacterial blooms, including common aquatic phyla such as *Proteobacteria, Bacteroidetes, Actinobacteria*, and *Planctomycetes* [10][11]. Some unidentified and potentially new species or genera were also located within these samples, which could indicate organisms that specifically interact with cyanobacteria [10]. However, most often, the bacteria that make up these communities are also found living independently of the cyanobacteria [11]. The makeup of the cyanobacterial-associated community varies based on the

location and type of cyanobacterium, as well as the environmental conditions such as nutrient availability and temperature [11][12][13]. The heterotrophic bacterial community does appear to be directly influenced by the cyanobacteria, however, in that the community will change over the progression of a bloom [14]. In fact, if the cyanobacteria are eliminated by viral infection, the heterotrophic bacterial community drastically shifts [15]. These heterotrophic bacteria have conversely also been shown to affect the growth of cyanobacteria. Various strains of bacteria found living with a *Nodularia* cyanobacterium were co-cultured with *Nodularia* and were found to significantly increase or decrease the growth of the cyanobacterium [16]. Further studies of various heterotrophic bacteria associated with cyanobacterial blooms have corroborated that co-culturing with heterotrophic bacteria can increase or decrease cyanobacterial growth [17]. This is likely due to specific interactions of carbon and nutrient exchange [8][18]. However, since the interactions between cyanobacteria and heterotrophic bacteria involve a large number and variety of organisms, the relationships are certainly more complex than the aforementioned symbioses with two partners, and more inquiry into the communities surrounding cyanobacteria is required.

Cyanobacteria are also notable for their production of natural products (secondary metabolites and/or toxins) [19][20]. However, there is evidence that the bacterial communities present amongst the cyanobacteria may affect this process in different ways. On one hand, genetic analysis of natural

product assembly pathways is complicated by the presence of diverse heterotrophic bacteria within a cyanobacterial sample. These bacteria provide “contaminating” DNA that makes it difficult to locate the assembly pathways for the natural products [21]. However, heterotrophic bacteria may also play a more active role in affecting cyanobacterial secondary metabolite production. The heterotrophic bacterial communities surrounding the cyanobacteria can not only change cyanobacterial growth, but may also have the potential to break down cyanobacterial toxins [17] or modulate their production. For example, toxic *Microcystis* blooms with different associated heterotrophic bacteria produce different microcystins of varying toxicity [22]. It has conversely been proposed that secondary metabolites produced by cyanobacteria may select for certain organisms from the surrounding bacterial community [8]. Additionally, certain cyanobacterial and algal secondary metabolites have been shown to inhibit quorum sensing, a common mechanism of bacteria-to-bacteria communication, indicating that these secondary metabolites can have distinctive ecological effects [23].

Additionally, in some cases there are uncertainties about which organism is the true producer of a natural product compound. Considering the complexity of these compounds and their assembly pathways, it is highly unlikely that the pathways evolved separately in such divergent organisms as cyanobacteria and heterotrophic bacteria. For example, the lyngbyatoxins, a class of potent skin irritants and tumor promoters isolated from field collections

of *M. producens*, show high structural and pharmacological similarity to teleocidin, a metabolite which is produced by *Streptomyces* species [24][25]. Similarly, an extract from an assemblage of the cyanobacteria *Moorea producens* and *Tolypothrix* sp. yielded the toxin kalkipyrone, which proved to be structurally related to the actinopyrones and iromycins produced by *Streptomyces* species [26][27]. Another example is given by swinholide A, an actin-binding toxin originally isolated from, and assumed to be produced by, the sponge *Theonella swinhoei* [28]. However, confusion about the true producer of swinholide A occurred when this compound as well as a glycosylated derivative was also isolated from a field collection of a marine cyanobacterium [29]. It was later discovered that swinholide A in fact originates in the sponge from a member of the complex community of heterotrophic bacteria growing within *T. swinhoei*, rather than the sponge itself [28], and this may be the case for the cyanobacterial occurrence of swinholide A as well. These examples illustrate the uncertainty of whether bacteria associated with macroorganisms presumed to produce natural products could be the actual producers of these compounds. Overall, the study of the heterotrophic communities surrounding cyanobacteria that produce natural products could provide insights into the ecological roles or the production of these natural products.

Moorea producens is a filamentous tropical cyanobacterium, capable of photosynthesis but unable to fix nitrogen. It is a known producer of natural

products, with many polyketide synthase and non-ribosomal peptide synthetase genes present in its genome [30]. Similarly to other filamentous cyanobacteria, *M. producens* cells are surrounded by a complex polysaccharide sheath, which hosts a large community of associated bacteria [30]. Since *M. producens* was fairly recently defined as a distinct species, there has not been extensive research on the cyanobacterium in general, much less its heterotrophic bacterial community or its relationships with this community. The following experiments illustrate attempts to study a single member of the heterotrophic bacterial community of *M. producens.*

REFERENCES FOR THEORETICAL BACKGROUND

Note: This section has been adapted from material currently being prepared for publication, of which the thesis author is the primary author: Cummings SL, Barbé D, Leao TF, Korobeynikov A, Engene N, Glukhov E, Gerwick WH, Gerwick L. “A Novel Uncultured Heterotrophic Bacterial Associate of the Cyanobacterium Moorea producens JHB.”

1. Kardish N, Kessel M, and Galun M. (1989) Characterization of Symbiotic and Cultured *Nostoc* of the Lichen *Nephroma laevigatum* Ach. *Symbiosis,* 7:257-266.

2. O’Brien HE, Miadlikowska J, and Lutzoni F. (2005) Assessing host specialization in symbiotic cyanobacteria associated with four closely related species of the lichen fungus *Peltigera*. *Eur. J. Phycol*. 40:363-378.

3. Ray TB, Peters GA, Toia RE Jr, and Mayne BC. (1978) *Azolla-Anabaena* Relationships: Distribution of Ammonia-assimilating Enzymes, Protein, and Chlorophyll between Host and Symbiont. *Plant Physiol.* 62(3):463-467.

4. Bergman, B, Ran, L and Adams, DG. (2008) Cyanobacterial–plant symbioses: Signalling and development. From *The Cyanobacteria: Molecular Biology, Genomics and Evolution*, pp447-473. A. Herrero and E. Flores, eds. (Caister Academic Press. Wymondham, U.K.)

5. Amin SA, Parker MS, and Armbrust EV. (2012). Interactions Between Diatoms and Bacteria. *Microbiol. Molecul. Biol. Rev.*, 76(3): 667-684.

6. Foster RA, Kuypers MMM, Vagner T, Paerl RW, Musat N, and Zehr JP. (2011) Nitrogen fixation and transfer in open ocean diatom-cyanobacterial symbioses. *ISME J.*, 5:1484-1493.

7. Thompson AW, Foster RA, Krupke A, Carter BJ, Musat N, Vaulot D, Kuypers MMM, and Zehr JP. (2012) Unicellular Cyanobacterium Symbiotic with a Single-Celled Eukaryotic Alga. *Science*, 337:1546-1550.

8. Paerl HW and Pinckney JL. (1995) A Mini-review of Microbial Consortia: Their Roles in Aquatic Production and Biogeochemical Cycling. *Microb. Ecol.*, 31:225-247.

9. Paerl HW. (1996) A comparison of cyanobacterial bloom dynamics in freshwater, estuarine and marine environments. *Phycologica*, 35(6):25-35.

10. Eiler A and Bertilsson S. (2004) Composition of freshwater bacterial communities associated with cyanobacterial blooms in four Swedish lakes. *Environ. Microbiol.*, 6(12):1228-1248.

11. Hube AE, Heyduck-Söller B, and Fischer U. (2009) Phylogenetic classification of heterotrophic bacteria associated with filamentous marine cyanobacteria in culture. *Syst. Appl. Microbiol.* 32:256-265.

12. Brauer VS, Stomp M, Bouvier T, Fouilland E, Leboulanger C, Confurius-Guns V, Weissing FJ, Stal LJ, Huisman J. (2015) Competition and facilitation between the marine nitrogen-fixing cyanobacterium *Cyanothece* and its associated bacterial community. *Front. Microbiol.*, 5:795.

13. Limei S, Yuanfeng C, Hualin Y, Peng X, Pengfu L, Lingdong K, and Fanxiang K. (2009) Phylogenetic diversity and specificity of bacteria associated with *Microcystis aeruginosa* and other cyanobacteria. *J. Environ. Sci.*, 21:1581-1590.

14. Tuomainen J, Hietanen S, Kuparinen J, Martikainen PJ, and Servomaa K. (2006) Community Structure of the Bacteria Associated with *Nodularia* sp. (Cyanobacteria) Aggregates in the Baltic Sea. *Microb. Ecol.* 52:513-522.

15. Van Hannen EJ, Zwart G, Van Agterveld MP, Gons HJ, Ebert J, and Laanbroek HJ. (1998) Changes in Bacterial and Eukaryotic Community Structure after Mass Lysis of Filamentous Cyanobacteria Associated with Viruses. *Appl. Environ. Microbiol.*, 65(2):795-801.

16. Salomon PS, Janson S, and Granéli E. (2003) Molecular identification of bacteria associated with filaments of *Nodularia spumigena* and their effect on the cyanobacterial growth. *Harmful Algae* 2:261-272.

17. Berg KA, Lyra C, Sivonen K, Paulin L, Suomalainen S, Tuomi P, and Rapala J. (2009) High diversity of cultivable heterotrophic bacteria in association with cyanobacterial water blooms. *ISME J.*, 3:314-325.

18. Beliaev AS, Romine MF, Serres M, Bernstein HC, Linggi BE, Markillie LM, Isern NG, Chrisler WB, Kucek LA, Hill EA, Pinchuk GE, Bryant DA, Wiley HS, Fredrickson JK, and Konopka A. (2014) Inference of interactions in cyanobacterial-heterotrophic co-cultures via transcriptome sequencing. *ISME J.*, 8:2243-2255.

19. Wiegand C, Pflugmacher S. (2004) Ecotoxicological effects of selected cyanobacterial secondary metabolites, a short review. *Toxicol. Appl. Pharmacol.* 203:201-218.

20. Gerwick WH, Moore BS. (2012) Lessons from the Past and Charting the Future of Marine Natural Products Drug Discovery and Chemical Biology. *Chem. Biol.* 19:85-98.

21. Grindberg RV, Ishoey T, Brinza D, Esquenazi E, Coates RC, Liu WT, Gerwick L, Dorrestein PC, Pevzner P, Lasken R, and Gerwick WH. (2011) Single Cell Genome Amplification Accelerates Identification of the Apratoxin Biosynthetic Pathway from a Complex Microbial Assemblage. *PLoS One* 6(4):e18565.

22. Dziallas C and Grossart HP. (2012) Microbial interactions with the cyanobacterium *Microcystis aeruginosa* and their dependence on temperature. *Mar. Biol.*, 159:2389-2398.

23. Gerwick L, Boudreau P, Choi H, Mascuch S, Villa FA, Balunas MJ, Malloy KL, Teasdale ME, Rowley DC, Gerwick WH. (2013) Interkingdom signaling by structurally related cyanobacterial and algal secondary metabolites. *Phytochemistry Reviews*, 12(3):459-465.

24. Fujiki H, Mori M, Terada M, Sugimura T, and Moore RE. (1981) Indole alkaloids: dihydroteleocidin B, teleocidin, and lyngbyatoxin A as members of a new class of tumor promoters. Proc. Natl. Acad. Sci., 78(6):3872-3876.

25. Edwards DJ and Gerwick WH. (2004) Lyngbyatoxin Biosynthesis: Sequence of Biosynthetic Gene Cluster Identification of a Novel Aromatic Prenyltransferase. *J. Am. Chem. Soc.*, 126:11432-11433.

26. Graber MA and Gerwick WH. (1998) Kalkipyrone, a Toxic Gamma-Pyrone from an Assemblage of the Marine Cyanobacteria *Lyngbya majuscula* and *Tolypothrix* sp. *J. Nat. Prod.*, 61:677-680.

27. Surup F, Wagner O, von Frieling J, Schleicher M, Oess S, Muller P, Grond S. (2007) The Iromycins, a New Family of Pyridone Metabolites from *Streptomyces* sp. I. Structure, NOS Inhibitory Activity, and Biosynthesis. *J. Org. Chem.,* 72:5085-5090.

28. Bewley CA, Holland ND, Faulkner DJ. (1996) Two classes of metabolites from *Theonella swinhoei* are localized in distinct populations of bacterial symbionts. *Experientia*, 52(7):716-722.

29. Andrianasolo EH, Gross H, Goeger D, Musafija-Girt M, McPhail K, Leal RM, Mooberry SL, Gerwick WH. (2005). Isolation of swinholide A and related glycosylated derivatives from two field collections of marine cyanobacteria. Org Lett. 7(7):1375-1378.

30. Engene N, Rottacker EC, Kastovsky J, Byrum T, Choi H, Ellisman MH, Komarek J, and Gerwick WH. (2012) *Moorea producens* gen. nov., sp. nov. and *Moorea bouillonii* comb. nov., tropical marine cyanobacteria rich in bioactive secondary metabolites. *Int. J. of Syst. and Evol. Microbiol.* 62:1171-1178.

1.2: PREVIOUS RESEARCH ON THE UNKNOWN HETEROTROPHIC BACTERIUM

The study of this particular unknown bacterium originated from efforts to sequence the genome of *Moorea producens* strain JHB. The Gerwick laboratory culture of *M. producens* JHB was originally collected in Hector Bay, Jamaica in 1996, and has been maintained as a monoculture in the laboratory since then. DNA was extracted from the *M. producens* JHB monoculture and sequenced. During the assembly and binning of the sequences by % GC content, two large contigs thought to belong to an unknown associated bacterium were discovered. Through the PCR amplification of a missing 16S rRNA gene, these contigs were assembled into one contig of 5.99 Mb total size. This contig does not constitute a complete genome, as it is still missing approximately 2820 nucleotides of the 16S-23S ITS region and the 23S rRNA gene. However, the genome appears to be circular since the 16S rRNA gene is located on the 3’ end and the partial 23S rRNA gene is on the 5’ end. This genome was later deposited into GenBank under the accession number CP011806.

Given this genome sequence information, further examination of the bacterium could be performed. The genome has 66.8% GC content and contains 4792 protein-coding genes and 48 RNA-encoding genes. Examination of genes of interest was performed using the online program called RAST [31], and the genome was additionally scanned for secondary

metabolite assembly pathways using the online program called antiSMASH [32]. The bacterium was determined to be heterotrophic and non nitrogen-fixing, and had two secondary metabolite pathways present, a terpene pathway and a polyketide synthase type III pathway. Some other interesting features noted via RAST were genes for flagellar motility, ammonification and ammonia assimilation, beta-lactamase, cobalt-zinc-cadmium resistance, and inorganic sulfur assimilation.

In order to taxonomically identify the bacterium, a 16S rRNA gene was located and analyzed via comparison to NCBI's BLAST database and submission to the online program RDP Classifier [33]. The BLAST comparison revealed <95% similarity to various unknown, uncultured samples, and only 85% similarity to the closest known and cultured strain, *Desulfobacca acetoxidans.* The RDP Classifier analysis determined that the organism belongs to the phylum *Acidobacteria*, subgroup 22. There are currently 26 subgroups of *Acidobacteria* with few cultured representatives, but the 16S rRNA genes of *Acidobacteria* have recently been identified in various environmental samples, especially from sediments [34][35]. Considering the largely unstudied nature of *Acidobacteria* subgroup 22, and the limited similarity to other samples within the BLAST database, the organism is likely previously unidentified, and certainly belongs to an as yet unnamed genus and species. The bacterium will thus be further referred to as Mor1. A phylogenetic tree comparing the 16S rRNA sequence of Mor1 to other bacteria, including

Acidobacteria, Proteobacteria, and *Cyanobacteria* is depicted in FIGURE 1 (page 16).

As a final component to the genetic analysis of Mor1, a gene was selected from the genome that could be isolated through PCR to serve as a molecular identifier for the unknown organism within various cyanobacterial and bacterial cultures. This gene was located via RAST [31] and was annotated as L-seryl tRNA selenium transferase, known as *selA*. *selA* encodes for the tRNA incorporation of selenium-containing cysteine residues in proteins; it is present in some heterotrophic bacteria, but is not present in cyanobacteria [36]. Analysis with RAST confirmed that *selA* was contained within the genome of Mor1 but not *M. producens* JHB, and so specific PCR primers were designed based on the sequence of this gene. These primers, further referred to as the *selA* primers, were integral for further examination of Mor1 in various culture experiments.

The relative abundance of Mor1 compared to *M. producens* JHB was estimated using the sequencing coverage of single-copy genes within each genome. *selA* was selected as the single-copy gene within the genome of Mor1, and a single-copy gene of similar length, *argC*, was selected from the genome of *M. producens* JHB. The average coverage of *selA* was 25.1 ± 6.1 times, whereas the average coverage of *argC* was 106.0 ± 11.0 times. This indicates that the cells of *M. producens* JHB are approximately 4 times more abundant in culture than the cells of Mor1.

After genetic analysis, attempts were made to culture Mor1 separately from the filaments of *M. producens* JHB, using a variety of different marine media and nutrient enrichments. However, despite the fact that dozens of bacterial strains were successfully isolated from the *M. producens* JHB filaments, none were Mor1. This was determined by extracting DNA samples from the various bacterial cultures and testing them using PCR with the *selA* primers. Given this, it is not currently known how Mor1 can be cultured separately from *M. producens* JHB, or if it is possible to culture it separately.

Further examination of Mor1 sought to determine where it was located on *M. producens* JHB. TEM images of the outside of *M. producens* JHB sheaths as well as cross-sections of the sheaths suggested that the heterotrophic bacterial community of *M. producens* JHB is present on the exterior of the polysaccharide sheaths rather than inside the sheath or intracellularly. To confirm that Mor1 was located on the outside surface of the sheaths of *M. producens* JHB, a semi-quantitative PCR of the *selA* gene was performed. For this PCR, two samples of *M. producens* JHB filaments were used: one containing the intact external bacterial community (unwashed), and one that had been treated with a detergent wash designed to remove a substantial fraction of associated bacteria. Given equal amounts of DNA from each sample, a PCR was run using the *selA* primers. The *selA* signal decreased in the washed sample, confirming that Mor1 is present on the outside surface of *M. producens* JHB sheaths.

In order to demonstrate that Mor1 was a specific associate of *M. producens* and not generally associated with the laboratory cyanobacterial cultures, several other cyanobacterial cultures were tested for the presence of Mor1 using the *selA* primers. The cyanobacterial cultures tested included *Moorea producens* JHB, *Moorea producens* 3L, *Moorea bouillonii*, 3L *Oscillatoria*, *Scytonema hoffmani* "2846 axenic and xenic," *Leptolyngbya* sp. (coded ISBN3Nov94-8), and *Phormidium* sp. (coded PAP25Jun12-2). Interestingly, the signal for *selA* only appeared within *M. producens* JHB (originally collected from Jamaica) and *M. producens* 3L, a separate strain of *M. producens* collected in Curaçao; however, the signal did not appear in *M. bouillonii* or any other cyanobacterial species. On the basis of this observation, Mor1 was deduced to not be a general laboratory bacterial contaminant in the cultures, and was speculated to be a highly specific associate of *M. producens*.

At the culmination of these experiments, it was determined that this unknown bacterium was likely a novel organism, located on the outside of the polysaccharide sheaths of *M. producens* JHB. The unsuccessful attempts to culture the organism and the finding that the bacterium only appeared in cultures of *M. producens* suggested a potentially specific relationship with *M. producens*. The specificity of this relationship would be further tested with the following co-culturing experiment.

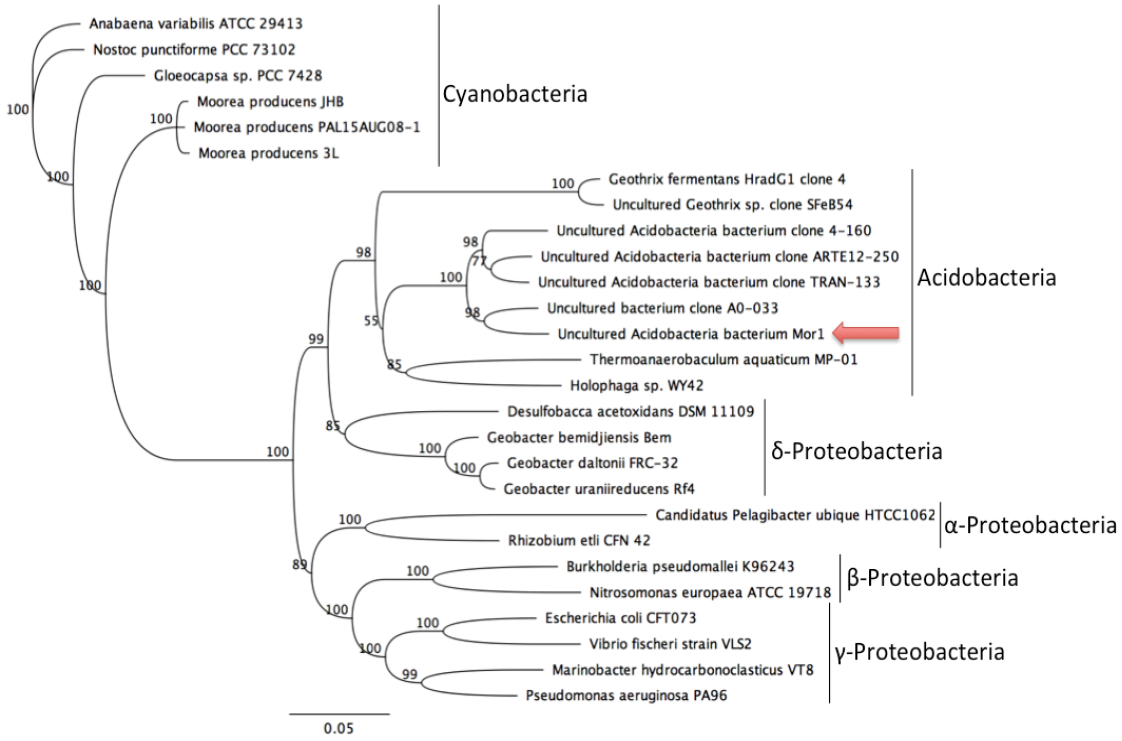

FIGURE 1. Phylogenetic tree comparing the 16S rRNA sequence of Mor1 to those of other bacteria. *Anabaena variabilis* ATCC 29413 was used as the outgroup. Mor1, indicated by an arrow, clusters with uncultured *Acidobacteria* strains, indicating that it likely belongs to a novel clade of phylum *Acidobacteria*.

REFERENCES FOR PREVIOUS RESEARCH

Note: This section has been adapted from material currently being prepared for publication, of which the thesis author is the primary author: Cummings SL, Barbé D, Leao TF, Korobeynikov A, Engene N, Glukhov E, Gerwick WH, Gerwick L. “A Novel Uncultured Heterotrophic Bacterial Associate of the Cyanobacterium Moorea producens JHB.” This paper contains more detailed methodology on the previous experiments performed to examine the unknown heterotrophic bacterium. FIGURE 1 in this thesis was directly duplicated from Figure 3 of the aforementioned paper.

31. Overbeek R, Olson R, Pusch GD, Olsen GJ, Davis JJ, Disz T, Edwards RA, Gerdes S, Parrello B, Shukla M, Vonstein V, Wattam AR, Xia F, and Stevens R. (2014) The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). *Nucleic Acids Res.*, 42(D1):D206-D214.

32. Blin K, Medema MH, Kazempour D, Fischbach MA, Breitling R, Takano E, and Weber T. (2013) antiSMASH 2.0 - a versatile platform for genome mining of secondary metabolite producers. *Nucleic Acids Research,* 41, W204-W212.

33. Wang Q, Garrity GM, Tiedje JM, and Cole JR. (2007) Naïve Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy. *Appl. Environ. Microbiol.,* 73(16):5261-7.

34. Barns SM, Cain EC, Sommerville L, and Kuske CR. (2007) *Acidobacteria* Phylum Sequences in Uranium-Contaminated Subsurface Sediments Greatly Expand the Known Diversity within the Phylum. *Appl. Environ. Microbiol.*, 73(9):3113-3116.

35. Navarrete AA, Kuramae EE, de Hollander M, Pijl AS, van Veen JA, and Tsai SM. (2012) Acidobacterial community responses to agricultural management of soybean in Amazon forest soils. *FEMS Microbiol. Ecol.*, 83(3):607-621.

36. Romero H, Zhang Y, Gladyshev VN, Salinas G. (2005) Evolution of selenium utilization traits. *Genome Biol.,* 6:R66.

CHAPTER 2: CO-CULTURING *MOOREA PRODUCENS* JHB WITH OTHER CYANOBACTERIA: HOW SPECIFIC IS THE ASSOCIATION OF THE UNKNOWN BACTERIUM WITH *MOOREA PRODUCENS*?

Based on the previous examinations of Mor1, it was determined that the bacterium was found within laboratory cultures of *Moorea producens*, but not other species of cyanobacteria. This suggested that there is a potentially specific association between Mor1 and *M. producens*. In order to determine whether Mor1 was capable of living with other species of cyanobacteria, a co-culturing experiment was designed in which *M. producens* JHB filaments containing Mor1 were cultured together with different strains of cyanobacteria. If Mor1 is capable of transferring to and living with these other cyanobacteria, the relationship between it and *M. producens* is likely not very specific; conversely, if the bacterium fails to transfer to and live with other cyanobacteria, it could suggest a more specific relationship between Mor1 and *M. producens*. The methodology and results of this experiment are laid out in the following sections.

2.1: METHODS

In order to determine whether Mor1 could be transferred from *M. producens* JHB to other strains of cyanobacteria, a co-culturing experiment was performed. This co-culturing experiment involved four laboratory cyanobacterial cultures: *3L Oscillatoria*, *Leptolyngbya sp*. (previously coded ISBN3Nov94-8), *Phormidium* sp. (previously coded PAP25Jun12-2), and *Moorea producens* JHB. The cultures selected for co-culture with *M. producens* were previously confirmed not to contain Mor1; when DNA samples from each of the cultures were tested via PCR with the *selA* primers, the *selA* signal was absent for each. Additionally, each of the cyanobacterial cultures is morphologically distinct to aid in the separation step of the co-culturing: *M. producens* JHB forms floating star-shaped clumps of thick red-brown filaments; *3L Oscillatoria* forms mats of thin dark-brown filaments; *Leptolyngbya sp.* forms mats of thin magenta filaments; and *Phormidium* sp. forms floating clusters of greenish-brown filaments.

Three main categories of co-cultures were performed: single-culture controls, with one cyanobacterial strain per flask; absent unknown bacterium controls, with two different strains without Mor1 (for example, *3L Oscillatoria* and *Leptolyngbya sp.*); and finally, the experimental co-cultures, with the three different cyanobacterial cultures being cultured individually with *M. producens* JHB. This co-culturing schematic is outlined in TABLE 1 (page 23). Each co-culture or control was grown in intimate contact in 250-mL flasks with SWBG-

11 media for two weeks until separation of the cultures, and each was also performed in duplicate flasks. In co-cultures involving two separate cyanobacteria, the two strains were separated after two weeks. The different strains were separated based on differing morphologies, using a dissecting microscope under sterile conditions. After separation, the cultures were grown for variable amounts of time to acquire several grams of wet biomass.

When enough biomass was obtained, DNA was extracted from each of the cultures. The cyanobacterial filaments were first prepared by drying with vacuum filtration and freezing with liquid nitrogen; then they were ground with mortar and pestle and transferred to microcentrifuge tubes. DNA was then extracted from these prepared samples, following the Joint Genome Institute's DNA extraction protocol for bacteria using phenol-chloroform and CTAB [37]. DNA concentration and quality for each sample was checked by UV spectrophotometry (NanoDrop 2000, Thermo Scientific).

Using the DNA samples obtained from each co-culture, two different PCR reactions were run. First, a PCR was run using general 16S rRNA primers called 27F and 781R (sequences shown in TABLE 2, page 23). These two primers produce a product of length 754 bp. Second, a PCR was run using the Mor1-specific *selA* primers, called *selA* Fw 428 and *selA* Rv 1180 (sequences shown in TABLE 2, page 23). These two primers produce a product of 775 bp in length.

PCR for both reactions was run with 20 µL total volumes, containing 10 µL of of 2x *Taq* Master Mix, 0.5 µL $MgCl_2$ (25 mM), 1.0 µL each of respective forward and reverse primers (10 µM), 1.0 µL of DNA template, and 6.5 µL of sterile water. Each reaction was also run with a negative control, using 1 µL of sterile water in place of DNA. The amplification conditions were as follows: initial denaturation at 95ºC for 4 min, followed by 30 cycles of 95ºC for 30 s, 50ºC with 16S rRNA primers or 55ºC with *selA* primers for 30 s, and 72ºC for 60 s, followed by a final extension step at 72ºC for 7 min.

The results of each PCR were visualized on 1% agarose gels containing 1:10,000 diluted Gel Red (Biotium Inc.). The samples were run with the Invitrogen 1 KB Plus DNA Ladder as a molecular weight marker. Gels for the 16S rRNA and *selA* PCR reactions are depicted in Section 1.2: Results and Discussion.

TABLE 1. Matrix of co-culturing experiments involving *M. producens* JHB and various other filamentous tropical marine cyanobacteria. Each entry indicates the intent of the co-culturing combination.

	M. producens JHB	*3L Oscillatoria*	*Leptolyngbya sp.*	*Phormidium* sp.
M. producens JHB	Single culture control	Potential Mor1 transfer	Potential Mor1 transfer	Potential Mor1 transfer
3L Oscillatoria	X	Single culture control	Absent Mor1 control	Absent Mor1 control
Leptolyngbya sp.	X	X	Single culture control	Absent Mor1 control
Phormidium sp.	X	X	X	Single culture control

TABLE 2. Primer sequences used in the co-culturing experiment.

Primer name	Primer sequence	T_m in ºC
27 F	5’ -AGAGTTTGATCCTGGCTCAG- 3’	54.3
781 R	5’ -GACTACAGGGGTATCTAATCC- 3’	52.0
selA Fw 428	5’ -ACTATCGCAAGGCGATCAACAAGA- 3’	58.6
selA Rv 1180	5’ -CTAGCTCATCGCTCCTATCAG- 3’	58.3

2.2: RESULTS AND DISCUSSION

In order to analyze the results of the co-culturing experiment, two different PCR reactions were run using the same co-culturing samples. The PCR using 16S rRNA primers served as a positive control for sample quality. With similar amounts of high quality DNA in each sample, the 16S rRNA bands should have the same brightness and intensity on the gel. As shown in FIGURE 2 (page 26), this appears to be the case. The negative control (Lane 2) shows no band, as expected, and the other lanes (Lanes 3-18) show bright bands around 754 bp, the expected size for the 16S rRNA product. This ensures that equal amounts of DNA were added into each reaction, and that the DNA samples were all of high quality.

A PCR reaction was run using the *selA* primers, with the same amounts of DNA used in each tube as the 16S rRNA reaction. This served to show whether Mor1 was present within any of the co-culture samples. The positive control for this reaction was the *M. producens* JHB single-culture control. Any other bands appearing on the gel would signify that Mor1 successfully transferred to an “acceptor” strain of cyanobacteria. The results of this PCR reaction are shown in FIGURE 3 (page 27). The negative control (Lane 2) shows no band, as expected, and the lane corresponding to the *M. producens* JHB single-culture sample (Lane 3) shows a band around 775 bp, as expected. The co-cultures of different cyanobacteria without Mor1, in Lanes 10-18, do not show any bands around 775 bp. This is expected, and ensures

that the *selA* PCR signal was not produced by the co-culturing experiment in itself. Lanes 4-9, which represent the three different cyanobacteria that had been co-cultured with *M. producens* JHB in duplicate, do not show any bands for *selA* around 775 bp. This means that Mor1 was not detectably present in the cyanobacterial samples that had been co-cultured with *M. producens* JHB. Mor1 did not appear to transfer to and grow on cyanobacteria other than *M. producens* JHB, indicating that Mor1 was not transferrable to these strains, and that it is a specific associate of *M. producens*.

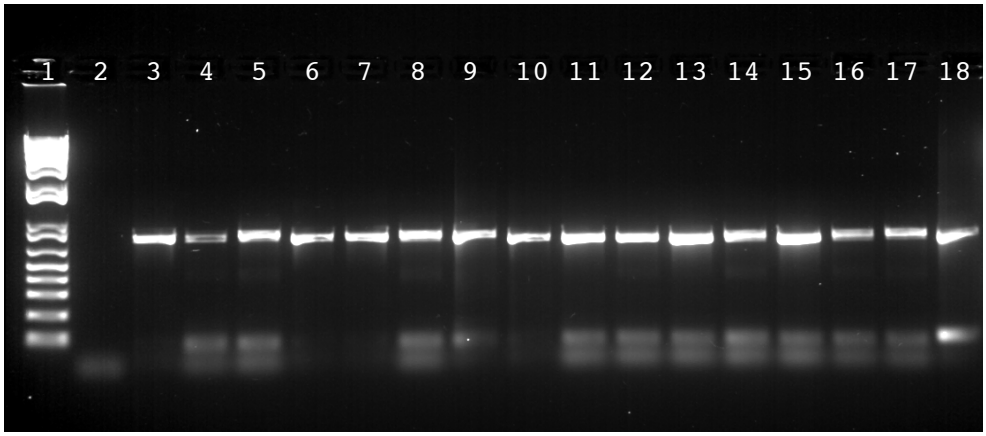

FIGURE 2. 16S rRNA PCR using DNA from the co-culturing of *Moorea producens* JHB with other cyanobacteria.
The size of the 16S rRNA PCR product is 754 bp, as indicated. Lane 1: Molecular weight marker (Invitrogen 1 kb Plus DNA Ladder). Lane 2: Negative control, sterile water. Lane 3: *Moorea producens* JHB. Lane 4: 3L *Oscillatoria*. Lane 5: *Phormidium* sp.. Lane 6: *Leptolyngbya* sp. Lanes 7 and 8: 3L *Oscillatoria* from co-culture with JHB, duplicate co-cultures. Lanes 9 and 10: *Phormidium* sp. from co-culture with JHB, duplicate co-cultures. Lanes 11 and 12: *Leptolyngbya* sp. from co-culture with JHB, duplicate co-cultures. Lane 13: *Phormidium* sp. from co-culture with *Leptolyngbya* sp. Lane 14: *Phormidium* sp. from co-culture with 3L *Oscillatoria*. Lane 15: *Leptolyngbya* sp. from co-culture with *Phormidium* sp.. Lane 16: *Leptolyngbya* sp. from co-culture with 3L *Oscillatoria*. Lane 17: 3L *Oscillatoria* from co-culture with *Phormidium* sp.. Lane 18: 3L *Oscillatoria* from co-culture with *Leptolyngbya* sp.

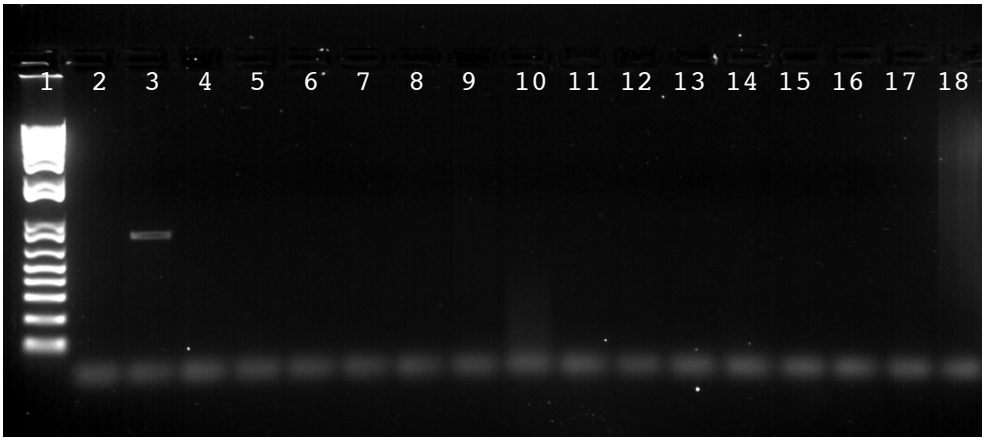

FIGURE 3. *selA* PCR using DNA from the co-culturing of *Moorea producens* JHB with other cyanobacteria.

The size of the *selA* PCR product is 775 bp, as indicated. Lane 1: Molecular weight marker (Invitrogen 1 kb Plus DNA Ladder). Lane 2: Negative control, sterile water. Lane 3: *Moorea producens* JHB. Lane 4: 3L *Oscillatoria*. Lane 5: *Phormidium* sp.. Lane 6: *Leptolyngbya* sp. Lanes 7 and 8: 3L *Oscillatoria* from co-culture with JHB, duplicate co-cultures. Lanes 9 and 10: *Phormidium* sp. from co-culture with JHB, duplicate co-cultures. Lanes 11 and 12: *Leptolyngbya* sp. from co-culture with JHB, duplicate co-cultures. Lane 13: *Phormidium* sp. from co-culture with *Leptolyngbya* sp. Lane 14: *Phormidium* sp. from co-culture with 3L *Oscillatoria*. Lane 15: *Leptolyngbya* sp. from co-culture with *Phormidium* sp.. Lane 16: *Leptolyngbya* sp. from co-culture with 3L *Oscillatoria*. Lane 17: 3L *Oscillatoria* from co-culture with *Phormidium* sp.. Lane 18: 3L *Oscillatoria* from co-culture with *Leptolyngbya* sp.

CHAPTER 2 REFERENCES

Note: This section has been adapted from material currently being prepared for publication, of which the thesis author is the primary author: Cummings SL, Barbé D, Leao TF, Korobeynikov A, Engene N, Glukhov E, Gerwick WH, Gerwick L. “A Novel Uncultured Heterotrophic Bacterial Associate of the Cyanobacterium Moorea producens JHB.” The experiment outlined in this section was designed and performed exclusively by the thesis author. FIGURES 2 and 3 in this thesis were adapted from Figure 8 of the aforementioned paper.

37. Feil WS, Feil H, and Copeland A. “Bacterial genomic DNA isolation using CTAB” (2012). US Department of Energy’s Joint Genome Institute. Web. <http://1ofdmq2n8tc36m6i46scovo2e.wpengine.netdna-cdn.com/wp-content/uploads/2014/02/JGI-Bacterial-DNA-isolation-CTAB-Protocol-2012.pdf>

CHAPTER 3: ANALYSIS OF THE UNKNOWN BACTERIUM AND PROPOSED FURTHER EXPERIMENTS

3.1: GENOMIC INFORMATION

Most of the current knowledge of Mor1 is based on the assembled 5.99 Mb genome obtained from the efforts to sequence the *Moorea producens* JHB monoculture. This genome shows that the bacterium does not have genes for photosynthesis or nitrogen fixation, indicating a heterotrophic and non-diazotrophic lifestyle. As previously mentioned, the genome also contains genes for flagellar motility, ammonification and ammonia assimilation, beta-lactamase, cobalt-zinc-cadmium resistance, and inorganic sulfur assimilation. Further analysis of Mor1's genome could be performed to locate particular genes of interest or compare to the genomes of other organisms.

When laboratory cyanobacterial cultures were tested for the presence of the *selA* gene indicative of Mor1, Mor1 was also detected within the culture of *M. producens* strain 3L. Thus, it would be valuable to sequence the genome of Mor1 derived from strain 3L. As a result of the previous sequencing of *M. producens* 3L, there were many contigs that were binned out and identified as belonging to associated heterotrophic bacteria. However, when these contigs were compared to the full genome of Mor1 obtained from *M. producens* JHB, none of them matched. Thus, it appears that in the previous sequencing effort of *M. producens* 3L, sequences from Mor1 were in low abundance. Since the *selA* gene is present within the cultures of *M. producens* 3L, Mor1 is present, but the previous sequencing effort incidentally did not yield a large number of

Mor1-like sequences. Thus, re-sequencing efforts could allow a genome of Mor1 associated with *M. producens* 3L to be obtained, and this could be compared to the current Mor1 genome derived from JHB. *M. producens* 3L was collected in Curaçao [30], whereas *M. producens* JHB was collected in Jamaica [38], and the two cyanobacteria differ in their genomes and secondary metabolite production. Thus, the genomes of Mor1 associated with these two distinct cyanobacteria would likely also show differences.

The size of the current Mor1 genome is also notable. Heterotrophic marine bacteria living in coastal environments with plentiful nutrients (copiotrophic) tend to have larger genome sizes than marine bacteria in low-nutrient (oligotrophic) environments [39]; since *M. producens* JHB was collected from a Jamaican reef, the cyanobacterium and its associated bacteria are copiotrophic. However, the copiotrophic bacterium exemplified in Lauro *et al.* has a “large” genome of 4.80 Mb [39], whereas Mor1 has a genome even larger than that, at 5.99 Mb. Additionally, it has been discovered that heterotrophic bacterial symbionts tend to have reduced genomes, though this genome reduction process occurs in obligate symbionts in “very stable environments” [40]. It is unclear though, what conclusions can be made about the genome size in relation to Mor1’s association with *M. producens* JHB. If Mor1 shows genome reduction and is missing common genes present in related bacteria, this is an indicator of an obligate symbiosis [41]. However, since there is still limited genomic information on bacteria within phylum

Acidobacteria, it is not known how Mor1's genome size compares to related organisms.

Analysis of Mor1's 16S rRNA gene enabled identification of the organism as a member of phylum *Acidobacteria*, subgroup 22. Further taxonomic classification of subgroup 22 does not currently exist [34][35], and so this organism belongs to a yet unnamed genus and species. In general, *Acidobacteria* is a poorly characterized phylum, containing few cultured representatives, and the known genetic information on *Acidobacteria* is primarily 16S rRNA sequences [42][43]. However, these 16S rRNA sequences have been detected in soil and sediment samples throughout the world, and members of the phylum are thought to play significant roles in these ecosystems [34][35][43]. Additionally, *Acidobacteria* have been discovered living within the associated communities of marine sponges and zoanthids [44][45], suggesting not only that *Acidobacteria* are present within marine and reef environments, but also that they are capable of forming complex, even symbiotic relationships with other organisms. Thus, although the phylum *Acidobacteria* is still poorly characterized, there is still some information that can be gained about the capabilities and ecology of Mor1 from studying related bacteria. Conversely, the addition of this complete *Acidobacteria* genome to GenBank has the potential to greatly expand the knowledge of a phylum that is significantly underrepresented in genomic information.

3.2: CULTIVATION-DEPENDENT INFORMATION

When laboratory cyanobacterial cultures were tested for the presence of *selA,* a gene specific to Mor1, the *selA* signal only appeared in cultures of *M.* producens. Additionally, the results of co-culturing *M. producens* JHB with other cyanobacteria indicate that Mor1 does not transfer to cyanobacteria other than *M. producens* JHB. According to the results of these experiments, it was concluded that Mor1 has a specific relationship with *M. producens*. However, information on Mor1 is currently limited because of the inability to culture it separately from the filaments of *M. producens* JHB. This could be indicative of the relationship between the two organisms, but culturing an organism individually can provide crucial microbiological information. Thus, an important further step in examining this bacterium is, if possible, obtaining a pure culture of Mor1 separate from *M. producens* JHB.

One potential route for the separation of *M. producens* JHB and Mor1 involves the differential sensitivity of the organisms to antibiotics. Comparison of the genomes of *M. producens* JHB and Mor1 using RAST [31] reveals that though both Mor1 and *M. producens* JHB have genes for beta-lactamase and several multi-drug efflux pumps, Mor1 also has genes for colicin E2 resistance and fosfomycin resistance. Thus, the use of either of these antibiotics could select against the cyanobacterium but allow Mor1 to live. However, the antibiotic resistances of the other heterotrophic bacteria that are associated with *M. producens* JHB are not currently known. Therefore, experiments along

these lines would simply be a first step in separating the cyanobacterium from Mor1.

Considering that Mor1 appears to be attached to the outer surface of sheaths of *M. producens*, another method of separating Mor1 and *M. producens* could involve washing the filaments of *M. producens* with detergent to remove associated bacteria. This procedure was previously performed along with a semi-quantitative PCR using *selA* primers in order to verify that Mor1 was largely present on the outside of the sheaths of *M. producens* JHB; because the bacterium appeared to partially wash off, this is a plausible method for separating the cyanobacterium and Mor1. However, previous attempts to culture Mor1 from directly plating this wash buffer were unsuccessful. Thus, this method could be used in conjunction with other methods of culturing “unculturable” bacteria; for example, using dilution to extinction of the wash buffer to reduce the number of competing bacteria being plated [46][47], and incubating for extended periods of time (up to 12 weeks) in case Mor1 is slow-growing [47].

Previous culturing attempts may also have been missing essential nutrients for the growth of Mor1. One strategy may be to use the media described by Joseph *et al.,* who successfully cultured various “unculturable” *Acidobacteria* strains from soil [42]. However, this method may not be successful for a marine *Acidobacteria* strain, or may need to be modified. Mor1 could also be missing nutrients specifically provided by *M. producens*.

The cyanobacterial compounds that Mor1 is most likely in contact with are the polysaccharides of the external sheath of *M. producens* [48]. Mor1 may utilize these for its growth, and thus purification of these polysaccharides to add to the growth media could provide more success in culturing Mor1 independently. However, during previous culturing attempts, whole *M. producens* filaments were frozen and powdered by grinding, and then added to the agar. The culturing of Mor1 from these trials was still unsuccessful, which could suggest that Mor1 requires nutrients from the living *M. producens* filaments. Comparison of genes between *M. producens* and Mor1 may give further insights into what Mor1 is “missing” that *M. producens* may provide. For example, comparison of amino acid synthesis and utilization genes between the two organisms using RAST [31] reveals that Mor1 contains genes for histidine degradation, but only *M. producens* JHB contains genes for histidine biosynthesis. Thus, supplementation of the media with histidine could allow for the growth of Mor1. Further, if this culturing strategy is successful, this could suggest an essential nutrient exchange that occurs between the two organisms.

Additional examinations of the relationship between *M. producens* and Mor1 could develop evidence for a symbiosis between the two organisms. Nutrient exchange is one of the clearest indicators of symbiotic interaction; however, demonstrating nutrient exchange often requires separate and axenic cultures of the two organisms. Evidence of nutrient exchange between Mor1

and *M. producens*, which would further suggest a symbiosis, could be provided if the two organisms can be cultured separately. If separate cultures of *M. producens* JHB and Mor1 can be obtained, co-culturing experiments between the two organisms could be performed, comparing the growth rates individually and together [16][17]. If *M. producens* and Mor1 grow better when co-cultured than when separated, this would suggest beneficial interactions between the two organisms. To better examine these interactions, transcriptome analysis could be carried out on the individual cultures and the co-culture; genes that are more highly expressed during co-culturing could indicate nutrient exchange or communication between the two organisms [18]. Direct exchange of molecules between Mor1 and *M. producens* could also be visualized using imaging mass spectrometry [49]. Additionally, since it has been shown previously that associated heterotrophic bacteria can affect the natural products produced by a cyanobacterium [22], this could be examined between *M. producens* and Mor1. LCMS (liquid chromatography–mass spectrometry) profiles of *M. producens* with and without Mor1 could be compared to examine whether *M. producens* produces differing secondary metabolites in the presence of Mor1. Overall, if interactions between the two organisms, including nutrient exchange, can be established, this provides further evidence for a symbiosis between Mor1 and *M. producens*, a novel symbiosis between two prokaryotes.

CHAPTER 3 REFERENCES

16. Salomon PS, Janson S, and Granéli E. (2003) Molecular identification of bacteria associated with filaments of *Nodularia spumigena* and their effect on the cyanobacterial growth. *Harmful Algae* 2:261-272.

17. Berg KA, Lyra C, Sivonen K, Paulin L, Suomalainen S, Tuomi P, and Rapala J. (2009) High diversity of cultivable heterotrophic bacteria in association with cyanobacterial water blooms. *ISME J.*, 3:314-325.

18. Beliaev AS, Romine MF, Serres M, Bernstein HC, Linggi BE, Markillie LM, Isern NG, Chrisler WB, Kucek LA, Hill EA, Pinchuk GE, Bryant DA, Wiley HS, Fredrickson JK, and Konopka A. (2014) Inference of interactions in cyanobacterial-heterotrophic co-cultures via transcriptome sequencing. *ISME J.*, 8:2243-2255.

22. Dziallas C and Grossart HP. (2012) Microbial interactions with the cyanobacterium *Microcystis aeruginosa* and their dependence on temperature. *Mar. Biol.*, 159:2389-2398.

31. Overbeek R, Olson R, Pusch GD, Olsen GJ, Davis JJ, Disz T, Edwards RA, Gerdes S, Parrello B, Shukla M, Vonstein V, Wattam AR, Xia F, and Stevens R. (2014) The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). *Nucleic Acids Res.*, 42(D1):D206-D214.

34. Barns SM, Cain EC, Sommerville L, and Kuske CR. (2007) *Acidobacteria* Phylum Sequences in Uranium-Contaminated Subsurface Sediments Greatly Expand the Known Diversity within the Phylum. *Appl. Environ. Microbiol.*, 73(9):3113-3116.

35. Navarrete AA, Kuramae EE, de Hollander M, Pijl AS, van Veen JA, and Tsai SM. (2012) Acidobacterial community responses to agricultural management of soybean in Amazon forest soils. *FEMS Microbiol. Ecol.*, 83(3):607-621.

38. Edwards DJ, Marquez BL, Nogle LM, McPhail K, Goeger DE, Roberts MA, Gerwick WH. (2004) Structure and Biosynthesis of the Jamaicamides, New Mixed Polyketide-Peptide Neurotoxins from the Marine Cyanobacterium Lyngbya majuscula. *Chem. Biol.*, 11(6):817-833.

39. Lauro FM, McDougald D, Thomas T, Williams TJ, Egan S, Rice S, DeMaere MZ, Ting L, Ertan H, Johnson J, Ferriera S, Lapidus A, Anderson

I, Kyrpides N, Munk AC, Detter C, Han CS, Brown MV, Robb FT, Kjelleberg S, and Cavicchioli S. (2009) The genomic basis of trophic strategy in marine bacteria. *Proc. Natl. Acad. Sci.,* 106(37):15527-15533.

40. Moya A, Gil R, Latorre A, Pereto J, Garcillan-Barcia MP, and De La Cruz F. (2009) Toward minimal bacterial cells: evolution vs. design. *FEMS Microbiol. Rev.,* 33(1):225-235.

41. Liu Z, Muller J, Alvey RM, Vogl K, Frigaard N, Rockwell NC, Boyd ES, Tomsho LP, Schuster SC, Henke P, Rohde M, Overmann J, and Bryant DA. (2013) Genomic analysis reveals key aspects of prokaryotic symbiosis in the phototrophic consortium "*Chlorochromatium aggregatum.*" *Genome Biol.,* 14:R127.

42. Joseph SJ, Hugenholtz P, Sangwan P, Osborne CA, Janssen PH. (2003) Laboratory Cultivation of Widespread and Previously Uncultured Soil Bacteria. *Appl. Environ. Microbiol.*, 69(12):7210-7215.

43. Quaiser A, Ochsenreiter T, Lanz C, Schuster SC, Treusch AH, Eck J, and Schleper C. (2003) Acidobacteria form a coherent but highly diverse group within the bacterial domain: evidence from environmental genomics. *Mol. Microbiol*., 50(2):563-575.

44. Webster NS, Luter HM, Soo RM, Botte ES, Simister RL, Abdo D, and Whalan S. (2013) Same, same but different: symbiotic bacterial associations in GBR sponges. *Front. Microbiol.,* 3:444.

45. O'Connor-Sanchez A, Rivera-Dominguez AJ, De los Santos-Briones C, Lopez-Aguiar LK, Pena-Ramirez YJ, Prieto-Davo A. (2014) *Acidobacteria* appear to dominate the microbiome of two sympatric Caribbean Sponges and one Zoanthid. *Biol. Res.*, 47:67.

46. Stewart EJ. (2012) Growing Unculturable Bacteria. *J. Bacteriol.*, 194(16):4151-4160.

47. Vartoukian SR, Palmer RM, Wade WG. (2010) Strategies for culture of 'unculturable' bacteria. *FEMS Microbiol. Lett.,* 309:1-7.

48. De Philippis R, Vincenzini M. (1998) Exocellular polysaccharides from cyanobacteria and their possible applications. *FEMS Microbiol. Rev.,* 22:151-175.

49. Esquenazi E, Dorrestein PC, Gerwick WH. (2009) Probing marine natural product defenses with DESI-imaging mass spectrometry. *Proc. Natl. Acad. Sci.,* 106(18):7269-7270.

CHAPTER 4: CONCLUSION

During the efforts to sequence the genome of *Moorea producens* strain JHB, the genome of a yet unknown and uncultured bacterium emerged. This genome provides valuable information about the bacterium, which was named Mor1. Mor1 is a heterotrophic bacterium belonging to phylum *Acidobacteria*, subgroup 22, and because of its low similarity to other known and cultured bacteria, Mor1 likely represents a novel clade of *Acidobacteria*. Since there are still few sequenced genomes of members of phylum *Acidobacteria*, acquiring the genome of Mor1 is in itself an exciting development.

Additional studies on Mor1, outlined in this thesis, have suggested a specific relationship between Mor1 and *Moorea producens*. Mor1 was located within laboratory cultures of *M. producens,* but no other species of cyanobacteria. It was also unable to be cultured independently of *M. producens* JHB, which could suggest an interdependence of the two organisms. Further, the co-culturing experiments outlined in this thesis show that Mor1 is unable to transfer to cyanobacteria other than *M. producens*, which additionally supports the specificity between Mor1 and *M. producens*.

These data substantiate a specific relationship between Mor1 and *M. producens*, but further studies are also needed. Efforts to culture the two organisms independently could further elucidate the relationship between Mor1 and *M. producens*, especially if there appears to be nutrient exchange between the two. Exchange of nutrients would provide strong evidence for a

symbiosis between Mor1 and *M. producens*. Therefore, not only can further research on Mor1 provide more information about the understudied phylum *Acidobacteria*, but it can also elucidate some of the interactions between filamentous cyanobacteria and heterotrophic bacteria, and characterize a potential novel symbiosis between two prokaryotic organisms.

FULL REFERENCE LIST

1. Kardish N, Kessel M, and Galun M. (1989) Characterization of Symbiotic and Cultured *Nostoc* of the Lichen *Nephroma laevigatum* Ach. *Symbiosis,* 7:257-266.

2. O'Brien HE, Miadlikowska J, and Lutzoni F. (2005) Assessing host specialization in symbiotic cyanobacteria associated with four closely related species of the lichen fungus *Peltigera*. *Eur. J. Phycol*. 40:363-378.

3. Ray TB, Peters GA, Toia RE Jr, and Mayne BC. (1978) *Azolla-Anabaena* Relationships: Distribution of Ammonia-assimilating Enzymes, Protein, and Chlorophyll between Host and Symbiont. *Plant Physiol.* 62(3):463-467.

4. Bergman, B, Ran, L and Adams, DG. (2008) Cyanobacterial–plant symbioses: Signalling and development. From *The Cyanobacteria: Molecular Biology, Genomics and Evolution*, pp447-473. A. Herrero and E. Flores, eds. (Caister Academic Press. Wymondham, U.K.)

5. Amin SA, Parker MS, and Armbrust EV. (2012). Interactions Between Diatoms and Bacteria. *Microbiol. Molecul. Biol. Rev.*, 76(3): 667-684.

6. Foster RA, Kuypers MMM, Vagner T, Paerl RW, Musat N, and Zehr JP. (2011) Nitrogen fixation and transfer in open ocean diatom-cyanobacterial symbioses. *ISME J.*, 5:1484-1493.

7. Thompson AW, Foster RA, Krupke A, Carter BJ, Musat N, Vaulot D, Kuypers MMM, and Zehr JP. (2012) Unicellular Cyanobacterium Symbiotic with a Single-Celled Eukaryotic Alga. *Science*, 337:1546-1550.

8. Paerl HW and Pinckney JL. (1995) A Mini-review of Microbial Consortia: Their Roles in Aquatic Production and Biogeochemical Cycling. *Microb. Ecol.*, 31:225-247.

9. Paerl HW. (1996) A comparison of cyanobacterial bloom dynamics in freshwater, estuarine and marine environments. *Phycologica*, 35(6):25-35.

10. Eiler A and Bertilsson S. (2004) Composition of freshwater bacterial communities associated with cyanobacterial blooms in four Swedish lakes. *Environ. Microbiol.*, 6(12):1228-1248.

11. Hube AE, Heyduck-Söller B, and Fischer U. (2009) Phylogenetic classification of heterotrophic bacteria associated with filamentous marine cyanobacteria in culture. *Syst. Appl. Microbiol.* 32:256-265.

12. Brauer VS, Stomp M, Bouvier T, Fouilland E, Leboulanger C, Confurius-Guns V, Weissing FJ, Stal LJ, Huisman J. (2015) Competition and facilitation between the marine nitrogen-fixing cyanobacterium *Cyanothece* and its associated bacterial community. *Front. Microbiol.*, 5:795.

13. Limei S, Yuanfeng C, Hualin Y, Peng X, Pengfu L, Lingdong K, and Fanxiang K. (2009) Phylogenetic diversity and specificity of bacteria associated with *Microcystis aeruginosa* and other cyanobacteria. *J. Environ. Sci.*, 21:1581-1590.

14. Tuomainen J, Hietanen S, Kuparinen J, Martikainen PJ, and Servomaa K. (2006) Community Structure of the Bacteria Associated with *Nodularia* sp. (Cyanobacteria) Aggregates in the Baltic Sea. *Microb. Ecol.* 52:513-522.

15. Van Hannen EJ, Zwart G, Van Agterveld MP, Gons HJ, Ebert J, and Laanbroek HJ. (1998) Changes in Bacterial and Eukaryotic Community Structure after Mass Lysis of Filamentous Cyanobacteria Associated with Viruses. *Appl. Environ. Microbiol.*, 65(2):795-801.

16. Salomon PS, Janson S, and Granéli E. (2003) Molecular identification of bacteria associated with filaments of *Nodularia spumigena* and their effect on the cyanobacterial growth. *Harmful Algae* 2:261-272.

17. Berg KA, Lyra C, Sivonen K, Paulin L, Suomalainen S, Tuomi P, and Rapala J. (2009) High diversity of cultivable heterotrophic bacteria in association with cyanobacterial water blooms. *ISME J.*, 3:314-325.

18. Beliaev AS, Romine MF, Serres M, Bernstein HC, Linggi BE, Markillie LM, Isern NG, Chrisler WB, Kucek LA, Hill EA, Pinchuk GE, Bryant DA, Wiley HS, Fredrickson JK, and Konopka A. (2014) Inference of interactions in cyanobacterial-heterotrophic co-cultures via transcriptome sequencing. *ISME J.*, 8:2243-2255.

19. Wiegand C, Pflugmacher S. (2004) Ecotoxicological effects of selected cyanobacterial secondary metabolites, a short review. *Toxicol. Appl. Pharmacol.* 203:201-218.

20. Gerwick WH, Moore BS. (2012) Lessons from the Past and Charting the Future of Marine Natural Products Drug Discovery and Chemical Biology. *Chem. Biol.* 19:85-98.

21. Grindberg RV, Ishoey T, Brinza D, Esquenazi E, Coates RC, Liu WT, Gerwick L, Dorrestein PC, Pevzner P, Lasken R, and Gerwick WH. (2011) Single Cell Genome Amplification Accelerates Identification of the

Apratoxin Biosynthetic Pathway from a Complex Microbial Assemblage. *PLoS One* 6(4):e18565.

22. Dziallas C and Grossart HP. (2012) Microbial interactions with the cyanobacterium *Microcystis aeruginosa* and their dependence on temperature. *Mar. Biol.*, 159:2389-2398.

23. Gerwick L, Boudreau P, Choi H, Mascuch S, Villa FA, Balunas MJ, Malloy KL, Teasdale ME, Rowley DC, Gerwick WH. (2013) Interkingdom signaling by structurally related cyanobacterial and algal secondary metabolites. *Phytochemistry Reviews*, 12(3):459-465.

24. Fujiki H, Mori M, Terada M, Sugimura T, and Moore RE. (1981) Indole alkaloids: dihydroteleocidin B, teleocidin, and lyngbyatoxin A as members of a new class of tumor promoters. Proc. Natl. Acad. Sci., 78(6):3872-3876.

25. Edwards DJ and Gerwick WH. (2004) Lyngbyatoxin Biosynthesis: Sequence of Biosynthetic Gene Cluster Identification of a Novel Aromatic Prenyltransferase. *J. Am. Chem. Soc.*, 126:11432-11433.

26. Graber MA and Gerwick WH. (1998) Kalkipyrone, a Toxic Gamma-Pyrone from an Assemblage of the Marine Cyanobacteria *Lyngbya majuscula* and *Tolypothrix* sp. *J. Nat. Prod.*, 61:677-680.

27. Surup F, Wagner O, von Frieling J, Schleicher M, Oess S, Muller P, Grond S. (2007) The Iromycins, a New Family of Pyridone Metabolites from *Streptomyces* sp. I. Structure, NOS Inhibitory Activity, and Biosynthesis. *J. Org. Chem.,* 72:5085-5090.

28. Bewley CA, Holland ND, Faulkner DJ. (1996) Two classes of metabolites from *Theonella swinhoei* are localized in distinct populations of bacterial symbionts. *Experientia*, 52(7):716-722.

29. Andrianasolo EH, Gross H, Goeger D, Musafija-Girt M, McPhail K, Leal RM, Mooberry SL, Gerwick WH. (2005). Isolation of swinholide A and related glycosylated derivatives from two field collections of marine cyanobacteria. Org Lett. 7(7):1375-1378.

30. Engene N, Rottacker EC, Kastovsky J, Byrum T, Choi H, Ellisman MH, Komarek J, and Gerwick WH. (2012) *Moorea producens* gen. nov., sp. nov. and *Moorea bouillonii* comb. nov., tropical marine cyanobacteria rich in bioactive secondary metabolites. *Int. J. of Syst. and Evol. Microbiol.* 62:1171-1178.

31. Overbeek R, Olson R, Pusch GD, Olsen GJ, Davis JJ, Disz T, Edwards RA, Gerdes S, Parrello B, Shukla M, Vonstein V, Wattam AR, Xia F, and Stevens R. (2014) The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). *Nucleic Acids Res.*, 42(D1):D206-D214.

32. Blin K, Medema MH, Kazempour D, Fischbach MA, Breitling R, Takano E, and Weber T. (2013) antiSMASH 2.0 - a versatile platform for genome mining of secondary metabolite producers. *Nucleic Acids Research,* 41, W204-W212.

33. Wang Q, Garrity GM, Tiedje JM, and Cole JR. (2007) Naïve Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy. *Appl. Environ. Microbiol.,* 73(16):5261-7.

34. Barns SM, Cain EC, Sommerville L, and Kuske CR. (2007) *Acidobacteria* Phylum Sequences in Uranium-Contaminated Subsurface Sediments Greatly Expand the Known Diversity within the Phylum. *Appl. Environ. Microbiol.*, 73(9):3113-3116.

35. Navarrete AA, Kuramae EE, de Hollander M, Pijl AS, van Veen JA, and Tsai SM. (2012) Acidobacterial community responses to agricultural management of soybean in Amazon forest soils. *FEMS Microbiol. Ecol.*, 83(3):607-621.

36. Romero H, Zhang Y, Gladyshev VN, Salinas G. (2005) Evolution of selenium utilization traits. *Genome Biol.,* 6:R66.

37. Feil WS, Feil H, and Copeland A. “Bacterial genomic DNA isolation using CTAB” (2012). US Department of Energy’s Joint Genome Institute. Web. <http://1ofdmq2n8tc36m6i46scovo2e.wpengine.netdna-cdn.com/wp-content/uploads/2014/02/JGI-Bacterial-DNA-isolation-CTAB-Protocol-2012.pdf>

38. Edwards DJ, Marquez BL, Nogle LM, McPhail K, Goeger DE, Roberts MA, Gerwick WH. (2004) Structure and Biosynthesis of the Jamaicamides, New Mixed Polyketide-Peptide Neurotoxins from the Marine Cyanobacterium Lyngbya majuscula. *Chem. Biol*., 11(6):817-833.

39. Lauro FM, McDougald D, Thomas T, Williams TJ, Egan S, Rice S, DeMaere MZ, Ting L, Ertan H, Johnson J, Ferriera S, Lapidus A, Anderson I, Kyrpides N, Munk AC, Detter C, Han CS, Brown MV, Robb FT, Kjelleberg S, and Cavicchioli S. (2009) The genomic basis of trophic strategy in marine bacteria. *Proc. Natl. Acad. Sci.,* 106(37):15527-15533.

40. Moya A, Gil R, Latorre A, Pereto J, Garcillan-Barcia MP, and De La Cruz F. (2009) Toward minimal bacterial cells: evolution vs. design. *FEMS Microbiol. Rev.,* 33(1):225-235.

41. Liu Z, Muller J, Alvey RM, Vogl K, Frigaard N, Rockwell NC, Boyd ES, Tomsho LP, Schuster SC, Henke P, Rohde M, Overmann J, and Bryant DA. (2013) Genomic analysis reveals key aspects of prokaryotic symbiosis in the phototrophic consortium "*Chlorochromatium aggregatum.*" *Genome Biol.,* 14:R127.

42. Joseph SJ, Hugenholtz P, Sangwan P, Osborne CA, Janssen PH. (2003) Laboratory Cultivation of Widespread and Previously Uncultured Soil Bacteria. *Appl. Environ. Microbiol.*, 69(12):7210-7215.

43. Quaiser A, Ochsenreiter T, Lanz C, Schuster SC, Treusch AH, Eck J, and Schleper C. (2003) Acidobacteria form a coherent but highly diverse group within the bacterial domain: evidence from environmental genomics. *Mol. Microbiol*., 50(2):563-575.

44. Webster NS, Luter HM, Soo RM, Botte ES, Simister RL, Abdo D, and Whalan S. (2013) Same, same but different: symbiotic bacterial associations in GBR sponges. *Front. Microbiol.,* 3:444.

45. O'Connor-Sanchez A, Rivera-Dominguez AJ, De los Santos-Briones C, Lopez-Aguiar LK, Pena-Ramirez YJ, Prieto-Davo A. (2014) *Acidobacteria* appear to dominate the microbiome of two sympatric Caribbean Sponges and one Zoanthid. *Biol. Res.*, 47:67.

46. Stewart EJ. (2012) Growing Unculturable Bacteria. *J. Bacteriol.*, 194(16):4151-4160.

47. Vartoukian SR, Palmer RM, Wade WG. (2010) Strategies for culture of 'unculturable' bacteria. *FEMS Microbiol. Lett.,* 309:1-7.

48. De Philippis R, Vincenzini M. (1998) Exocellular polysaccharides from cyanobacteria and their possible applications. *FEMS Microbiol. Rev.,* 22:151-175.

49. Esquenazi E, Dorrestein PC, Gerwick WH. (2009) Probing marine natural product defenses with DESI-imaging mass spectrometry. *Proc. Natl. Acad. Sci.,* 106(18):7269-7270.