UC San Diego
UC San Diego Electronic Theses and Dissertations

**Title**
The diversity, distribution, and biological activity of brominated natural products in the genus Pseudoalteromonas

**Permalink**
https://escholarship.org/uc/item/78t2h9kt

**Author**
Busch, Julia

**Publication Date**
2018

Peer reviewed|Thesis/dissertation

eScholarship.org Powered by the California Digital Library
University of California

UNIVERSITY OF CALIFORNIA SAN DIEGO

# The diversity, distribution, and biological activity of brominated natural products in the genus *Pseudoalteromonas*

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Marine Biology

by

Julia Busch

Committee in charge:

Professor Paul Jensen, Chair
Professor Eric Allen
Professor Lihini Aluwihare
Professor Bradley Moore
Professor Joseph Pogliano

2018

Copyright
Julia Busch, 2018
All rights reserved.

The Dissertation of Julia Busch is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

__________________________________________________

__________________________________________________

__________________________________________________

__________________________________________________

__________________________________________________

Chair

University of California San Diego

2018

TABLE OF CONTENTS

Signature page ........ iii

Table of contents ........ iv

List of abbreviations ........ vi

List of figures ........ vii

List of tables ........ ix

Acknowledgements ........ x

Vita ........ xi

Abstract of the dissertation ........ xii

Chapter 1: Introduction to the dissertation ........ 1
- 1.1 Marine natural products ........ 2
  - 1.1.1 History of natural products ........ 2
  - 1.1.2 Marine sources of medicine ........ 5
  - 1.1.3 Halogenated marine natural products ........ 9
- 1.2 Bacteria in the ocean ........ 12
  - 1.2.1 Marine microbial ecology ........ 12
  - 1.2.2 Chemotaxonomy ........ 15
- 1.3 Biosynthetic gene cluster evolution in marine bacteria ........ 17
- 1.4 *Pseudoalteromonas*: a genus of marine bacteria ........ 18
  - 1.4.1 *Pseudoalteromonas* diversity, distribution, and abundance ........ 18
  - 1.4.2 Bioactive metabolites produced by *Pseudoalteromonas* ........ 22
- 1.5 Overview of the dissertation ........ 23

Chapter 2: Diversity and distribution of the *bmp* gene cluster and pentabromopseudilin production in *Pseudoalteromonas* ........ 28
- 2.1 Abstract ........ 29
- 2.2 Introduction ........ 29
- 2.3 Materials and methods ........ 33
  - 2.3.1 Bacterial isolation ........ 33
  - 2.3.2 Genome mining, primer design and PCR ........ 33
  - 2.3.3 Phylogenetic analyses ........ 39
  - 2.3.4 Pentabromopseudilin analysis ........ 39
  - 2.3.5 Comparative genomics ........ 39
- 2.4 Results ........ 40
  - 2.4.1 Pseudoalteromonas species phylogeny ........ 40
  - 2.4.2 Distribution of the bmp gene cluster among *Pseudoalteromonas* spp ........ 42
  - 2.4.3 Pentabromopseudilin production ........ 44
  - 2.4.4 Evolutionary history of the bmp gene cluster ........ 46

2.5 Discussion....................50
2.6 Acknowledgements....................53

Chapter 3: The cellular target of pentabromopseudilin....................55
3.1 Abstract....................56
3.2 Introduction....................56
3.3 Materials and methods....................58
3.3.1 Minimal inhibitory concentration determination....................58
3.3.2 Bacterial Cytological Profiling (BCP)....................59
3.3.3 Viability counts....................59
3.3.4 Membrane permeability quantification....................60
3.3.5 Cytotoxicity assay....................60
3.4 Results and discussion....................60
3.4.1 Cytological profiling of cells treated with pentabromopseudilin....................60
3.4.2 Quantification of cell membrane permeability....................61
3.4.3 Pentabromopseudilin-treated cell viability....................64
3.4.4 The potentiation of pentabromopseudilin....................65
3.5 Acknowledgements....................69

Chapter 4: Genome mining and bioactive metabolite production in *Pseudoalteromonas* ....................70
4.1 Abstract....................71
4.2 Introduction....................71
4.3 Materials and methods....................73
4.3.1 Strain growth and chemical extractions....................73
4.3.2 Fractionation of crude extracts....................74
4.3.3 Disc diffusion assays....................74
4.3.4 Chemical analyses of crude extracts and fractions....................75
4.4 Results....................77
4.4.1 Bioactivity of *Pseudoalteromonas* extracts....................77
4.4.2 LC-MS analysis of *Pseudoalteromonas* crude extracts....................80
4.4.3 Bioactivity of fractions vs. PY79....................89
4.4.4 LC-MS analysis of *Pseudoalteromonas* crude extracts....................90
4.4.5 Chemical analyses of crude extracts and fractions....................94
4.5 Discussion....................97
4.6 Acknowledgements....................98

Chapter 5: Concluding remarks....................99

References....................103

# LIST OF ABBREVIATIONS

| | |
|---|---|
| BCP | bacterial cytological profiling |
| BGC | biosynthetic gene cluster |
| BLAST | basic local alignment search tool |
| *bmp* | brominated marine pyrrole/phenol gene cluster |
| DCM | dichloromethane |
| DGGE | denaturing gradient gel electrophoresis |
| EtOAc | ethyl acetate |
| *gyrB* | gene encoding DNA gyrase, subunit B |
| HGT | horizontal gene transfer |
| KBr | potassium bromide |
| LC-MS | liquid chromatography-mass spectrometry |
| MeOH | methanol |
| PBP | pentabromopseudilin |
| PCR | polymerase chain reaction |
| *pyrH* | gene encoding |
| qPCR | real-time quantitative PCR |
| *recA* | gene encoding |
| *rpoB* | gene encoding |
| rRNA | ribosomal RNA |
| ZOI | zone of inhibition |

LIST OF FIGURES

**Chapter 1**

**Figure 1.1** The maximum likelihood phylogeny of type strains for the 42 *Pseudoalteromonas* named species....................21

**Figure 1.2** Chapter 2 overview....................23

**Figure 1.3** Chapter 3 overview....................25

**Figure 1.4** Chapter 4 overview....................26

**Chapter 2**

**Figure 2.1** Brominated natural product biosynthesis in marine bacteria....................32

**Figure 2.2** *Pseudoalteromonas* species tree and *bmp* gene cluster distribution..........41

**Figure 2.3** ANI analysis....................42

**Figure 2.4** Detection of pentabromopseudilin by LCMS....................45

**Figure 2.5** *Bmp* gene phylogeny....................46

**Figure 2.6** Genomic environment of the *bmp* gene cluster....................49

**Figure 2.7** Evidence for partial loss of the *bmp* cluster....................50

**Figure 2.8** Evolutionary history of the *bmp* gene cluster in *Pseudoalteromonas* .......52

**Chapter 3**

**Figure 3.1** Pentabromopseudilin....................57

**Figure 3.2** Bacterial cytological profiling of *E. coli* ATCC 25922 treated with pentabromopseudilin at concentrations greater than or equal to the MIC value....................62

**Figure 3.3** Bacterial cytological profiling of *E. coli* ATCC 25922 treated with pentabromopseudilin at concentrations less than the MIC value..............63

**Figure 3.4** SYTOX Green intensity of *E. coli* ATCC 25922 cells treated with pentabromopseudilin over time....................64

**Figure 3.5** The viability of PBP-treated cells represented as log(CFU/mL) vs. time in minutes....................65

**Figure 3.6** Vancomycin potentiation assay of *E. coli* ATCC 25922....................67

**Figure 3.7** Erythromycin potentiation assay of *E. coli* ATCC 25922....................68

**Figure 3.8** Percent growth based on $OD_{600}$ ratio of untreated *E. coli* ATCC 25922 cells to cells treated with PBP....................68

## Chapter 4


**Figure 4.1** Disc diffusion assay results for *Pseudoalteromonas* crude extracts at 10 mg/mL concentration ....79

**Figure 4.2** Disc diffusion assay results for strain 2ta16 crude extracts at lower concentration ....80

**Figure 4.3** Molecular network of crude extracts from strains 2ta16, O-BC30, PS5, A757, and 2ta6 acquired in negative mode ....82

**Figure 4.4** Molecular network of crude extracts from strains 2ta16, O-BC30, PS5, A757, and 2ta6 acquired in positive mode ....83

**Figure 4.5** Molecular network of crude extracts from strains 2ta16, O-BC30, PS5, A757, and 2ta6 acquired in negative mode ....84

**Figure 4.6** Molecular network of crude extracts from strains 2ta16, O-BC30, PS5, A757, and 2ta6 acquired in positive mode ....85

**Figure 4.7** Molecular network of crude extracts from strains 2ta16, O-BC30, PS5, A757, and 2ta6 acquired in negative mode ....86

**Figure 4.8** Molecular network of crude extracts from strains 2ta16, O-BC30, PS5, A757, and 2ta6 acquired in positive mode ....87

**Figure 4.9** Disc diffusion assay results for 2ta16 active fractions at 2 mg/mL concentration ....90

**Figure 4.10** Molecular network of fractions from strain 2ta16, PS5, 2ta6 acquired in negative mode ....92

**Figure 4.11** Molecular network of fractions from strain 2ta16, PS5, 2ta6 acquired in positive mode ....93

## LIST OF TABLES

### Chapter 1

**Table 1.1** *Pseudoalteromonas* species ....................................................................20

### Chapter 2

**Table 2.1** Type strains without published genome sequences ..................................34

**Table 2.2** *Pseudoalteromonas* genomes....................................................................35

**Table 2.3** PCR primer sequences ...............................................................................38

### Chapter 3

**Table 3.1** SYTOX Green intensity cell counts ........................................................63

**Table 3.2** Average SYTOX Green intensity values..................................................63

### Chapter 4

**Table 4.1** Pseudoalteromonas strains used in study..................................................77

**Table 4.2** GNPS library hits found in *Pseudoalteromonas* crude extracts ................88

**Table 4.3** Genome mining analysis summary for five *Pseudoalteromonas* genomes ..........................................................................................................95

**Table 4.4** Similar known biosynthetic gene clusters to those found in strain O-BC30 ..........................................................................................................96

**Table 4.5** Similar known biosynthetic gene clusters to those found in strain A757..96

**Table 4.6** Similar known biosynthetic gene clusters to those found in strain PS5 ....96

**Table 4.7** Similar known biosynthetic gene clusters to those found in strain 2ta16..96

**Table 4.8** Similar known biosynthetic gene clusters to those found in strain 2ta6....97

ACKNOWLEDGEMENTS

I'd like to thank my advisor, Paul Jensen, for giving me the opportunity to be a student at SIO. I would also like to thank the rest of my dissertation committee for all of the help and guidance throughout my graduate studies. I'd like to acknowledge the other members of the Scripps Center for Oceans and Human Health (SCOHH) members including Bill Fenical, Eunha Hoh, Vinayak Agarwal, Abrahim El Gamal, Tracey Somera, Michelle Schorn, and Abdhesh Kumar.

Thanks to all the past and present members of the Jensen lab who have provided valuable input on my research; I've learned something from every individual who has been a part of our group over the years. I especially want to acknowledge Leesa Klau, Henrique Ramalho Machado, and Kaitlin Creamer for their support and encouragement. I also want to thank Christian McDonald and Rich Walsh for making the diving program at SIO such a valuable resource.

Chapter 2, in full, has been formulated into a manuscript that will be submitted for publication in 2018. Julia Busch, Vinayak Agarwal, Michelle Schorn, Henrique Machado, Bradley S. Moore, Greg W. Rouse, Lone Gram, Paul R. Jensen. The dissertation author was the primary investigator and author on these studies.

Chapter 3 is in prep and coauthored with Elizabeth Montano, Joseph Sugie, Abdhesh Kumar, William Fenical, Paul Jensen, and Joe Pogliano. The dissertation author was the primary investigator on these studies.

Chapter 4 is coauthored with Leesa Klau, and Paul Jensen. The dissertation author was the primary investigator on these studies.

VITA

| | |
|---|---|
| 2006 | Bachelor of Science, University of California Davis |
| 2006-2008 | Research Assistant, The Scripps Research Institute |
| 2008-2009 | Research Assistant, University of California San Diego AVRC |
| 2012 | Master of Science, San Diego State University |
| 2018 | Doctor of Philosophy, University of California San Diego |

## ABSTRACT OF THE DISSERTATION

The diversity, distribution, and biological activity of brominated natural products in the genus *Pseudoalteromonas*

by

Julia Busch

Doctor of Philosophy in Marine Biology

University of California San Diego, 2018

Professor Paul R. Jensen, Chair

Secondary metabolite production plays an important role in the interaction of bacteria and their environments. While many marine microbes have been found to produce bioactive molecules, relatively little is known about the diversity and distribution of biosynthetic gene clusters with known products. This dissertation includes five chapters that explore these topics in the genus *Pseudoalteromonas.* The first chapter introduces marine natural products and the group of bacteria, followed by three research chapters and a summary chapter.

Chapter 2 explores the evolution of the *bmp* gene cluster in the genus *Pseudoalteromonas*. A well-supported species phylogeny was generated and the

distribution and diversity of the biosynthetic gene cluster was mapped onto the tree. Four distinct versions of the *bmp* cluster were found in the genus, with evidence supporting gene loss in certain cases. It is uncertain how many times the gene cluster was acquired within *Pseudoalteromonas*, but the conservation of the biosynthetic genes within some lineages suggests that the metabolites produced are relevant to the ecology of these bacteria.

Chapter 3 presents the results from the bacterial cytological profiling of pentabromopseudilin in an *E. coli* strain. The primary phenotype observed in treated cells was membrane permeability, which increases over time and concentration. Despite its apparent ability to disrupt the cell membrane of Gram-negative bacteria and increase the potency of two tested antibiotics, pentabromopseudilin is not likely a good candidate for therapeutic uses due its cytotoxicity.

The final research chapter investigates the bioactivity and biosynthetic potential of five *Pseudoalteromonas* species. The crude extracts and fractions of some of the strains included in the study were bioactive against Gram-positive and Gram-negative test strains. Preliminary genome mining results reveal that *Pseudoalteromonas* are rich in biosynthetic gene clusters, most of which do not have known products or similar clusters.

# Chapter 1: Introduction to the dissertation

## 1.1 Marine natural products

### 1.1.1 History of natural products

Secondary metabolites, also known as natural products, are biologically synthesized molecules that are not essential for the immediate survival of an organism. Unlike primary metabolites, these molecules are generally not involved in the growth or reproduction of an organism but rather serve to increase the overall fitness of the producer in a specific niche. Some secondary metabolites have been identified as defense mechanisms against predators or competing organisms and have proven to be a rich source of drugs such as antibiotics. Historically, illnesses were viewed from a magical, superstitious, and religious viewpoint. Hippocrates, the "father of medicine" (460-370 BC), established the first scientific basis of western medicine by forming rational diagnoses based on direct clinical observations (Voultsiadou Eleni 2010).

Plants are rich sources of therapeutic natural products and have been used in medicines for thousands of years. Because they've had to adapt to survive pests (e.g. bacteria, fungi, insects) and other environmental pressures, they have evolved to produce a plethora of structurally diverse secondary metabolites. Early societies have experimented with the therapeutic potential of plants by simple trial and error. Aspirin is derived from salicin, a natural product isolated from willow tree bark (*Salix alba*) and research involving opium poppies (*Papaver somniferum*) led to the discovery of morphine (Dias, Urban, and Roessner 2012). Additionally, animal products have also been used for their medicinal properties, however these have been overwhelmingly from terrestrial sources. Perhaps the most well known terrestrial natural product is penicillin, from the fungus, *Penicillium notatum*. Alexander Fleming discovered the potent

antibiotic by accident in 1929. It has since saved countless human lives (Dias, Urban, and Roessner 2012). Most natural products-based medicines originate from terrestrial sources due to ease of access. For many early civilizations, the ocean was inaccessible. But even for those living near the coast, the marine environment was often viewed as dangerous and inhospitable. While there is ample evidence that some early civilizations used marine algae and organisms as a primary source of food, there are also accounts of their use as ancient medicines dating back thousands of years.

Specific research on the drug discovery application of marine natural products began about 60 years ago, but the use of marine organisms in traditional medicine originated long before that. Records of medicinal seaweeds appeared in Chinese literature over two thousand years ago and they are still being used today for a variety of medical purposes. *Laminaria japonica* and *Sargassum fusiforme* have been used for more than 20 centuries to cure ailments such as goiters, which we now know is due to the high iodine content of brown seaweeds (Chengkui and Junfu 1984). Additionally, the red algae *Chondrus crispus* and *Mastocarpus stellatus* were used to treat colds, sore throats, and chest infections such as tuberculosis (Dias, Urban, and Roessner 2012).

It was found that inhabitants of Monte Verde, the archaeological site in southern Chile, used seaweed for both food and medicine (Dillehay et al. 2008). The seaweeds were combined with other known medicinal terrestrial plants in masticated cuds, suggesting their medicinal application. *Additionally*, two nonedible genera, *Gigartina* and *Sargassum*, were found at the site and are still used medicinally today by local indigenous populations.

Records of the medicinal uses of marine invertebrates including shellfish, cephalopods, and sponges date back to the 5$^{th}$ century BC and include authors such Hippocrates. Classical Greek texts report the pharmaceutical properties of dozens of marine invertebrates, primarily in treatments of the digestive system, genitourinary system, and skin. Marine invertebrates were used as medicines so extensively and expertly that in some cases, specific body parts or organs of the animals were proposed as therapeutic (Voultsiadou Eleni 2010).

Traditional Indian medicine also employs marine animals as a source of treatments. Corals have been used to treat tuberculosis, sponges used to treat dysentery, oil from the flesh of crabs is used to ear infections, and sea turtle shells are used to treat cataracts and other eye disorders (Gopal et al. 2008).

Natural product discovery traditionally involved thorough screening of crude extracts generated from plants, fungi, bacteria, and other organisms. Subsequently, the extracts were fractionated and screened for bioactivity, and finally structures were elucidated. However, the discovery of novel compounds using this classic 'grind and find' method rapidly plateaued as the rediscovery of known natural products became more common.

Scientists have had to become more innovative and exhaustive in their efforts to identify new molecules. As whole genome sequencing became more efficient and cost effective in the 2000s, the field shifted focus to natural product isolation based on *in silico* predictions. This practice of genome mining includes sequencing the genome of a fungus or bacterium then searching for biosynthetic gene clusters. It was quickly discovered that these organisms have a greater number of gene clusters than reported

molecules, suggesting their untapped potential to produce additional pharmaceutically relevant metabolites (Winter, Behnken, and Hertweck 2011).

In addition to their human health applications, natural products presumably have ecological roles in order to make it worth it for the organism to expend energy synthesizing these molecules. In addition to exploiting natural sources for potential antibiotics and other drugs, understanding their ecological functions can facilitate the discovery process. Using bioactivity guided isolation limits discovery to compounds with specific activities (e.g. antibacterial, anticancer), but having a greater understanding of the ecological roles of secondary metabolites by the producing organism can be in credibly valuable information when it comes to determining their activities. While this approach also presents a number of significant challenges, the structural complexity and diversity of natural products suggests that they each have a biologically relevant role for the organism.

### 1.1.2 Marine sources of medicines

The ocean is the largest environment on the planet, covering roughly 70% of the earth. The oceans are home to a great number and diversity of organisms, including microbes. The harsh conditions of living in the ocean may lead to the evolution of distinct defense mechanisms and novel biosynthetic pathways. Some organisms use chemical defenses to avoid predation because they don't have structural protection. It is also possible that because of the dilution of seawater, marine natural products that are bioactive have evolved to be more potent. This means that the marine environment may still be a mostly untapped source of secondary metabolite diversity. So it's worth the extra work to find these compounds.

Natural product drug discovery techniques can be challenging, especially from the marine environment. One reason for this is because access to the samples can be difficult. Before the introduction of SCUBA in the 1970s, researchers were restricted to samples they could collect by wading or snorkeling in shallow intertidal waters. Later, the development of underwater submersibles, both manned and remotely operated has also led to the discovery of novel bioactive marine natural products (Dias, Urban, and Roessner 2012). Additionally, advancements in analytical chemistry have increased instrument sensitivity so only a few micrograms of a sample can be sufficient to identify some novel molecules (Molinski et al. 2009). Despite these advances increasing the access to marine natural product sources, there are still often supply problems. The chemical and biological characterizations are difficult because the compounds are often only isolated in small amounts. The acquisition of sustainable supplies of marine natural products for clinical trials is often a bottleneck in the process in drug development.

Marine bacteria have proven to be a rich source of natural products with potential therapeutic uses. Microbes tend to organize genes into clusters, which make them easier to find and define. Bacteria can be genetically manipulated and grow relatively quickly, making then ideal organisms to manipulate and exploit for drug discovery. Additionally, some groups of microbes have stringent relationships between their taxonomy and secondary metabolite production (Jensen et al. 2007). Furthermore, the relationship between the presence of certain biosynthetic gene clusters and the biogeography of certain strains is crucial to our ability to understand patterns of natural product discovery.

One group of marine bacteria that is of particular interest in terms of natural product discovery is the actinobacteria. Examples of compounds discovered from marine

actinobacteria include the manzamines, a class of compounds isolated from some species of *Micromonospora* (Bull and Stach 2007). These bioactive molecules are synthesized by sponge symbionts and have no known terrestrial equivalents. Salinosporamide A is an important anticancer agent produced by *Salinispora tropica* strains isolated from marine sediments (Feling et al. 2003). Its cytotoxic activity is attributed to the ability of the compound to inhibit all three active proteolytic sites of the 20S proteasome. The natural product is currently in clinical trials for the treatment of multiple myeloma but has also been reported to have potential in combination treatment potentiating the standard therapies for lung, pancreas, colorectal, and other cancers. Although the total synthesis of salinosporamide A was recently worked out, culturing remains the most cost-effective and dependable source of the molecule (Marx and Burton 2018).

Marine-derived drugs have also been isolated from a variety of marine invertebrates . Cytarabine, one of the first FDA-approved drugs from a marine organism, is an anticancer agent originally isolated from the Caribbean sponge *Tethya crypta* (Montaser and Luesch 2011). Other tumor suppressant metabolites include halichondrin B isolated from *Halichondria okadai* and hemiasterlin from *Hemiasterella minor* (Molinski et al. 2009). In 1990, discodermolide was isolated from the rare deep water sponge, *Discodermia dissolute*. The metabolite functions as an immunosuppressant and because of the incredibly limited natural supply, a total synthesis was developed. Unfortunately the drug was discontinued from Phase I trials due to its toxicity and lack of efficacy, but the potential remains for discodermolide to be used in combination drug therapy (Molinski et al. 2009). Vidarabine, an anti-herpes agent, was developed from natural products also isolated from the Caribbean sponge *Tethya crypta* and was more

recently discovered to prevent catecholamine-induced atrial fibrillation or ventricular arrhythmia in mice without negatively affecting normal cardiac function (Montaser and Luesch 2011; Suita et al. 2018).

Extracts from the bryozoan *Bugula neritina* exhibited high activity against leukemia cells in mice, which was later attributed to bryostatin 1 (Montaser and Luesch 2011; Molinski et al. 2009). Fortunately the total synthesis of the natural product was recently achieved; collecting sufficient amounts of the compound for therapeutic use presented a great challenge due to the limited access to the natural source (Wender et al. 2017). Tunicates such as *Trididemnum solidum* and *Ecteinascidia turbinate* also produce cytotoxic molecules including didemnin B and ecteinascidin-743 respectively (Xing et al. 2017; Molinski et al. 2009). The cytotoxic peptide dolastatin 10 was isolated from the sea hare *Dolabella auricularia.* Consistent with the major hurdle facing most marine natural products as therapeutic agents, the yields of the natural product were extremely low requiring 2 tons of animal to obtain the first milligram of pure compound. The total synthesis was achieved in 1989 but clinical trials for the drug were discontinued after a phase II study failed to elicit a treatment response (Molinski et al. 2009; Xing et al. 2017). Prialt® was the first FDA approved marine natural product for the management of severe chronic pain. The drug is the synthetic equivalent of a natural product originally isolated from the cone snail *Conus magus* (Montaser and Luesch 2011; Molinski et al. 2009). The anticancer agent kahalalide F, isolated from the sea slug *Elysia rufescens*, was not actually produced by the animal but by the alga on which it feeds (Molinski et al. 2009). As of 2017, the molecule is in phase II clinical trials for prostate and breast cancers.

Some marine natural products have the same mechanisms of action as terrestrial secondary metabolites. However, they can have the most potent activity of all similarly active molecules. For example, salinosporamide A, is one of the most potent proteasome inhibitors (Montaser and Luesch 2011). Additionally, some marine natural products have unique mechanisms of action. Apratoxin A, originally isolated from a marine cyanobacterium, was the first reported antitumor compound to reversibly inhibit the secretory pathway for several receptors by interfering with co-translational translocation, a distinct mechanism of action (Montaser and Luesch 2011).

To date, over 32,000 distinct marine natural products have been described (http://pubs.rsc.org/marinlit/). Not all of the described molecules are bioactive but a select few are FDA approved or are currently in clinical trials. It is incredibly difficult to bring a new drug to market and while many marine-derived drugs fail at various stages in the clinical trial process, they are still a great source of novel treatment options. Effective collaborations between academic research and pharmaceutical companies will continue to be critical to achieve the success of marine natural product therapies.

### 1.1.3 Halogenated marine natural products

Halogenated natural products are metabolites that contain chlorine, bromine, iodine, and fluorine. Halogenated chemicals are also produced for industrial purposes via chemical synthesis. These anthropogenic compounds include the pesticide DDT and CFCs from refrigerants often end up in the ocean from runoff and pollution. However, there is also substantial evidence that some halogenated compounds structurally identical to the manmade ones found in the marine environment are naturally produced (Reddy et al. 2004; Teuten and Reddy 2007).

One group of organisms that are a particularly rich source of halogenated natural products is marine sponges. Brominated metabolites have been isolated in large quantities from *Dysidea herbacea*, *D. chloren*, *D. dendyi*, and *P. folioscens* samples (Carte and Faulkner 1980; Utkina et al. 2001). These molecules are produced in such great amounts that they can contribute up to 6% of the dry weight of some sponge species (Faulkner, Unson, and Bewley 1994). Halogenated natural products have been isolated from sponge-eating nudibranchs. A complex mixture of polybrominated diphenyl ethers and sesquiterpenes were isolated from *Chromodoris funereal* collected in Iwayama Bay, Palau. The metabolite profile is essentially identical to the *Dysidea* spp. on which it feeds (Carté et al. 1986). This suggests that the nudibranchs not only tolerate the molecules produced by sponges but are also able to sequester the compounds and use them for their own defense.

Another well-studied source of halogenated marine natural products is algae. Extracts from *Asparagopsis taxiformis*, an edible red seaweed favored by native Hawaiians, contain over 100 halogenated metabolites (Gribble 2003). *Bonnemaisonia* spp., do not produce halomethanes such as those found in the closely related *Asparagopsis* spp., but halogenated ketones, alcohols, and carboxylic acids have been isolated from the alga (McConnell and Fenical 1980). Haolgenated terpenes and fucophlorethol derivatives have also been isolated from the brown algae *Laurencia* sp. and *Cystophora retroflexa* respectively (Sailler and Glombitza 1999). It is hypothesized that these molecules function as a chemical defense for the algae, possibly as feeding deterrents or antifouling agents (Gribble 2003).

While it’s possible that sponges and other marine macroorganisms are themselves synthesizing these halogenated natural products, it’s widely hypothesized, and in some cases proven, that microbial symbionts are responsible for compound production. However, in many cases, this hypothesis is not supported by direct experimental evidence (Faulkner, Unson, and Bewley 1994). The biosynthesis of some brominated metabolites in sponges can be attributed to cyanobacterial symbionts. The first evidence of this was when crystals of 2-(2’,4’-dibromophenyl)-4,6- dibromophenol were observed to be in contact with cyanobacteria filaments yet did not consistently occur in the sponge tissue (Faulkner, Unson, and Bewley 1994). More conclusively, the biosynthetic machinery needed to produce PBDEs was discovered in sponge metagenomic data (Agarwal et al. 2017). Biosynthesis carried out by symbiotic bacteria or microalgae living in the internal tissue of the animal is particularly evident in in *Dysidea herbacea* (Gribble 2003). Some members of this group of marine sponges have been found to be morphologically indistinguishable but chemically diverse, suggesting that specific populations of symbionts are in fact responsible for the variety of secondary metabolites found in different sponge samples (Carte and Faulkner 1980; Norton, Croft, and Wells 1981). Additionally, a large number of halogenated molecules have been isolated from *Lyngbya majuscule*, providing supporting evidence that multiple cyanobacterial species are capable of producing these molecules. (Burja et al. 2001; Gribble 2003). Other marine bacteria that have been shown to produce halogenated metabolites, some of which display high levels of antibiotic activity, are in the genus *Pseudoalteromonas* (Ross et al. 2015; Isnansetyo and Kamei 2009).

## 1.2 Bacteria in the ocean

### 1.2.1 Marine microbial ecology

Marine microbes are globally important organisms having complex interactions with the biological and geochemical systems of the ocean. One-half of the primary production on earth occurs in the oceans and a large portion of that primary production becomes dissolved organic matter (DOM), which is almost exclusively utilized by heterotrophic bacteria and archaea (Azam and Malfatti 2007). Historically, marine microbes were generally ignored because they were essentially invisible and early research conducted in the oceans focused on the health and significance of larger scale systems such as coral reefs and kelp forests. However, we now have a much greater understanding of the intimate interactions between bacteria and the whole ocean ecosystem, and can appreciate the importance of their significant roles in the carbon cycle.

Studies have shown that despite their significant and intricate roles, bacterial community composition in the marine environment follows fairly predictable patterns (Fuhrman, Cram, and Needham 2015). While some microorganisms prefer specific environmental conditions, they are remarkably dynamic and resilient. Marine microbes have evolved internal feedback mechanisms to maintain generally steady community structures despite the wide range of external forces they face, such as ocean mixing, temperature changes, and nutrient fluctuations (Fuhrman, Cram, and Needham 2015). As a result, the interactions and relationships between marine microbes and their processes are particularly complex (Duarte, Gasol, and Vaqué 1997). These relationships are also likely dependent on factors such as the presence of other organisms and seasonal

changes. Correlations between environmental conditions and taxa suggest that the majority of microorganisms in the ocean occupy well-defined niches, which define their relationships with one another and with other organisms (Fuhrman, Cram, and Needham 2015).

However, microbial communities in the ocean also can change over various timescales in response to the biological and physical forces of the marine environment. Some examples include the primary productivity cycle, the diel pattern of cyanobacterial abundance, and increased nutrient levels induced by upwelling from storms or run-off from land (Fuhrman, Cram, and Needham 2015). Seasonal variation in microbial communities is observed more frequently in the photic zone of surface waters compared to darker deep waters (Fuhrman, Cram, and Needham 2015). Additionally, many marine bacteria are motile, which may enable them to more readily couple with DOM sources (Azam and Malfatti 2007; Dinsdale et al. 2008). Studying these dynamic microbial systems helps us understand how organisms interact and change in relation to one another and to conditions in the ocean.

In addition to naturally occurring factors in the ocean causing changes in populations of bacteria, large-scale human activities can also influence the marine environment on the microbial level. For example, removal of the top predators in an ecosystem either indirectly or as a result of overfishing, slows the rate of energy turnover of the environment. This has been observed on Kiritimati, where the removal of both predatory and herbivorous fish has led to an increase in macroalgae growth (Dinsdale et al. 2008). The food web structure change resulted in an excess of dissolved organic carbon (DOC), which is almost exclusively available for heterotrophic microbes. The

effects of these environmental factors caused an increase in microbial growth as well as changes in bacterial community composition, which led to an increase in coral diseases and death. This is a clear example of the complex interactions between fish, algae, microorganisms, and coral health.

While it's important to study the top-down and bottom-up interactions of marine microbial populations, it's also imperative that we understand competitive interactions among bacteria. A great deal is still unknown about the competitive controls that function to regulate community composition as well as the driving forces behind the diversity and dynamics of bacterial communities in the ocean (Fuhrman, Cram, and Needham 2015). Positive correlations between certain microorganisms may result from parasitism or similar environmental preferences while negative correlations may suggest competition, allelopathy, or a preference for different environmental conditions (Fuhrman, Cram, and Needham 2015). While we want to understand the collective role of microbial processes in the marine environment, the interactions at the scale of individual microorganisms must also be understood.

Most published studies addressing marine bacteria report results of microbial abundance and growth in the ocean. However, research interests are shifting more towards elucidation of interactions between microbial taxa (Duarte, Gasol, and Vaqué 1997). The vast majority of marine microbial ecology experiments are conducted in the laboratory, but these highly controlled environments often exaggerate responses to treatments and can lead to results that may have no real significance in nature. Our current understanding of the intimate microbial interactions in the ocean is largely based on these small-scale experiments, which are extrapolated to explain larger ecosystem

dynamics. However, designing the necessary field experiments to test key large-scale hypotheses is extremely complicated.

In addition to improved experimental design, advancements in instrumentation and other technologies may offer a greater understanding of microbial population diversity and dynamics (Fuhrman, Cram, and Needham 2015). To sufficiently evaluate ecological interactions, we must go further than simply identifying who is there by investigating the functional capacity of the community using techniques such as metagenomic, transcriptomics, and other related analyses (Fuhrman, Cram, and Needham 2015). While these comparative analyses can provide clues about the biochemical potential of marine bacteria and how they obtain and process nutrients in the ocean, they must be experimentally validated (Azam and Malfatti 2007; Duarte, Gasol, and Vaqué 1997).

**1.2.2 Chemotaxonomy**

The secondary metabolite profiles of bacteria, also known as chemotypes, can play an important role in their taxonomy. Chemotaxonomy refers to the correlation between who the bacteria are and the molecules they make. While metabolite profiles can help assign taxonomic similarity, the two are not always in complete agreement. Nonetheless, chemotyping may be used as a supplementary approach to traditional classification methods for bacteria. Efforts are being made to establish a database of the chemical fingerprints of marine bacteria based on LC-MS data to provide another measurement of their classification (Lu et al. 2014). In some cases, the production of certain secondary metabolites is species-specific and a strain's chemical profile may be considered a diagnostic phenotype for its classification (Jensen et al. 2007). However, the

alternative can also be true. For example, *Streptomyces* strains that are the same species may have distinct secondary metabolite profiles (Jensen 2010). Additionally, the pathogenicity among strains from the same species of *Bacillus* can be determined based on their chemotypes (Bundy et al. 2005). Fatty acid and phospholipid compositions are commonly used to help classify bacteria, with some fatty acids considered genus-specific chemotaxonomic markers (Elena P. Ivanova et al. 2000). The production of certain fatty acids is not entirely sufficient to delineate species of bacteria, however, the results are useful for distinguishing between phenotypically similar genera.

*Salinispora* is a genus of marine bacteria with three names species: *S. tropica*, *S. arenicola*, and *S. pacifica* (Maldonado et al. 2005; Ahmed et al. 2013). The 16S rRNA sequences of *Salinispora* species are incredibly similar, making classification particularly challenging using this marker. However, five distinct chemotypes were reported, 1 from *S. tropica*, 1 from *S. arenicola*, and 3 from *S. pacifica* (Jensen et al. 2007). There was no commonality observed between the three *S. pacifica* metabolite profiles despite the fact that the 16S sequences for some of the strains only differed by a single nucleotide. This is evidence that regardless of how similar *Salinispora* species are based on 16S sequence identity, their secondary metabolite production may be entirely different. In this genus, chemotype is an important phenotype to consider when classifying strains. However, the correlation between the chemotaxonomic markers found in *Salinispora* cannot necessarily be extrapolated and applied to all marine bacteria. Unlike most bacterial taxa, the species in this genus are highly similar based on sequence identity and classification based on chemotype is not always appropriate for groups with greater intraspecific diversity. A bacterium's secondary metabolite profile is not the only characteristic that

should be considered when classifying species, however, it a valuable tool that often reflects phylogenetic relationships.

### 1.3 Biosynthetic gene cluster evolution

The genes that encode secondary metabolites in bacteria are typically found in clusters known as biosynthetic gene clusters (BGCs). These BGCs are incredibly diverse and represent some of the most rapidly evolving genetic elements (Fischbach, Walsh, and Clardy 2009). The diversity of BGCs is also reflected in the structural diversity of the metabolites they produce, which likely have distinct biological functions (Ruzzini and Clardy 2016). Biosynthetic gene clusters are frequently transferred horizontally between bacteria. The lateral transfer of genetic material is well established in microbes and is widely recognized to play a significant role in the classification, pathogenicity, and ecological relationships of bacteria (Ruzzini and Clardy 2016). Some gene clusters are only found in very closely related organism while nearly identical BGCs may be observed in distantly related groups (Fischbach, Walsh, and Clardy 2009). Thus, the taxonomic distribution of gene clusters provides important insight into their evolutionary history.

Gene clusters undergo changes that affect the small molecules whose biosynthesis they encode (Fischbach, Walsh, and Clardy 2009). For example, point mutations in the catalytic residues can inactivate an entire KS domain. However, mutations can result in either the loss or the gain of function, both of which have important evolutionary implications increasing the diversity of secondary metabolite production (Fischbach, Walsh, and Clardy 2009). Interestingly, the biosynthesis of some molecules requires more than one biosynthetic gene cluster. For example, four genetic loci are responsible

the production of sioxanthin by *Salinispora tropica* (Richter, Hughes, and Moore 2015). It is unknown how many secondary metabolites are synthesized by genes that are not contiguously clustered, but it's likely more common that currently represented in the literature.

The complexity of the small molecules produced by bacteria is astounding to chemists and the identification and characterization of the genes responsible is critical to discovering how the compounds are synthesized (Fischbach, Walsh, and Clardy 2009). Secondary metabolites have diverse functions and play important roles in the relationships between bacterial species as well as between microbes and eukaryotic organisms.

## 1.4 *Pseudoalteromonas*: a genus of marine bacteria

### 1.4.1 *Pseudoalteromonas* distribution, and abundance

The first 11 species of *Pseudoalteromonas* were established as part of a new genus over 20 years ago after previously being included in *Alteromonas* (G. Gauthier, Gauthier, and Christen 1995). *Pseudoalteromonas haloplanktis* ATCC 14393 was named the type species of the genus because it was the first to be described and has been extensively studied. *Pseudoalteromonas* are Gram-negative, non-spore-forming, strictly aerobic, obligate marine bacteria. The cells are straight or curved rods ranging from 0.2-1.5 by 1.8-3 μm in size and most species are motile enabled by a single polar flagellum. Over half of *Pseudoalteromonas* species are pigmented but none are luminescent (G. Gauthier, Gauthier, and Christen 1995). Since being defined as a genus, *Pseudoalteromonas* has expanded to include 42 named species (Table 1.1). The species are quite diverse when it comes to both primary and secondary metabolism; however,

many are over 99% similar based on 16S rRNA sequence data (Figure 1.1). Less than half of the species have available genome sequences, making further taxonomic assignments difficult.

*Pseudoalteromonas* strains are ubiquitous in the marine environment and have been isolated from nearly every sample type including seawater, algal surfaces, and marine animals (Skovhus et al. 2007). While found in the water column, they are also frequently associated with eukaryotic hosts and abiotic surfaces. The relative abundance of *Pseudoalteromonas* on different marine surfaces has been determined using real-time quantitative PCR (qPCR) and denaturing gradient gel electrophoresis (PCR-DGGE) used to assess diversity in the genus (Skovhus et al. 2007). *Pseudoalteromonas* strains were identified in all of the samples that were analyzed ranging from 0.7-3.4% of the total abundance, with an average or 1.6% across the 11 samples tested. The six samples with the greatest species diversity including *F. serratus*, *D. sanguinea*, seawater, *M. edulis*, sediment, and rock samples also had the greatest degree of surface fouling (Skovhus et al. 2007). The heterogeneity of *Pseudoalteromonas* on some marine surfaces may be due to physical, chemical, and nutrient conditions on the macro and micro-scale. It is hypothesized that the samples with low *Pseudoalteromonas* diversity contain species with antifouling capabilities, which a microbial community shift and results in an overall lower level of fouling.

**Table 1.1** *Pseudoalteromonas* species

| Species | Reference |
|---|---|
| *P. agarivorans* | (Romanenko 2003a) |
| *P. aliena* | (Elena P Ivanova et al. 2004) |
| *P. antarctica* | (Bozal et al. 1997) |
| *P. arabiensis* | (Matsuyama et al. 2013) |
| *P. arctica* | (Al Khudary et al. 2008) |
| *P. atlantica* | (Akagawa-Matsushita et al. 1992) |
| *P. aurantia* | (M. J. Gauthier and Breittmayer 1979) |
| *P. byunsanensis* | (Park et al. 2005) |
| *P. carrageenovora* | (Akagawa-Matsushita et al. 1992) |
| *P. citrea* | (M. J. Gauthier 2018) |
| *P. denitrificans* | (Enger 1987) |
| *P. distincta* | (E P Ivanova et al. 2000) |
| *P. donghaensis* | (Oh et al. 2011) |
| *P. elyakovii* | (Sawabe et al. 2000) |
| *P. espejiana* | (Chan et al. 1978) |
| *P. flavipulchra* | (Elena P Ivanova, Shevchenko, et al. 2002) |
| *P. fuliginea* | (Machado et al. 2017) |
| *P. haloplanktis* | (G. Gauthier, Gauthier, and Christen 1995) |
| *P. issachenkonii* | (Elena P. Ivanova et al. 2002) |
| *P. lipolytica* | (Xu et al. 2010) |
| *P. luteoviolacea* | (M. J. Gauthier 1982) |
| *P. maricaloris* | (Elena P Ivanova, Shevchenko, et al. 2002) |
| *P. marina* | (Nam et al. 2007) |
| *P. mariniglutinosa* | (Romanenko 2003b) |
| *P. nigrifaciens* | (G. Gauthier, Gauthier, and Christen 1995) |
| *P. paragorgicola* | (Elena P Ivanova, Sawabe, Lysenko, Gorshkova, Hayashi, et al. 2002) |
| *P. peptidolytica* | (Venkateswaran and Dohmoto 2000) |
| *P. phenolica* | (Isnansetyo and Kamei 2003b) |
| *P. piratica* | (Beurmann et al. 2017) |
| *P. piscicida* | (Bein 1954) |
| *P. prydzensis* | (J. P. Bowman 1998) |
| *P. rubra* | (M. J. Gauthier et al. 1976) |
| *P. ruthenica* | (Elena P Ivanova, Sawabe, Lysenko, Gorshkova, Svetashev, et al. 2002) |
| *P. shioyasakiensis* | (Matsuyama et al. 2014) |
| *P. spongiae* | (Lau et al. 2005) |
| *P. tetradonis* | (Simidu et al. 1990) |
| *P. translucida* | (Elena P Ivanova, Sawabe, Lysenko, Gorshkova, Hayashi, et al. 2002) |
| *P. tunicata* | (Holmstromfl et al. 1998) |
| *P. ulvae* | (Egan, Holmström, and Kjelleberg 2001) |
| *P. undina* | (Chan et al. 1978) |
| *P. xiamenensis* | (Zhao et al. 2014) |
| *P. xishaensis* | (Luo et al. 2013) |

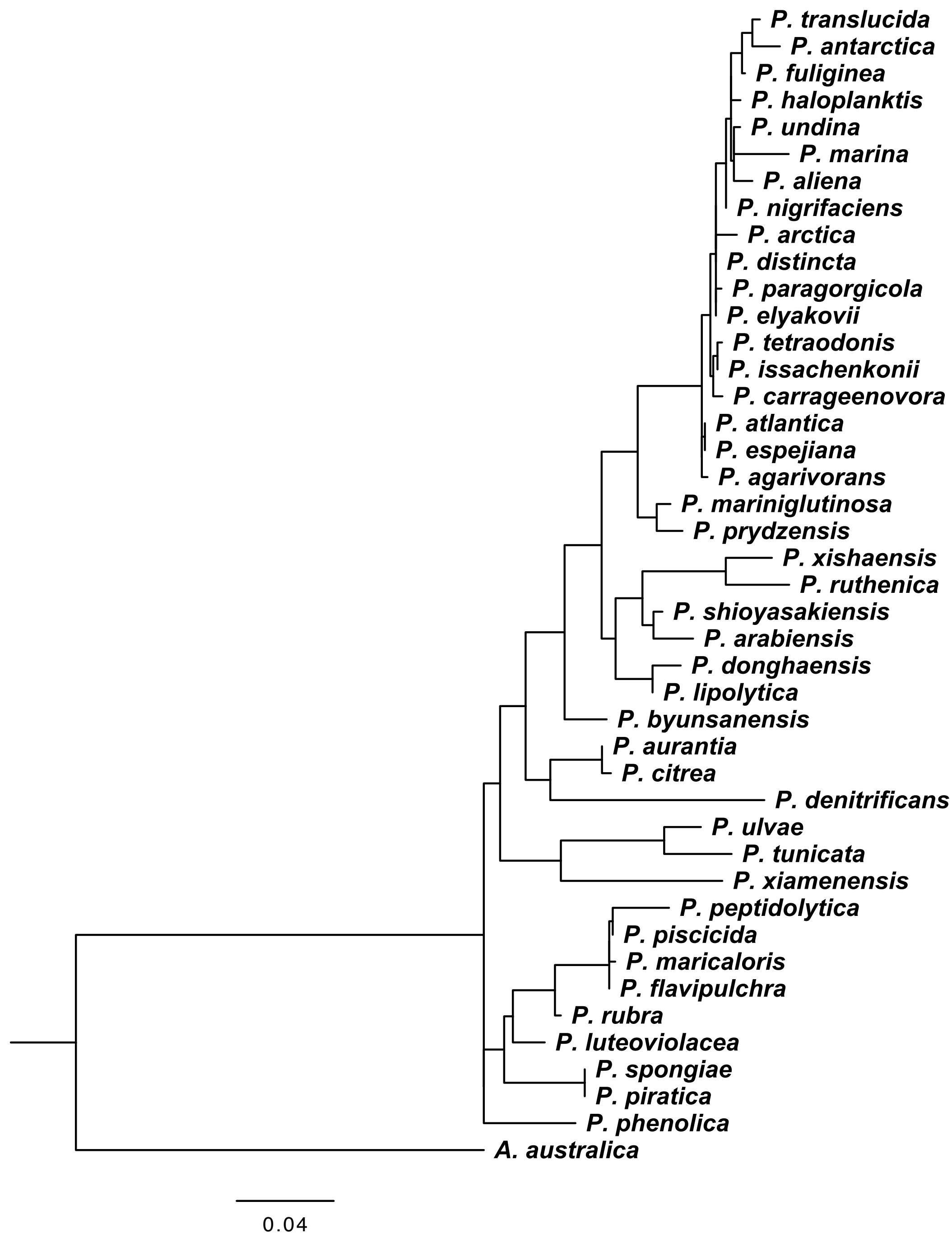


**Figure 1.1** The maximum likelihood phylogeny of type strains for the 42 *Pseudoalteromonas* named species. 16S rRNA sequences were aligned with MUSCLE and analyzed with RAxML.

### 1.4.2 Bioactive metabolites produced by *Pseudoalteromonas* strains

Many *Pseudoalteromonas* strains have been reported to produce bioactive molecules. Members of this genus are found in nearly every type of marine environment, often living on surfaces and forming biofilms (Dang and Lovell 2016; Skovhus et al. 2007). A variety of toxic metabolites from a number of *Pseudoalteromonas* species have been reported for their antifungal activity, antibiotic properties, and causing diseases in algae to name a few (Skovhus et al. 2007). Interestingly, there appears to be a correlation between pigmentation and the production of bioactive compounds in *Pseudoalteromonas* and, in the case of violacien, the pigment itself has antimicrobial activity (J. Bowman and P. 2007; Thøgersen et al. 2016).

*Pseudoalteromonas tetradonis* gets its name from the production of tetrodotoxin, a potent neurotoxin (J. Bowman and P. 2007). Another species, *P. tunicata*, was reported to produce a bioactive tambjamine compound, which had been previously isolated from marine invertebrates but never before from a bacterium (Burke et al. 2007). The BGC encoding the biosynthesis of this antifungal metabolite was also identified. Only a handful of other gene clusters observed in *Pseudoalteromonas* spp. have been assigned specific compounds including the previously mentioned violacien pigment, indolmycin, and alterochromide (Thøgersen et al. 2016; Ross et al. 2015). *Pseudoalteromonas* strains are also well known brominated metabolite producers, but it wasn't until recently that the *bmp* gene cluster responsible for some of these compounds was identified and characterized (Agarwal et al. 2014; M. J. Gauthier and Flatau 1976). The brominated compound MC21-B produced by the *P. phenolica* type strain O-BC30 was reported to have anti-MRSA activity. Additionally, the $IC_{50}$ value of pentabromopseudilin for MRSA

was determined to be 0.1 μM (±0.18) and bioactivity of the compound has also reported against the marine bacteria *Photobacterium phosphoreum* and *Chromobacter* sp. (Fehér et al. 2010; Andersen, Wolfe, and Faulkner 1974).

Because *Pseudoalteromonas* strains commonly reside in biofilms and are therefore living in very close proximity to other bacteria, their ability to produce bioactive compounds may be critical to their survival. Studies have shown that metabolites produced by *P. tunicata* are bioactive at ecologically relevant concentrations on the surfaces of marine organisms (Skovhus et al. 2007). These results suggest that *Pseudoalteromonas* species have a rich biosynthetic potential to produce bioactive molecules that are ecologically relevant but may also be useful therapeutic agents.

### 1.5 Overview of dissertation

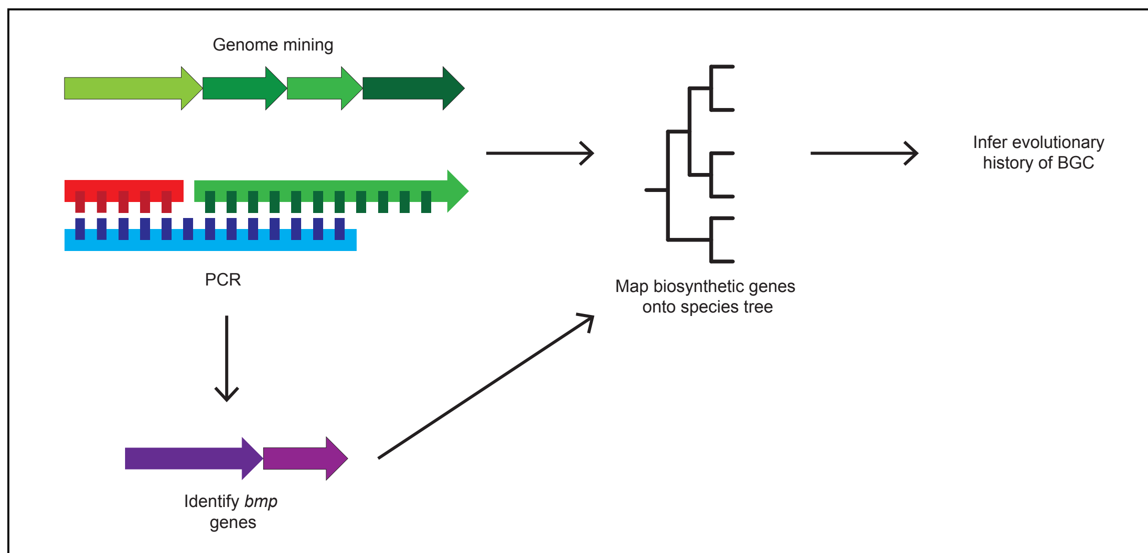


**Figure 1.2** Chapter 2 overview. A well-supported *Pseudoalteromonas* species phylogeny was constructed using MLSA data. The *bmp* gene cluster was mined from genomes or amplified using PCR and the distribution of the different versions of the BGC were mapped onto the species tree.

Chapter 2 of this dissertation includes a comprehensive *Pseudoalteromonas* species phylogeny. The data for this study was generated from publically available *Pseudoalteromonas* genomes (75), genomes sequences at SIO (3), sequence data from 10

genomes shared by Lone Gram, as well as 17 strains isolated as part of the study. Using ANI analysis for the 91 strains with genome sequences, we attempted to define the number of different species in the genus. Because only 16 of the 42 type strains have available genome sequences, the results were compared with those of the species phylogeny in an attempt to classify as many strains as possible. Based on the combined analyses, 17 candidate novel *Pseudoalteromonas* species were proposed.

Once a well-supported species phylogeny was generated, the next aim of this study was to explore a specific BGC of interest in *Pseudoalteromonas*. The *bmp* biosynthetic gene cluster was previously identified and experimentally characterized, but its genus-level distribution and diversity had not been explored. Four distinct versions of the *bmp* cluster were identified across multiple *Pseudoalteromonas* species. The production of pentabromopseudilin was evaluated for available strains and the literature was searched for previously published chemical data reporting the compound in bacterial extracts. The distribution and diversity of the *bmp* gene cluster as well as PBP production was mapped onto the species phylogeny to better understand the evolution of the BGC in *Pseudoalteromonas.* Finally, the genomic environment of the *bmp* cluster was investigated and found in three different locations. Two alternative hypotheses were made based on these results: 1) the BGC was acquired once by a common ancestor and inherited vertically, changing location within the genome or 2) there were three separate acquisition events of the BGC in *Pseudoalteromonas*. Without including additional genome sequences for strains with the BGC, it is difficult to tell which is more likely.

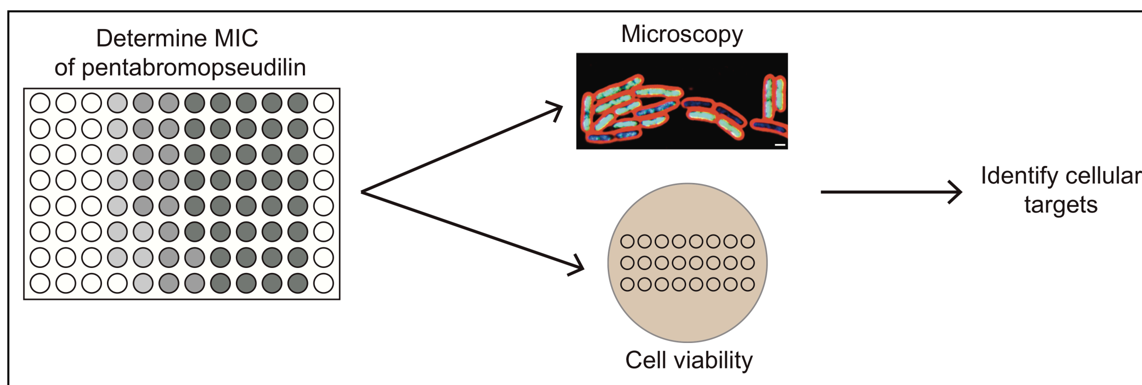


**Figure 1.3** Chapter 3 workflow. The overview of the workflow undertaken in Chapter 3, beginning with the determination of the MIC value of pentabromopseudilin. Fluorescence microscopy was then employed in parallel with cell viability assays to determine the effects of the compound on cells over time across increasing concentrations. Using the generated data, potential cellular targets of the bioactive molecule were identified.

In chapter 3, bacterial cytological profiling (BCP) to identify cellular targets and the mechanism of action (MOA) of pentabromopseudilin. This experimental technique can identify an antibiotic's MOA and distinguish between molecules with distinct cellular targets (Nonejuie et al. 2013). The results showed that SYTOX Green intensity increased with time and concentration of PBP in *E. coli* ATCC 25922 cells, indicating the cells were permeable to the dye. Based on the BCP results suggesting its ability to permeabilize membranes, PBP was also tested for potentiation activity. Because Gram-negative bacteria have a highly impermeable outer cell membrane, they are resistant to most antibiotics. Thus, many therapeutic treatments include a potentiator molecule, which is able to disrupt the outer membrane and allow the antibiotic to do its job. Pentabromopseudilin was found to function as a potentiator molecule, increasing the potency of vancomycin and erthryomycin against *E. coli* ATCC 25922. Unfortunately, its cytotoxicity does not make it a good applicant for therapeutic use. However, from an ecological standpoint, it is hypothesized that PBP may play an ecological role by making other antimicrobial compounds produced by *Pseudoalteromonas* strains more potent.

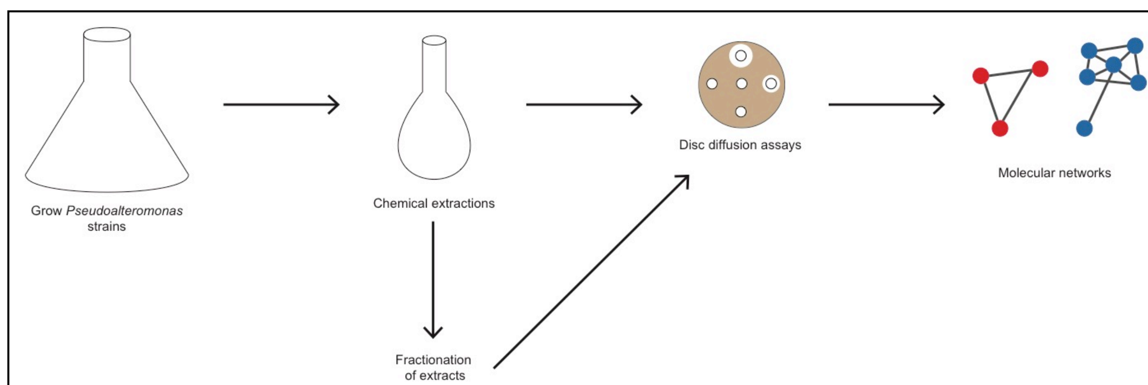


**Figure 1.4** Chapter 4 workflow. The overview of the workflow undertaken in Chapter 4, including growth of *Pseudoalteromonas* cultures, which were extracted and fractionated, then tested for bioactivity. The genomes of the strains used in this study were analyzed with antiSMASH to identify their BGCs and molecular networking was performed with the crude extracts and active fractions to identify potential novel structures.

Chapter 4 assesses the bioactivity and chemical analysis of extracts from five *Pseudoalteromonas* species. Crude extracts and fractions from the strains used in this study were tested for activity using disc diffusion assays with test strains *E. coli* ATCC 25922 and *B. subtilis* PY79 and analyzed with LC-MS. Molecular networks of the *Pseudoalteromonas* crude extracts and fractions were generated from the MS/MS data and screened for dependable library hits. For example, a group of spectra were matched with bromoaterochromide, sharing 19 peaks and having a cosine value of 0.92 when compared with the library spectrum. The bioactivity results were mapped onto molecular networks to identify known molecules and identify potential novel metabolites of interest. Several molecular families were found to be exclusive to specific strains, especially those with the highest bioactivity: 2ta16 and PS5.

The genomes for the strains were also run through antiSMASH v3.0 to identify all BGCs (Weber et al. 2015). A total of 147 biosynthetic gene clusters were identified, only a handful of which have known products. The genome mining data, chemical analyses, and bioactivity results combined suggest that *Pseudoalteromonas* species have great

potential to synthesize novel metabolites, some of which may be antibiotics. *Pseudoalteromonas* strains are ubiquitous in the ocean and are frequently found in high abundance in biofilms on marine surfaces. Because of limited space, strains producing antimicrobial molecules such as pentabromopseudilin (PBP) may have a selective advantage by preventing competing strains from settling on the same surfaces. The results of this chapter revealed the vast biosynthetic potential of five *Pseudoalteromonas* species and their capacity to produce novel bioactive metabolites.

# Chapter 2: Diversity and distribution of the *bmp* gene cluster and pentabromopseudilin production in *Pseudoalteromonas*

## 2.1 Abstract

The production of pentabromopseudilin and related brominated compounds by *Pseudoalteromonas* spp. has recently been linked to the *bmp* biosynthetic gene cluster. This study explored the distribution and evolution of this gene cluster in the genus *Pseudoalteromonas*. A phylogeny of the genus revealed numerous clades that do not contain type strains, suggesting some species level diversity has yet to be described. Comparative genomics revealed four distinct versions of *bmp*, which were distributed among 19 of the 101 genomes examined. These were largely localized to the least inclusive clades containing the *P. luteoviolacea* and *P. phenolica* type strains. There was clear evidence of gene and gene cluster loss within the *P. luteoviolacea* lineage, which included 17 candidate new species. The *bmp* gene phylogeny is highly congruent with the species tree, which is consistent with vertical inheritance within the genus. However, the gene cluster is found in three different genomic environments suggesting separate acquisition events or chromosomal rearrangement. While there is clear evidence of gene and gene cluster loss within certain lineages, the conservation of *bmp* within certain lineages suggests the products encoded are highly relevant to the ecology of these bacteria.

## 2.2 Introduction

The structure of the bromine rich antibiotic pentabromopseudilin was the first marine microbial natural product described (Lovell, 1966). It was isolated from an obligate marine bacterium identified at the time as *Pseudomonas* sp. (Burkholder et al., 1966). Subsequently, pentabromopseudilin and biosynthetically related brominated natural products (BNPs) have been reported from a single strain of *Marinomonas*

*mediterranea* (MMB-1) and multiple *Pseudoalteromonas* spp. (Agarwal et al. 2014; Vynne et al. 2011; Fehér et al. 2010; Vynne, Mansson, and Gram 2012; Whalen et al. 2015; Isnansetyo and Kamei 2003a; El Gamal, Agarwal, Rahman, et al. 2016; El Gamal, Agarwal, Diethelm, et al. 2016). In addition to pentabromopseudilin, brominated phenol and pyrrole monomers and dimers as well as polybrominated diphenyl ethers (PBDEs) have been reported in *Pseudoalteromonas* extracts. One brominated pyrrole monomer, tetrabromopyrrole, has been studied in depth and functions as a coral larvae settlement cue (El Gamal, Agarwal, Diethelm, et al. 2016). PBDEs are of particular interest because of their negative implications for human health such as disruption of the endocrine system, neurodevelopmental deficits, and cancer (Richardson et al. 2008; Hallgren et al. 2001; Zhou et al. 2002; Fernie et al. 2005). Starting in the late 1920s, polybrominated diphenyl ethers (PBDEs) were produced industrially and used as flame-retardants in a wide variety of products such as electronics and textiles (Johnson-Restrepo and Kannan 2009). While anthropogenic PBDEs can be introduced into the ocean via wastewater runoff, there is increasing evidence that these compounds are also produced naturally in the marine environment (Teuten and Reddy 2007; Reddy et al. 2004). These lipophilic molecules bioaccumulate in the blubber and tissues of marine mammals, thus suggesting that a human diet rich in seafood could lead to increased PBDE exposure (Teuten, Xu, and Reddy 2005; Malmvärn et al. 2005; Venkateswaran and Dohmoto 2000).

The recent identification of the *bmp* gene cluster as the biosynthetic origin of these compounds in MMB-1 and select *Pseudoalteromonas* strains (Agarwal et al. 2014; El Gamal, Agarwal, Diethelm, et al. 2016) provides a unique opportunity to more broadly assess the biosynthesis of these BNPs among marine bacteria. The *bmp* cluster encodes

the production of polybrominated phenol and pyrrole monomers (**2-4**), the phenolic homodimeric antibiotic bromophene (**5**), polybrominated diphenyl ethers (PBDES) (**6**), polybrominated biphenyls (**7**), and the brominated phenol/pyrrole heterodimer pentabromopseudilin (**1**) (Figure 2.1). The gene cluster encodes an ACP-TE di-domain protein (Bmp1) and two flavin-dependent brominases (Bmp2 and Bmp5) that catalyze the bromination of pyrrole and phenol monomers respectively (**2-4**). Bmp3, 4 and 8 play roles in bromopyrrole monomer synthesis and Bmp6 is involved in the biosynthesis of bromophene and PBDEs (**6-7**). Bmp7 catalyzes the oxidative coupling of bromophenol and bromopyrrole monomers in the presence of Bmp9, Bmp10, and NADH.

Genes similar to those found in the *bmp* cluster have been characterized in other bacterial strains. Bromopyrrole biosynthesis is achieved in a similar manner to that of pyoluteorin, an antifungal produced by a *Pseudomonas fluorescens* strain (Dorrestein et al. 2005). Additionally, the biosynthesis of pentachloropseudilin, which is incredibly similar to pentabromopseudilin with the only structural difference being the replacement of bromines with chlorines, also involves an $FADH_2$-dependent halogenase similar in function to Bmp5 (Van Pée 2001).

Previous studies of BNP production in *Pseudoalteromonas* spp. have focused on a few specific strains (Isnansetyo and Kamei 2003; Fehér et al. 2010). As a result, we lack a broader understanding of the bacterial taxa that either produce or have the potential to produce these compounds. Our results show that, with the exception of MMB-1, the *bmp* cluster and pentabromopseudilin production are restricted to marine bacteria in the genus *Pseudoalteromonas.* To better assess these traits within the genus, we generated a multilocus phylogeny of 144 *Pseudoalteromonas* strains using five genes: 16S, *gyrB*,

*pyrH*, *recA*, and *rpoD*. Mapping the distribution of the *bmp* genes onto this phylogeny suggests a complex evolutionary history and clues as to how this biosynthetic capacity evolved within the genus.

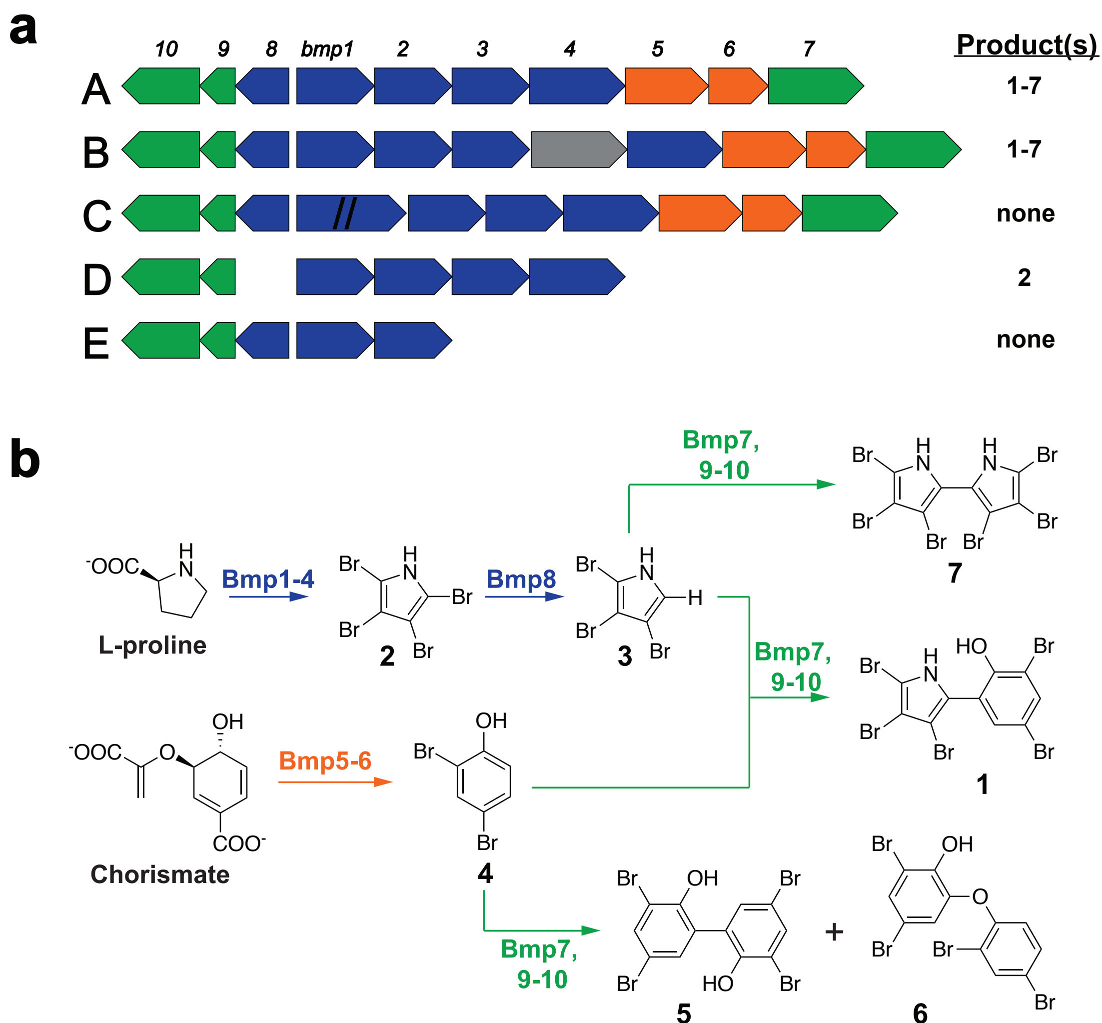


**Figure 2.1** Brominated natural product biosynthesis in marine bacteria. Five versions of the *bmp* gene cluster (a) and the brominated natural products whose biosynthesis they encode (b). Versions A and C-E are observed in various *Pseudoalteromonas* strains while version B is found in *Marinomonas mediterranea* MMB-1. Version C has an ~150 bp insertion in *bmp1* (indicated by hash marks), version D lacks *bmp5-7* and *bmp8,* and version E lacks *bmp3-7*.

## 2.3 Materials and Methods

### 2.3.1 Bacterial isolation

Seawater samples were collected off Scripps Institution of Oceanography (SIO), plankton tows (20 µm mesh size) were performed from the SIO pier, and algal samples were collected by SCUBA from Catalina Island, CA and the Coral Coast, Fiji. The algae were gently shaken to remove excess water before a small piece of tissue was added to 10 ml of 0.2 µm-filtered seawater. The algal samples were mixed and diluted 1:10, 1:50, and 1:100 in sterile seawater, and 100 µl of each dilution spread onto Difco 2216 media with 15 g/L agar added. Seawater and plankton tow samples were diluted 1:2, 1:10, and 1:50 and similarly plated. Bacterial colonies were isolated based on morphology, specifically selecting for dark purple and other pigmentation that is characteristic of *Pseudoalteromonas* spp. Strains were identified at the genus level based on 16S rRNA sequence analysis (see below) and glycerol stocks frozen (-80°C) in Difco 2216 broth with 20% glycerol.

### 2.3.2 Genome mining, primer design and PCR

Published genome sequences are available for 16 of 42 *Pseudoalteromonas* type strains while *gyrB* and 16S sequences are available for an additional 12 (Table 2.1). Our analysis also included an additional 75 published *Pseudoalteromonas* genomes (Table 2.2), 10 unpublished genomes, and 17 strains isolated as part of this study. Genome sequences were acquired from the NCBI and JGI public databases or sequenced at SIO. They were uploaded and annotated in RAST (Aziz et al. 2008) and mined for 16S, *gyrB*, *pyrH*, *recA*, and *rpoD* based on annotations. *Bmp1-10* gene sequences were identified using the internal BLAST search tool in RAST with gene sequences from *P.*

*luteoviolacea* strain 2ta16 as queries. Ten unpublished *Pseudoalteromonas* genomes were similarly mined. PCR was used to generate sequences for strains isolated as part of this study but for which genome sequences were not available. Degenerate PCR primers were designed based on the translated and MUSCLE aligned *gyrB*, *pyrH*, *recA*, *rpoD*, and *bmp* sequences (Edgar, Drive, and Valley 2004). The primers were validated across different *Pseudoalteromonas* spp. using Primaclad software and manual analyses (Table 2.3) (Gadberry et al. 2005).

**Table 2.1** Type strains without published genome sequences.

| Strain ID | Species | 16S accession number | *gyrB* accession number |
|---|---|---|---|
| DSM 14585 | *agarivorans* | AJ417594 | KF793928 |
| LMG 22059T | *aliena* | AY387858 | FR668563 |
| LMG 18002T | *antarctica* | X98336 | FR668564 |
| NCIMB 14688 | *arabiensis* | AB576636 | N/A |
| ATCC 19262 | *atlantica* | X82134 | AB100885 |
| ATCC 33046 | *aurantia* | X82135 | AF007275 |
| KCTC 12274 | *byunsanensis* | DQ011289 | N/A |
| DSM 6820T | *carrageenovora* | X82136 | FR668560 |
| ATCC 43337 | *denitrificans* | X82138 | N/A |
| KCTC 22219 | *donghaensis* | FJ754319 | N/A |
| LMG 2866T | *espejiana* | X82143 | FR668566 |
| LMG 19697T | *issachenkonii* | AF316144 | FR668568 |
| JCM 15903 | *lipolytica* | FJ404721 | N/A |
| LMG 19692 | *maricaloris* | AF144036 | N/A |
| NCIMB 1770 | *mariniglutinosa* | AJ507251 | N/A |
| LMG 2227T | *nigrifaciens* | X82146 | FR668569 |
| LMG 19696T | *paragorgicola* | AY040229 | FR668570 |
| DSM 14001T | *peptidolytica* | AF007286 | FR668573 |
| CIP 105820 | *prydzensis* | U85855 | N/A |
| LMG 19699 | *ruthenica* | AF316891 | N/A |
| SE3 | *shioyasakiensis* | AB720724 | N/A |
| ATCC 51193 | *tetradonis* | AF214730 | N/A |
| LMG 19694T | *translucida* | AY040230 | FR668571 |
| NCIMB 13762 | *ulvae* | AF172987 | N/A |
| Y2T | *xiamenensis* | JN188399 | N/A |
| E418 | *xishaensis* | JQ237129 | N/A |

**Table 2.2** *Pseudoalteromonas* genomes. The 91 genome sequences used in this study. Type strains indicated in bold. Roman numerals correspond to candidate ANI species designations assigned based on coherence between the species tree and ANI dendogram. Asterisks (*) indicate possible classifications for ANI species in cases where type strain genome sequences are unavailable.

| Strain ID | Species | GenBank accession number |
|---|---|---|
| 10-33 | *P. marina* | LOFI01000000 |
| 13-15 | *P.* sp. nov (VIII) | FSRF01000000 |
| 23 GOM-1509m | *P. issachenkonii** (X) | JADO01000000 |
| 2ta16 | P. sp. nov (XXV) | AUSV00000000 |
| 2ta6 | *P. elyakovii/piscicida* | JGI Taxon ID: 2505119014 |
| 520P1 No. 412 | *P. denitrificans** (I) | BBIN01000000 |
| 520P1 No. 423 | *P. denitrificans** (I) | BBZB01000000 |
| 6BO GOM-1096m | *P. issachenkonii** (X) | JIAM01000000 |
| A 37-1-2 | ***P. arctica*** | AHBY02000000 |
| A2 | *P. issachenkonii** (X) | JPMC01000000 |
| A757 | P. sp. nov (III) | QNQN00000000 |
| AC163 | *P. distincta/flavipulchra* | AUTK01000000 |
| ANT/505 | *P. distincta/flavipulchra* | ADOP01000000 |
| ATCC 14393 | ***P. haloplanktis*** | AHCA01000000 |
| ATCC 29581 | *P. xiamenensis** (XXVIII) | CAPN01000000 |
| ATCC 700518 | ***P. distincta*** | JWIG01000000 |
| ATCC 700519 | ***P. elyakovii*** | JWIH01000000 |
| BSi20311 | P. sp. nov (IX) | BADU01000000 |
| BSi20429 | *P. arctica* | BADV01000000 |
| BSi20439 | P. sp. nov (IX) | BADW01000000 |
| BSi20480 | *P. marina* | BADX01000000 |
| BSi20495 | *P. fuliginea* | BADY01000000 |
| BSi20652 | P. sp. nov (XI) | BADT01000000 |
| BSw20308 | *P. fuliginea* | AMYA01000000 |
| CGMCC 1.8499 | *P. donghaensis/lipolytica** (XVIII) | FPAZ01000000 |
| CP76 | *P. ruthenica** (V) | AOPM00000000 |
| CPMOR-1 | P. sp. nov (XXII) | AUYC00000000 |
| CPMOR-2 | *P. luteoviolacea* | AUYA01000000 |
| D2 | ***P. tunicata*** | AAOH01000000 |
| DSM 17587 | ***P. marina*** | AHCB02000000 |
| DSM 26666 | *P. issachenkonii** (X) | FPAR01000000 |
| DSM 6059 | P. sp. nov (II) | FOLO01000000 |
| DSM 6061 | ***P. luteoviolacea*** | AUYB01000000 |

**Table 2.2** *Pseudoalteromonas* genomes (continued).

| Strain ID | Species | GenBank accession number |
|---|---|---|
| DSM 6065 | ***P. undina*** | AHCF02000000 |
| DSM 6842 | ***P. rubra*** | AHCD02000000 |
| DSM 8771 | ***P. citrea*** | AHBZ02000000 |
| ECSMB14103 | *P. marina* | JWGY01000000 |
| H103 | *P. carrageenovora** (VII) | LOFG01000000 |
| H105 | P. sp. nov (XV) | LOFH01000000 |
| H33 | P. sp. nov (XXIV) | AUXZ01000000 |
| H33-S | P. sp. nov (XXIV) | AUXY01000000 |
| HI1 | P. sp. nov (XXII) | JWIC01000000 |
| JCM 20779 | ***P. piscicida*** | AHCC02000000 |
| JG1 | *P. elyakovii/piscicida* | AJMP01000000 |
| KMM 216 | ***P. fuliginea*** | JJNZ01000000 |
| NCIMB 2033 | ***P. flavipulchra*** | JTDZ01000000 |
| NCIMB 1942 | P. sp. nov (XXVII) | AUXT01000000 |
| NCIMB 1944 | P. sp. nov (XXV) | AUXS01000000 |
| NCIMB 2035 | P. sp. nov (XXVII) | JPWZ01000000 |
| ND6B | *P. issachenkonii** (X) | JQFL01000000 |
| NJ631 | *P. elyakovii/piscicida* | AKXJ01000000 |
| NW 4327 | *P. atlantica** (VI) | AZIO01000000 |
| O-BC30 | ***P. phenolica*** | RCWG00000000 |
| OCN003 | ***P. piratica*** | CP009888, CP009889 |
| OCN096 | P. sp. nov (XXI) | LFZX01000000 |
| PAMC 22718 | *P. issachenkonii** (X) | AJTK01000000 |
| PS5 | *P. peptidolytica** (IV) | RCSQ00000000 |
| R3 | P. sp. nov (XX) | LJDF01000000 |
| S2040 | *P. elyakovii/piscicida* | JXXW01000000 |
| S2292 | *P. fuliginea* | JXYD01000000 |
| S2471 | P. sp. nov (XX) | JXYA01000000 |
| S2607 | P. sp. nov (XXVI) | AUXV01000000 |
| S2724 | *P. elyakovii/piscicida* | JXXX01000000 |
| S3137 | *P. ruthenica** (V) | JXXZ01000000 |
| S3258 | *P. ruthenica** (V) | JXXY01000000 |
| S3431 | *P. fuliginea* | JJNY01000000 |
| S4047-1 | P. sp. nov (XXIII) | AUXU01000000 |
| S4054 | P. sp. nov (XXIII) | AUXW01000000 |

**Table 2.2** *Pseudoalteromonas* genomes (continued).

| Strain ID | Species | GenBank accession number |
|---|---|---|
| S4060-1 | P. sp. nov (XXVI) | AUXX01000000 |
| S8-38 | *P. arctica* | AUTS01000000 |
| S8-8 | *P. arctica* | AUTR01000000 |
| S816 | *P. atlantica** (VI) | APME01000000 |
| SCSIO 04301 | *P. donghaensis/lipolytica** (XVIII) | JGI Taxon ID: 2568526619 |
| SCSIO 11900 | *P. issachenkonii** (X) | JEMJ01000000 |
| SM9913 | *P. issachenkonii** (X) | CP001796, CP001797 |
| TAB23 | *P. arctica* | AUTP01000000 |
| TAC125 | *P. nigrifaciens** (XIII) | CR954246, CR954247 |
| TAE56 | *P. arctica* | AUTN01000000 |
| TAE79 | *P. distincta/flavipulchra* | AUTL01000000 |
| TAE80 | *P. distincta/flavipulchra* | AUTM01000000 |
| TB13 | *P. arctica* | AUTJ01000000 |
| TB25 | *P. distincta/flavipulchra* | AUTI01000000 |
| TB41 | *P. issachenkonii** (X) | AUTH01000000 |
| TB51 | *P. atlantica** (VI) | AUTO01000000 |
| TB64 | P. sp. nov (XII) | AUTQ01000000 |
| UCD-SED14 | P. sp. nov (XIV) | LHPH01000000 |
| UCD-SED8 | *P. undina* | LITK01000000 |
| UCD-SED9 | P. sp. nov (XIV) | LITL01000000 |
| UCD-33C | *P. arabiensis/shioyasakiensis** (XVII) | LJTB01000000 |
| UCD-48B | *P. arabiensis/shioyasakiensis** (XVI) | LJTC01000000 |
| UST010723-006 | ***P. spongiae*** | AHCE02000000 |

**Table 2.3** PCR primer sequences.

| Gene | Forward primer sequence | Reverse primer sequence | Tm (°C) |
|---|---|---|---|
| 16S | 5' GGCAGCAGTGGGGAATAT 3' | 5' GGTTTATCACCGGCAGTCTC 3' | 55 |
| *gyrB* | 5' THGGBGAYACNGAYGATG 3' | 5' GGRTCCATNGTBGTYTCCC 3' | 53 |
| *pyrH* | 5' GTAGARYTMGACRTAGAAGTRGGT 3' | 5' GTDCCYTCNTCTTCVCCCATR 3' | 52 |
| *recA* | 5' GGHCCWGARTCDTCRGGTAA 3' | 5' GGHGGNGCHACYTTGTTYT 3' | 52 |
| *rpoD* | 5' CVCCDGATGCYGATGARYT 3' | 5' GCYTTNGCTTCDATYTGA 3' | 55 |
| *bmp2* | 5' GAYGTHGTYATTATTGGYAGYGG 3' | 5' CATYTGYTTRCGRTCGTAYTCTT 3' | 52 |
| *bmp5* | 5' GCAGTSATHGGKGCTGGTTTATC 3' | 5' GGCCARGARCTDATRTTKGG 3' | 52 |
| *1942bmp1* | 5' AGGCGCCGGGTTTAAGCAGC 3' | 5' TCGCGGGTAACCACGACCAA 3' | 55 |
| *2035bmp1* | 5' AGGCGCCGGGTTTAAGCAGC 3' | 5' TCGTGGTTACCCGCGACAAACA 3' | 55 |

For genomic DNA extractions, strains were cultured overnight at room temperature in 10 ml of marine broth (Difco 2216) with shaking at 230 rpm. Cells were pelleted (16,000 x g, 2 min) from 1 ml of culture, re-suspended in 600 µl of Nuclei Lysis Solution (Promega), and incubated at 80°C for 5 min after which 200 µl of Protein Precipitate Solution (Promega) was added. The samples were vortexed at high speed for 20 sec, incubated on ice for 5 min, centrifuged (16,000 x g, 10 min), and the supernatant transferred into 600 µl of isopropanol. The samples were mixed by gentle inversion, centrifuged (13,000 rpm, 10 min), and the DNA pellet washed once with 70% ethanol, dried, and rehydrated in water. PCR thermocycling conditions were as follows: 5 min of initial denaturation at 95°C, 30 cycles of denaturation at 95°C for 1 min, annealing for 1 min at various temperatures depending upon primers, extension at 72°C for 1 min, and a

final extension at 72°C for 5 min. PCR products were sequenced using Sanger methods by Eton Biosciences (https://www.etonbio.com/) and trimmed for quality before analysis.

### 2.3.3 Phylogenetic analyses

Nucleotide sequences used to establish the species phylogeny were aligned using MAFFT (Katoh et al. 2002) then concatenated. Maximum likelihood analysis of the partitioned data was run using RAxML with 100 rapid bootstrap replicates and the GTR+G model (Stamatakis 2014). To assess the phylogeny of the *bmp* cluster, the MUSCLE alignments of the *bmp1*, *2, 9* and *10* gene nucleotide sequences were concatenated and a maximum likelihood analysis performed using RAxML with 100 rapid bootstrap replicates and GTR+G model (Edgar, Drive, and Valley 2004; Stamatakis 2014). All trees were visualized using FigTree v1.4.2.

### 2.3.4 Pentabromopseudilin analysis

Bacterial cultures were grown at room temperature with agitation (200 rpm) for 48 hours in 50 ml of Difco 2216 media with 1 g/L of KBr added. The cultures were extracted twice with 50 ml ethyl acetate and the organic fractions combined and dried *in vacuo*. The dried extracts were dissolved in 500 µL of MeOH and analyzed by LC/MS using previously described methods (Agarwal et al. 2014). Pentabromopseudilin production was verified in comparison to an authentic standard either as part of this study or based on previously reported results.

### 2.3.5 Comparative genomics

Average nucleotide identity (ANI) was determined for 91 *Pseudoalteromonas* genomes using custom scripts available at (https://github.com/juanu/ANI_analysis). A distance matrix was generated from all pairwise comparisons and ANI divergence (100 –

ANI) calculated. Pairwise comparisons between contigs containing the *bmp* BGC were preformed using the Artemis Comparison Tool (ACT) v13.0.0 (Carver et al. 2005).

## 2.4 Results

### 2.4.1 Pseudoalteromonas species phylogeny

The *Pseudoalteromonas* species phylogeny reveals three primary clades (Figure 2.2). Clade 3 distinguishes the least inclusive clade containing *P. ruthenica* and *P. xishaensis* from the remaining strains, which subsequently diverge into clades 1 and 2. The species tree reveals numerous clades at varying depths in the phylogeny that do not contain type strains, suggesting some species level diversity has yet to be described. To further assess this potential for taxonomic novelty, average nucleotide identity (ANI) was calculated among all genome sequences. At the suggested >95% ANI cut-off for species designations (Goris et al. 2007), 42 species are resolved of which 14 contain a type strain (Figure 2.3). We identified two instances where multiple type strains occurred within the same ANI species (*P. elyakovii*/*P. piscicida* and *P. flavipulchra*/*P. distincta*), suggesting these lineages have been over described. Genome sequences are unavailable for 26 of the *Pseudoalteromonas* type strains. Thus, at least two of the 28 candidate ANI species are unnamed. To better address taxonomic novelty, the candidate ANI species groupings were compared to the *Pseudoalteromonas* species tree. If the groupings were coherent and did not include a type strain, a new species was proposed (Figure 2.2). Using this approach, we identified 17 candidate new species (Table 2.2).

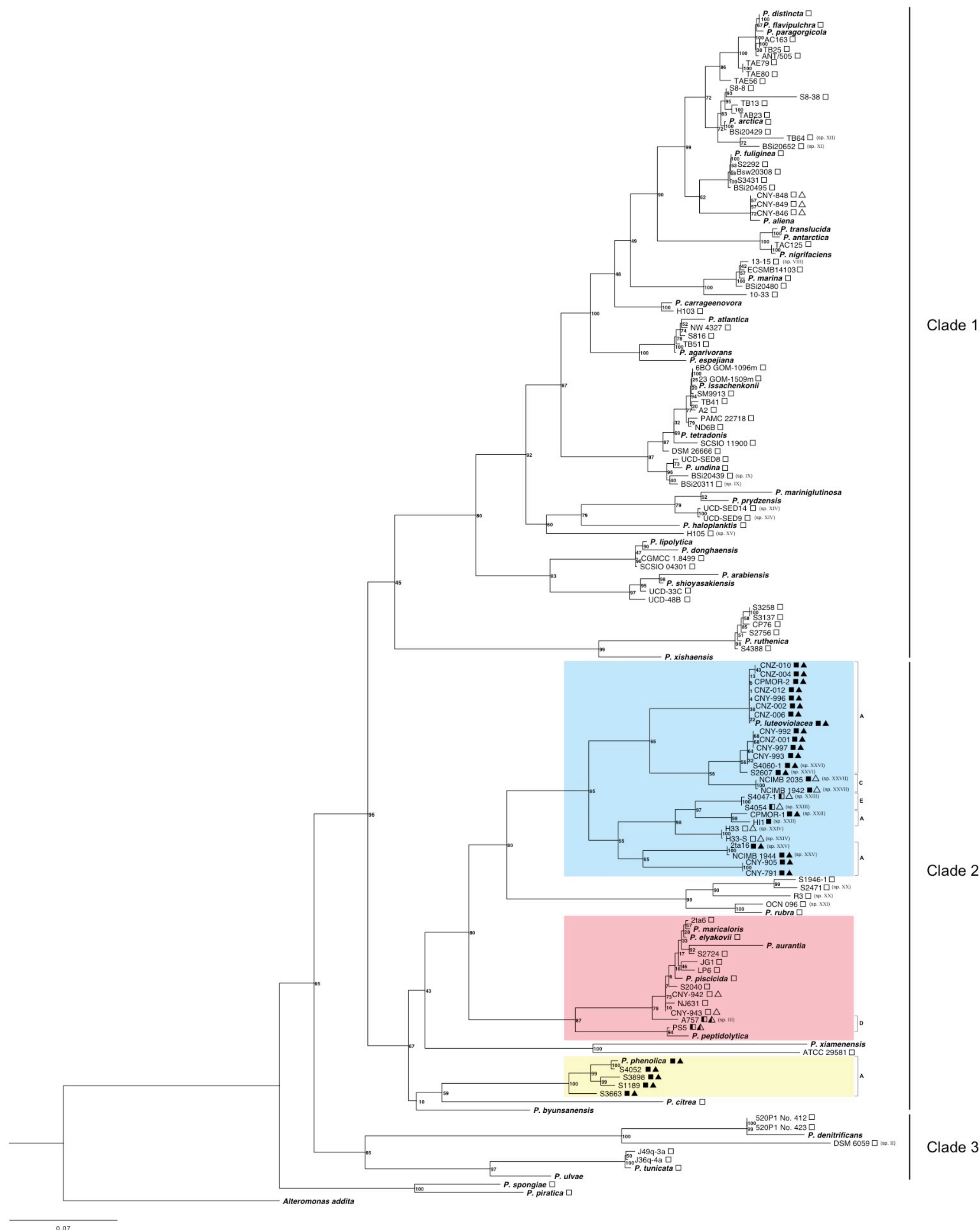


**Figure 2.2** *Pseudoalteromonas* species tree and *bmp* gene cluster distribution. A maximum likelihood phylogeny generated from concatenated 16S, *gyrB*, *pyrH*, *recA*, and *rpoD* nucleotide sequences reveals three primary clades. The three least inclusive clades containing strains with the *bmp* cluster are highlighted in color. Lettered brackets (A, C, D, and E) indicate the version of the *bmp* cluster. The 42 type strains are indicated in bold italics and strains isolated as part of this study are assigned CN numbers. Filled squares indicate the 10-gene *bmp* cluster, half-filled squares indicate a partial gene cluster. Filled triangles indicate the detection of pentabromopseudilin, half-filled triangles indicate that only tetrabromopyrrole was detected. Empty triangles indicate that none of the target brominated compounds were detected. Candidate novel species assigned based on the ANI analysis are indicated with roman numerals.

**Figure 2.3** ANI analysis. Average nucleotide identity (ANI) of 91 *Pseudoalteromonas* genome sequences. The vertical dashed line specifies 95% ANI and the grey dots designate candidate ANI species. Candidate species that do not include a type strain were assigned numbers (I-XXVIII). The matrix next to the right of the strains displays the distribution of the 10 genes in the *bmp* gene cluster. Hash marks indicate a modified *bmp1* sequence.

### 2.4.2 Distribution of the *bmp* gene cluster among *Pseudoalteromonas* spp.

We next mapped the distribution of the *bmp* gene cluster onto the *Pseudoalteromonas* species phylogeny. Of the 101 *Pseudoalteromonas* genomes mined (91 published, 10 unpublished), 19 contain some version of this gene cluster and all of these fall within clade 2 (Figure 2.2). Three least inclusive sub-clades containing the *bmp* BGC can be identified. The 12 strains that were PCR positive for the *bmp* cluster all fall

within the least inclusive clade that includes *P. luteoviolacea*. *Pseudoalteromonas luteoviolacea* and *P. phenolica* are the only named species whose type strains contain the BGC and these fall into different sub-clades. In addition, six candidate species containing the BGC were identified (sp. III, XXII, XXIII, XXV, XXVI, XXVII).

A comparative analysis of the *bmp* cluster revealed four versions (A, C-E) among the *Pseudoalteromonas* strains and a fifth version (B) in *Marinomonas* strain (MMB-1) (Figure 2.2). Version A contains 10 genes, has been experimentally characterized, and is linked to the production of pentabromopseudilin and related BNPs (Agarwal et al. 2014). It was observed in 25 of the Clade 2 strains including the *P. phenolica* and *P. luteoviolacea* type strains and five of the candidate ANI species (Figure 2.2). Version B was only observed in a single *Marinomonas mediterranea* strain (MMB-1) and has one additional gene annotated as a putative permease. Version C has an ~150 bp insertion in *bmp1* suggesting it may be non-functional. This insertion was verified by PCR and sequencing in the two strains (NCIMB 1942 and NCIMB 2035) in which it was observed. The genome sequences for PS5 (ANI sp. IV) and A757 (ANI sp. III) contain a partial *bmp* cluster that lacks *bmp*5-8 (version D) (Figure 2.3). These are the only two strains with any form of the BGC in the least inclusive clade containing 2ta6 and *P. peptidolytica* (Figure 2.2). Strains S4047 and S4054 (both ANI sp. XXIII) possess version E of the gene cluster, which lacks *bmp*3-7 (Figure 2.3). This truncated version, along with a pair of strains that lack the entire gene cluster (H33 and H33-S), are part of a well-supported sub-clade that is dominated by version A (Figure 2.2), suggesting they result from gene loss events. Strains isolated as part of this study were assigned version A if both *bmp2* and *bmp5* were detected by PCR and sequence verified. They were not

assigned version B, since this has only been detected in a single *Marinomonas* strain, or versions C-E based on subsequent metabolomics data.

### 2.4.3 Pentabromopseudilin production

*Pseudoalteromonas* strains possessing different versions of the *bmp* gene cluster were next analyzed for pentabromopseudilin (**1**) production (Figure 2.4). Pentabromopseudilin (**1**) is a heterodimer comprised of brominated pyrrole and phenol monomers. All 10 genes in version A of the gene cluster are required for the production of this compound in *Pseudoalteromonas* spp. (Agarwal et al. 2014). Therefore, any *Pseudoalteromonas* strains that produce this compound can be expected to contain *bmp* version A, since version B has not been observed in the genus. Of the 25 *Pseudoalteromonas* strains containing *bmp* version A, 24 produce pentabromopseudilin (**1**) (Figure 2.2) (Vynne et al. 2011; Agarwal et al. 2014) thus matching the metabolic predictions. The genome of HI1 contains version A of the BGC but chemical data has not been published for the strain, therefore, pentabromopseudilin production is unconfirmed. H33 and H33-S are the only strains in the *P. luteoviolacea* sub-clade missing the entire *bmp* gene cluster and as expected, no BNPs were detected (Vynne, Mansson, and Gram 2012). We also failed to detect brominated compounds from the strains that possess *bmp* version C (NCIMB 1942 and NCIMB 2035). The 150 bp insertion in the ACP-TE encoding *bmp1* gene distinguishes version C from A and may explain the lack of BNP production in these strains. Tetrabromopyrrole (**2**) was the only BNP detected from strains with *bmp* version D, which is consistent with the expected phenotype (El Gamal, Agarwal, Diethelm, et al. 2016). Additionally, BNPs were not detected from the two strains containing version E of the BGC (S4047 and S4054), which lacks *bmp3-7*. These

results are consistent with the functional characterization of the BGC, as the products of the missing genes are required to synthesize brominated pyrrole and phenol monomers (Vynne, Mansson, and Gram 2012).

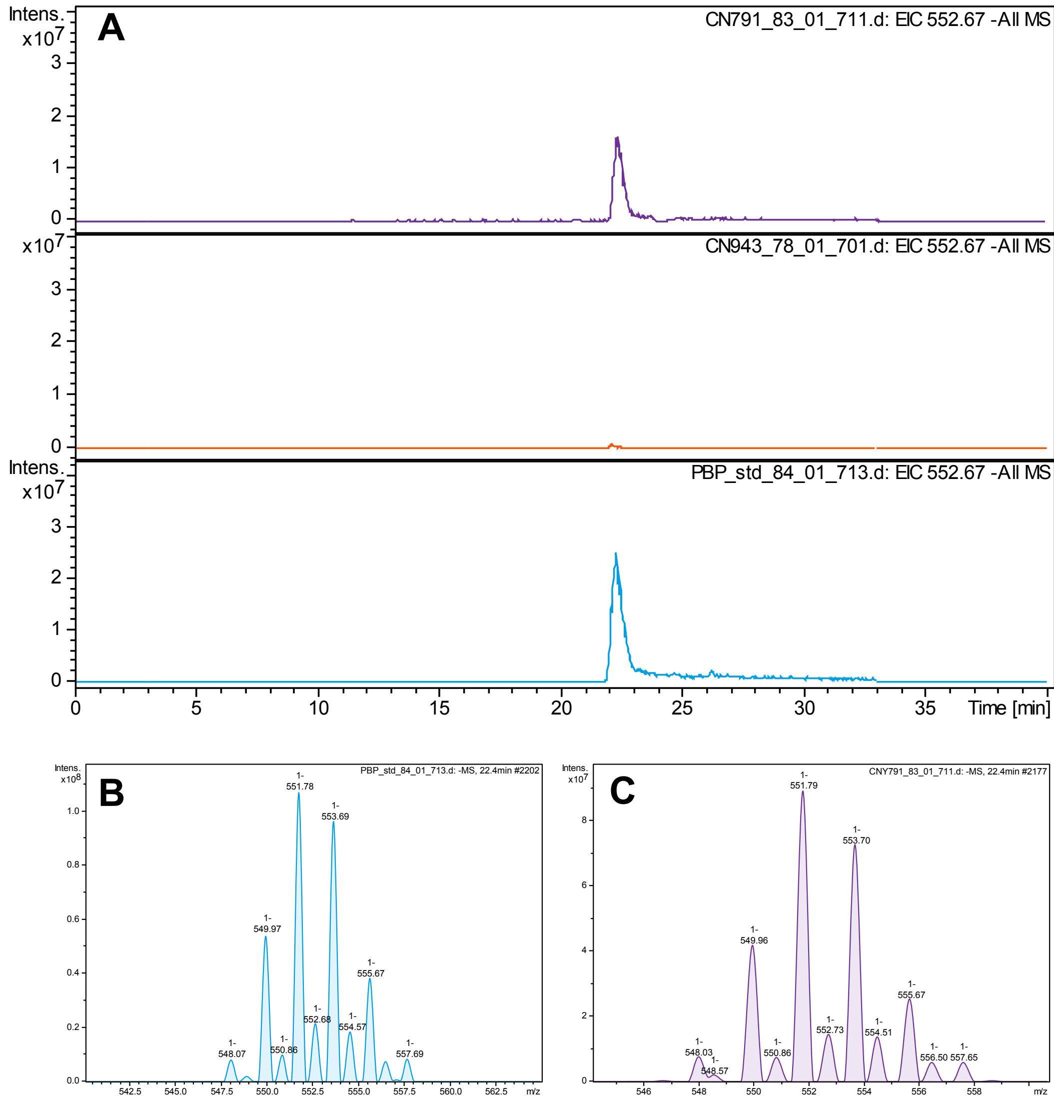


**Figure 2.4** Detection of pentabromopseudilin by LCMS. Strains isolated as part of this study were screened for pentabromopseudilin production. (A) Extracted ion chromatograms for the mass of pentabromopseudilin (553.67 g/mol) from a strain with version A of the BGC (CNY-791, top) and one that lacks the BGC (CNY-943, middle). Pentabromopseudilin standard (bottom). All strains containing *bmp* version A produced pentabromopseudilin with an absolute intensity of at least 1.4 x $10^6$ (B) Isotopic pattern of a pentabromopseudilin standard and the compound detected from strain CNY-791 (C).

### 2.4.4 Evolutionary history of the bmp gene cluster

A concatenated phylogeny of the *bmp* genes derived from all 19 *Pseudoalteromonas* genome sequences that contained some version of the BGC was compared to a species tree generated for the same strains (Figure 2.5). No outgroup was used in the *bmp* phylogeny; however, the sequences from *Marinomonas* strain MMB-1 were included and the tree was rooted consistently with the species tree (Figure 2.5a). With the exception of the position of MMB-1, the two trees are highly congruent. The *bmp* phylogeny shows that the sister clades containing *P. luteoviolacea* and A757 and the more distantly related *P. phenolica* clade are consistent with the species tree, supporting a single acquisition of the *bmp* cluster in the genus.

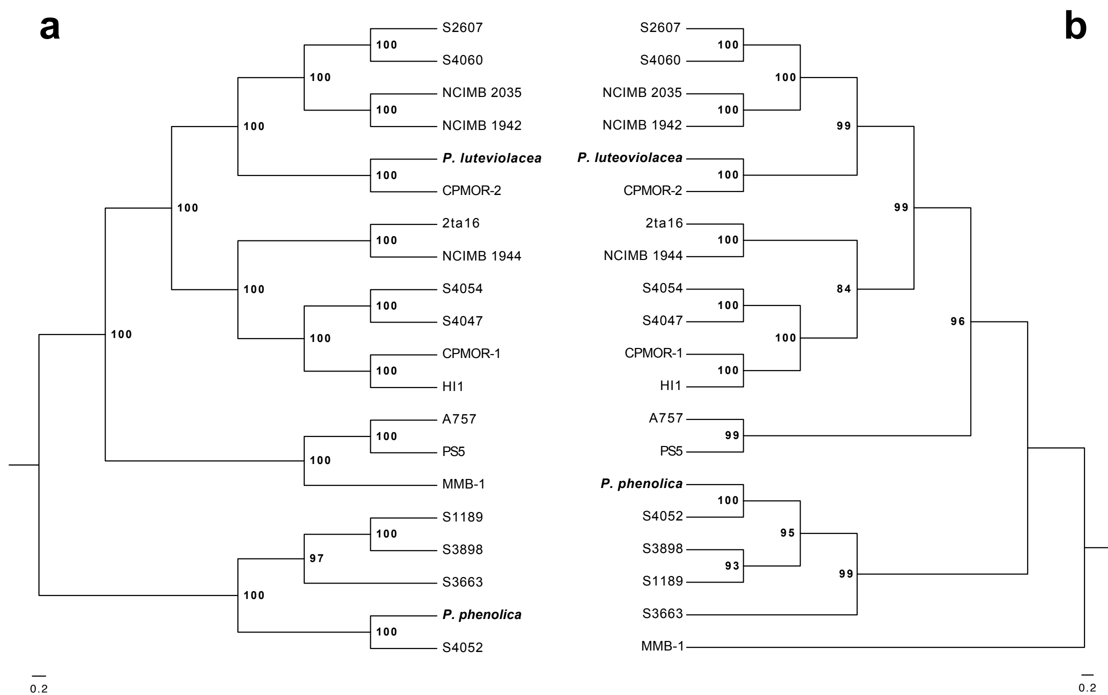


**Figure 2.5** *Bmp* gene phylogeny. Concatenated maximum likelihood phylogenies of the *bmp* gene sequences (a) and the 16S, *gyrB*, *pyrH*, *recA*, and *rpoD* sequences (b) derived from the 19 *Pseudoalteromonas* strains and the one *Marinomonas* strain (MMB-1) that contain any version of the *bmp* gene cluster. The *bmp* gene tree is rooted based on the species phylogeny while the species tree is rooted with MMB-1.

To further test a single acquisition event for the *bmp* gene cluster in *Pseudoalteromonas*, we explored the genomic environment in which it was observed, expecting that it would be similar. However, the *bmp* cluster is found in three different genomic environments, thus, if there was a single acquisition event, the BGC has subsequently moved (Figure 2.6). The genomic environment analysis suggests a single acquisition event of the gene cluster by the large clade containing strains CPMOR-2 and H33. The strains in the clade have high sequence identity of the *bmp* genes as well as synteny both upstream and downstream of the BGC, suggesting that once acquired it remained in the same genomic position. It is hypothesized that subsequent gene loss or modification resulted in versions C and E in strains NCIMB 1942 and S4054 respectively. Strain H33 lacks *bmp* genes yet is highly syntenic in this region with strain CPMOR-1, which possesses version A of the gene cluster. Additionally, strain S4054 contains the abbreviated *bmp* version E but the BGC is in the same genomic environment as *bmp* version A in strains NCIMB 1944 and HI1. The detection of a remnant of *bmp7* in strain S4054 supports the gene loss hypothesis (Figure 2.7). These results suggest that a common ancestor possessed the BGC with subsequent partial (S4054) and complete (H33) loss events in these two strains. The lack of synteny between more distantly related strains that possess the same version of the BGC (e.g., HI1 and *P. phenolica*) indicates that regardless of whether the same biosynthetic genes are present in a pair of more distantly related strains, the *bmp* gene cluster can be found in different genomic environments (Figure 2.6b). These results suggest that *P. phenolica* and HI1 did not inherit version A the *bmp* gene cluster from a common ancestor within the genus. When HI1 (version A) was compared with A757 (version D), aside from the *bmp* genes, very

little synteny was found, which does not support the hypothesis that the strains inherited the BGC from a common ancestor and version D is the result of a partial loss of the cluster (Figure 2.6c). The genomic analysis results provide evidence that the *bmp* gene cluster may have been independently acquired three times within the genus. Alternatively, the BGC could have also been acquired by a common ancestor then moved its position within some genomes. Regardless of which hypothesis is correct, there is clear evidence that the gene cluster has been maintained, modified, or lost following vertical inheritance within certain subclades (Figure 2.8).

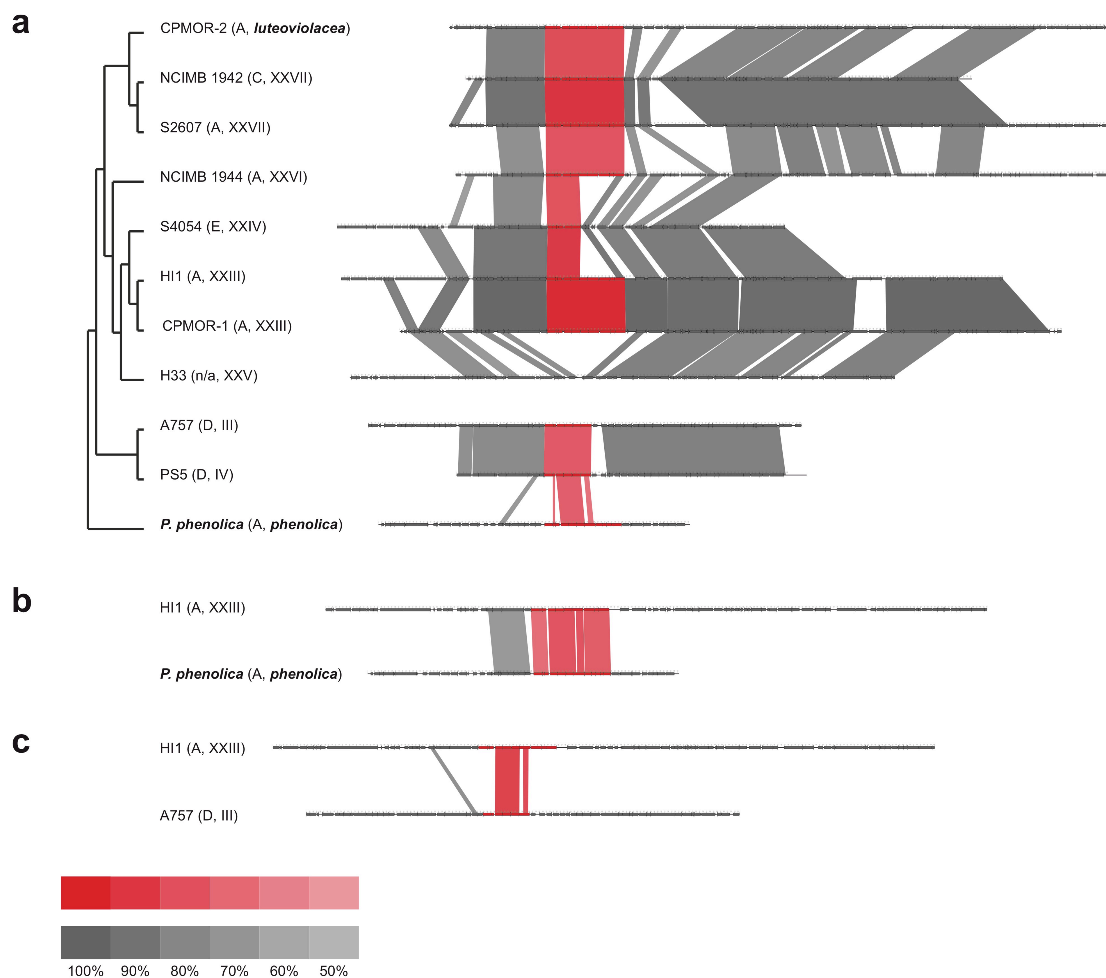


**Figure 2.6** Genomic environment of the *bmp* gene cluster. For each contig, the *bmp* gene cluster version (A, C, D and E) and ANI species identity is indicated in parentheses after the strain name. The opacity of shaded regions indicates percent sequence identity. (a) The phylogenetic relationships of the strains consistent with the species tree are shown on the left. The lack of synteny between *bmp* gene clusters (shown in red) and the surrounding genomic environments in *Pseudoalteromonas* strains supports independent acquisition events or movement of the BGC within the chromosome. (b) Sequence identity for *bmp* gene cluster version A is shown in two distantly related strains (HI1 and ***P. phenolica***), but no other synteny on the contigs is observed. (c) Strains HI1 (*bmp* version A) and A757 (*bmp* version D) have highly similar sequences for the biosynthetic genes that they share, however, there is no indication of a partial loss of the BGC.

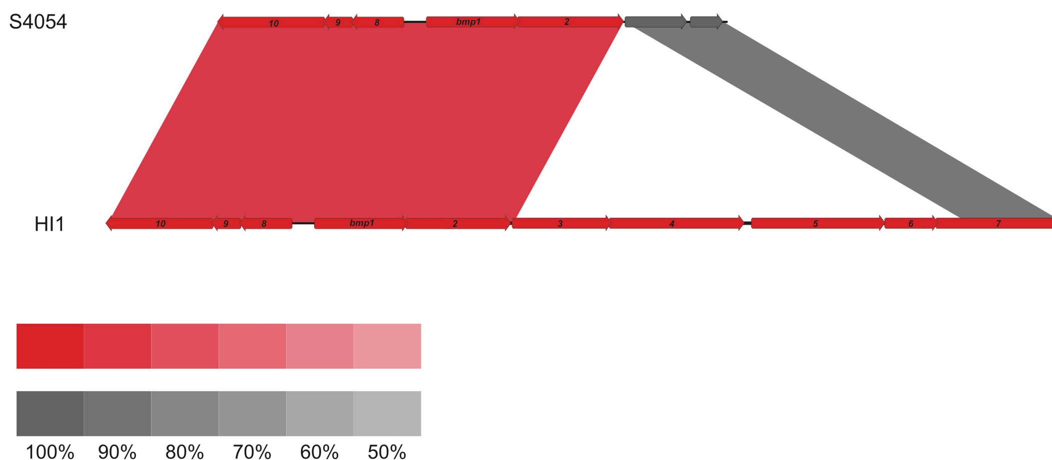


**Figure 2.7** Evidence for partial loss of the *bmp* cluster. A region immediately downstream of *bmp2* in strain S4054 (version E) has high sequence identity to a portion of *bmp7* in strain HI1 (version A), supporting gene loss in S4054.

The *bmp* cluster is found in three different genomic environments in *Pseudoalteromonas* strains, which could be attributed to separate acquisition events or post acquisition migration (Figure 2.6). While incomplete genome sequences prevented the precise mapping of BGC chromosomal positions, there is a lack of synteny among genes surrounding the different versions of the gene cluster, despite the close relationship among the strains based on ANI analysis (>82%).

## 2.5 Discussion

Characterization of the *bmp* biosynthetic gene cluster revealed it accounts for the production of the potent antibiotic pentabromopseudilin (Burkholder, Pfister, and Leitz 1966), the larval settlement cue tetrabromopyrrole (El Gamal, Agarwal, Diethelm, et al. 2016), and polybrominated diphenyl ethers whose industrial production and use as flame retardants has been banned due to human health implications (Johnson-Restrepo and Kannan 2009). Despite the potential ecological significance of these compounds, their production and the distribution of *bmp* among marine bacteria has not been examined.

This study addressed these questions within the genus *Pseudoalteromonas*, the primary bacterial source of pentabromopseudilin and biosynthetically related compounds reported to date. The results indicate a narrow distribution and complex evolutionary history that includes gene modifications and deletions that affect compound production. While congruence between the species tree and *bmp* gene phylogeny supports a single acquisition event for the gene cluster (Figure 2.5), this model requires eight complete or partial gene cluster losses to explain the current distributions. Based on these results, the possibility of multiple acquisition events cannot be ruled out. While the evolutionary history of the *bmp* gene cluster remains obscure at the genus level, there are three points from which this history can be confidently inferred (Figure 2.8). Even within these sub-lineages, there is clear evidence of gene cluster loss or modification, attesting to the complex evolutionary history of this gene cluster.

There is ample evidence that the gene clusters associated with secondary metabolism are exchanged by horizontal gene transfer. Despite this, there was a strong correlation between taxonomy, *bmp* composition, and compound production within the genus *Pseudoalteromonas*. For example, all *P. phenolica* strains analyzed maintained the same version of the BGC and produced the same secondary metabolites. The least inclusive clade that includes *P. luteoviolacea* is also highly conserved for these feature with the exception of a few loss events. BGC conservation associated with species level units of diversity suggests that the products encoded provide an important selective advantage for the producer and add to growing evidence that secondary metabolites can be useful taxonomic markers for bacteria (Hoffmann et al. 2018).

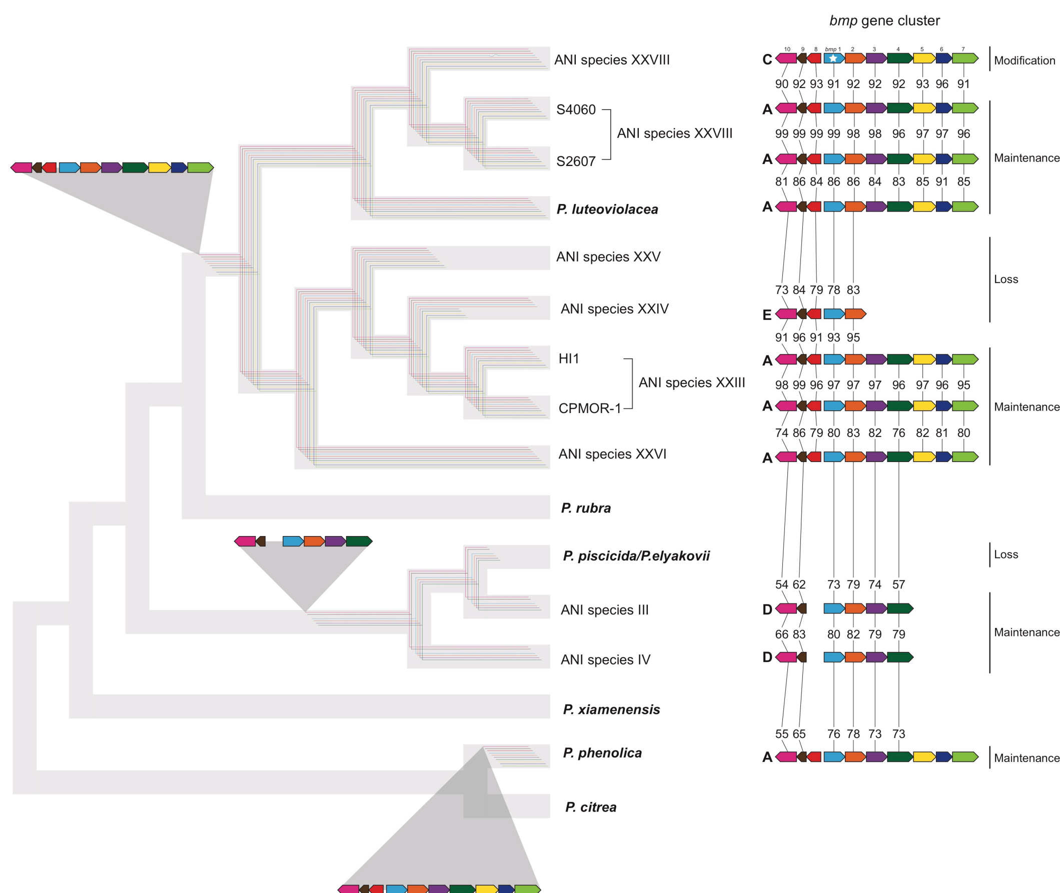


**Figure 2.8** Evolutionary history of the *bmp* gene cluster in *Pseudoalteromonas*. Abbreviated species phylogeny represented as a cladogram indicating ancestral BGC composition and inferred evolutionary history of each gene (colored lines). Versions of the BGC (A, C, D, or E) observed in different lineages with evolutionary events indicated as maintenance, loss, or modification. Loss includes complete or partial deletion of the BGC. An insertion in *bmp1* is the only modification observed (indicated with a star).

During the course of this study, gene clusters related to *bmp* were detected in sponge cyanobacterial symbionts (Agarwal et al. 2017). While these gene clusters have yet to be fully characterized, they include *bmp5-7* homologs, which encode the production of PBDEs, but not the genes required to make brominated pyrrole molecules or pentabromopseudilin. These results suggest that an even more complex evolutionary history remains to be resolved for the *bmp* gene cluster in marine bacteria.

Remarkable new insight is being gained into the evolutionary processes that drive the biosynthesis of specialized metabolites (Lind et al. 2017; Ruzzini and Clardy 2016; Medema et al. 2014). These studies have helped reveal the dynamic processes of gene gain, loss, and degradation (Letzel et al. 2017) and how subtle changes in regulatory genes can have substantial effects on both gene expression and compound production (Amos et al. 2017). This study adds to the growing number of examples in which the evolutionary history of a biosynthetic gene cluster provides insight into the processes that establish lineage specific chemical diversity (Freel et al. 2011). Biosynthetic gene clusters evolve rapidly and while there are multiple versions of the *bmp* cluster found in marine bacteria, selection seems to be favoring the maintenance of version A, which enables the biosynthesis of bioactive molecules such as pentabromopseudilin (**1**) (Fehér et al. 2010; Fischbach, Walsh, and Clardy 2008). It is also possible that loss of the ability to produce this bioactive compound is complemented by the products of an unrelated BGC, as was shown with siderophore biosynthetic gene clusters in marine actinomycetes (Bruns et al. 2017). Bioinformatic analyses have become powerful predictors of chemical phenotypes, as there was a perfect correlation between the version of the BGC detected and BNP production among the strains analyzed here. With continued access to more strains and genome sequences, it will likely become possible to infer the evolutionary events that led to the acquisition of the *bmp* gene cluster in *Pseudoalteromonas*.

### 2.6 Acknowledgements

This work was jointly supported by the US National Science Foundation (OCE-1313747) and the US National Institute of Environmental Health Sciences (P01-ES021921) through the Oceans and Human Health program.

Chapter 2, in full, has been formulated into a manuscript that will be submitted for publication in 2018. Julia Busch, Vinayak Agarwal, Michelle Schorn, Henrique Machado, Bradley S. Moore, Greg W. Rouse, Lone Gram, Paul R. Jensen. The dissertation author was the primary investigator and author on these studies.

# Chapter 3: The cellular target of pentabromopseudilin

## 3.1 Abstract

Pentabromopseudilin is a bioactive marine natural product isolated from several *Pseudoalteromonas* species. While its antimicrobial activity has been reported, the mechanism of action for the molecule remains unknown. Using bacterial cytological profiling techniques in conjunction with cell viability assays, we show that the primary phenotype observed in pentabromopseudilin-treated *E. coli* ATCC 25922 cells was membrane permeability, which increases over time and with compound concentration. Infections caused by Gram-negative bacteria present a therapeutic challenge because antibiotics cannot act on these pathogens due to their highly impermeable outer cell membrane. Therefore, potentiator molecules are often administered concurrently to increase the efficacy of antibiotics. Despite its observed potentiation of antibiotics against *E. coli* cells, pentabromopseudilin is highly cytotoxic and thus, is not a good candidate for therapeutic applications. However, this function may provide a selective advantage for strains that produce other antibiotics.

## 3.2 Introduction

The antimicrobial natural product pentabromopseudilin (PBP) was first discovered from a marine bacterium over 50 years ago (Burkholder, Pfister, and Leitz 1966) (Figure 3.1). However, it wasn't until recently that the biosynthetic gene cluster (BGC) attributed with the production of PBP, the *bmp* gene cluster, was identified (Agarwal et al. 2014). The *bmp* cluster encodes the biosynthesis of numerous compounds including brominated phenols, pyrroles, homodimers (both phenol and pyrrole), as well as the heterodimer PBP. The BGC and suite of brominated metabolites has been reported

in multiple *Pseudoalteromonas* strains as well as a single *Marinomonas mediterranea* strain (MMB-1) (Agarwal et al. 2014).

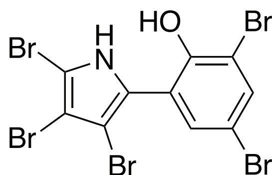


**Figure 3.1** Pentabromopseudilin

While the potent bioactivity of PBP has been reported, little is known about its mechanism of action (MOA). One study suggested that PBP inhibits myosin motor activity, thus possibly explaining the cytotoxicity, but there is no known mechanism of action for its antibiotic properties (Fedorov et al. 2009). The $IC_{50}$ value of PBP for MRSA was determined to be 0.1 µM (±0.18) and bioactivity of the compound has also reported against the marine bacteria *Photobacterium phosphoreum* and *Chromobacter* sp. (Fehér et al. 2010; Andersen, Wolfe, and Faulkner 1974).

To investigate the MOA of PBP, bacterial cytological profiling (BCP) was used. This technique can be used to identify the cellular targets of antimicrobial molecules in both Gram-positive and Gram-negative bacteria and can accurately predict their MOA (Nonejuie et al. 2013). Unlike other methods to identify the MOA of antibacterial compounds, BCP requires a very small amount of compound and results can be generated very quickly. In this study, BCP revealed increased membrane permeability in response to PBP concentration and exposure time, thus indicating that the major cellular target in *E. coli* ATCC 25922 is the cell membrane.

## 3.3 Materials and Methods

### 3.3.1 Minimal inhibitory concentration determination

The minimal inhibitory concentration (MIC) of PBP was determined against *E. coli* ATCC 25922 using the broth microdilution method according to the guidelines outlined by the Clinical and Laboratory Standards Institute. Abdhesh Kumar synthesized pentabromopseudilin with a shortest route by Suzuki coupling of Boc protected pyrrole boronic acid with anisole and then deprotection of Boc and methoxy group by sodium methoxide and anhydrous sodium sulfide respectively. And finally, bromination with pyridinium tribromide gave pentabromopseudilin.

A single colony was picked from a plate of *E. coli* ATCC 25922 (on LB incubated at 30°C overnight) and added to 5 mL of LB broth in a culture tube and incubated overnight at 30°C while rolling at maximum speed (80 rpm). A series of 100-fold dilutions were prepared from the overnight culture in a culture tube with 5 mL of LB then incubated at 30°C while rolling at max speed (80 rpm) until the $OD_{600}$ reached 0.2 – 0.4 (log phase growth). LB broth was added to sterile flat-bottom 96-well plates as follows: 200 µL in column 1 rows A-H, 100 µL in all other wells. A 2 mM stock of PBP in DMSO was added to wells corresponding to column 1 rows A-H to final concentrations of 22, 21.875, 21.6, 21.4, 21.2, 21, 20.6, and 20 µM respectively. A series of two-fold dilutions consisting of 100 µL from column 1 rows A-H, mixed with column 2 rows A-H were created to incorporate all wells except those in columns 11 and 12 rows A-H. Finally, a volume of 1 µL of log phase cells diluted to 0.05 $OD_{600}$ was added to every well except those in column 12 (negative control, LB only). Column 11 served as the positive control and contained only cells and media. The $OD_{600}$ at time 0 was recorded

using a TECAN plate reader. The plates were incubated at 30°C with shaking at 220 rpm for 18-24 hours. The $OD_{600}$ reading from T0 was subtracted from the T18-24 values and the lowest concentration at which there was no growth ($OD_{600} < 0.06$) was the MIC value.

### 3.3.2 Bacterial Cytological Profiling (BCP)

Cell cultures in early log phase ($OD_{600}$ 0.15-0.17) were treated with 1/8, 1/4, 1/2, 1, 2, 4, 8x MIC concentrations of pentabromopseudilin and incubated at 30°C while rolling at max speed (80 rpm). Three hundred microliters of cells were collected for imaging after 10 min, 30 min, 1 h, and 2 h of PBP treatment and stained with 1 µL of a dye mixture to achieve the following dye concentrations: 1 µg/mL FM-464 (red, membrane), 2 µg/mL DAPI (blue, DNA), and 0.5 µM SYTOX Green (green, DNA, impermeable to intact membranes) (Pogliano et al. 1999). Stained cells were then transferred to 1.6 mL microfuge tubes, centrifuged for 30 seconds at 3,300 x *g*, and 14 µl of the supernatant removed to concentrate the cells, which were re-suspended and transferred to an agarose pad (1.2 % agarose, 20% LB medium) for fluorescence microscopy. Six images were taken for each concentration at every time point. Fluorescence microscopy was performed as previously described with the same exposure time for each wavelength maintained throughout every experiment (Lamsa et al. 2016).

### 3.3.3 Viability counts

At each imaging time point, 10 µl of cells was added to 90 µl of 1X Tbase (10X Tbase = 20g $(NH_4)_2SO_4$, 140 g $K_2HPO_4$, 60 g $KH_2PO_4$, 10 g $Na_3Citrate \bullet H_2O$ per L) in a 96-well plate and serially diluted 1:10 to a final dilution of $10^{-8}$. Five µl of each dilution was pipetted onto LB agar media. The plates were incubated at room temperature

overnight, then checked for growth. Colony counts were multiplied by the volume plated and dilution factor to calculate colony forming units (CFU) per ml.

### 3.3.4 Membrane permeability quantification

Cell permeability was quantified using a custom script in MATLAB version R2017b. The SYTOX Green intensity per pixel was measured in a total of 25,288 cells across all pentabromopseudilin concentrations and time points. Intensity was determined using the membrane outline, and then subtracted by its own background intensity. The analysis only included measurements of the dye within intact cells.

### 3.3.5 Cytotoxicity assay

Cytotoxicity to H-460 human lung carcinoma cells was measured as cell viability using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction method (Alley, M.C., Scudiero, D.A., Monks, P.A., Hursey, M.L., M.J., Fine, D.L., Abbott, B.J., Mayo, J.G., Shoemaker, and M.R. 1988). Evgenia Glukhov performed these experiments.

## 3.4 Results & Discussion

### 3.4.1 Cytological profiling of cells treated with pentabromopseudilin

The MIC values for PBP against *E. coli* ATCC 25922, *Bacillus subtilis* PY79, and a *E. coli* Δtolc strain AD3644 were determined to be 5.47 μM, 0.093 μM, and 0.219 μM respectively. Only the wild type *E. coli* strain (ATCC 25922) was selected for BCP experiments. *E. coli* ATCC 25922 cultures were treated with 1/8X (0.68 μM), 1/4X (1.37 μM), 1/2X (2.73 μM) 1X (5.47 μM), 2X (10.94 μM), 4X (21.88 μM), and 8X (43.75 μM) MIC concentrations of PBP and harvested cells were stained with FM, DAPI, and SYTOX Green for imaging at 10 min, 30 min, 1 hr, and 2 hr time points. The primary

phenotype observed was permeability to SYTOX Green, which suggests that PBP increases membrane permeability (Figure 3.2, Figure 3.3). Interestingly, at the higher concentrations of PBP tested, permeability was observed after only 10 min. While membrane permeability is not a unique phenotype, PBP doesn't cause the cells to lyse as readily as other compounds with the same MOA such as detergents (Mohammad et al. 2017). Additionally, at the 8X MIC concentration (43.75 μM), it appears that the DAPI fluorescence decreases and the chromosomes appear to de-condense, suggesting that PBP may also be targeting DNA in cells.

**3.4.2 Quantification of cell membrane permeability**

In addition to identifying the predominant phenotype of PBP treated cells, we were interested to know if this phenotype developed in a dose-dependent manner and how membrane permeability changed over time. To answer these questions the SYTOX Green intensity was quantified in a total of 25,288 cells across eight different concentrations and four time points (Table 3.1). For all treatments, the SYTOX Green intensity in *E. coli* cells increased as concentrations of PBP increased (Figure 3.4). The cells that were incubated with less than the MIC concentration (<5.47 μM) showed no increase in membrane permeability over the course of two hours. The cells treated with the 1X MIC concentration of PBP displayed very low levels of permeability for the first hour, but after 2 hours, the SYTOX Green intensity increased. At the higher concentrations (greater than 1X MIC), the cells are permeable at the first time point (Figure 3.4). The dye only enters cells with compromised membranes, thus its intensity is a measurement of cell permeability. At concentrations greater than the MIC of PBP over longer periods of time, the cells began to lyse and released the dye. Since the SYTOX

Green intensity was only measured inside the boundaries of the cells, this is why the signal decreases over time at some of the higher concentrations. Overall, the quantification of the SYTOX Green intensity results suggest that membrane permeability increases with dose and time.

While the results of this study clearly show that PBP increases *E. coli* cell membrane permeability, the specific mechanism of this activity remains unclear. One explanation is that PBP is simply a highly lipophilic molecule and thus is capable of untargeted membrane disruption. The logarithm of the ratio of the partition coefficient (P) is one metric used to measure the lipophilicity or hydrophobicity of a compound; the higher the value the more lipophilic the molecule. The logP value of PBP is 4.8 (calculated using ChemDraw version 16.0). Because the molecule is lipophilic, it's possible that its mechanism of increasing membrane permeability is simple membrane disruption rather than targeting a specific cellular pathway. However, further studies are needed to support this hypothesis.

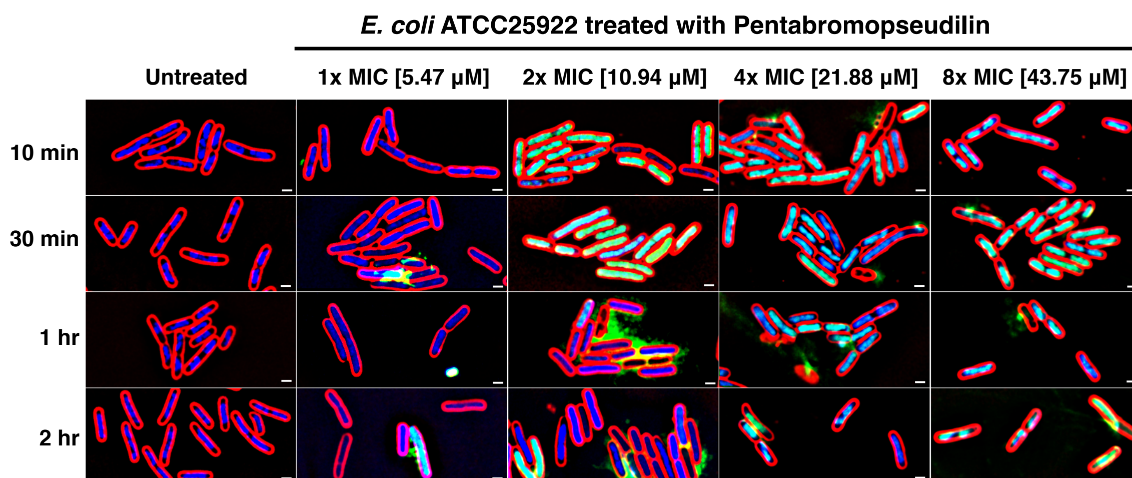


**Figure 3.2** Bacterial cytological profiling of *E. coli* ATCC 25922 treated with pentabromopseudilin at concentrations greater than or equal to the MIC value.

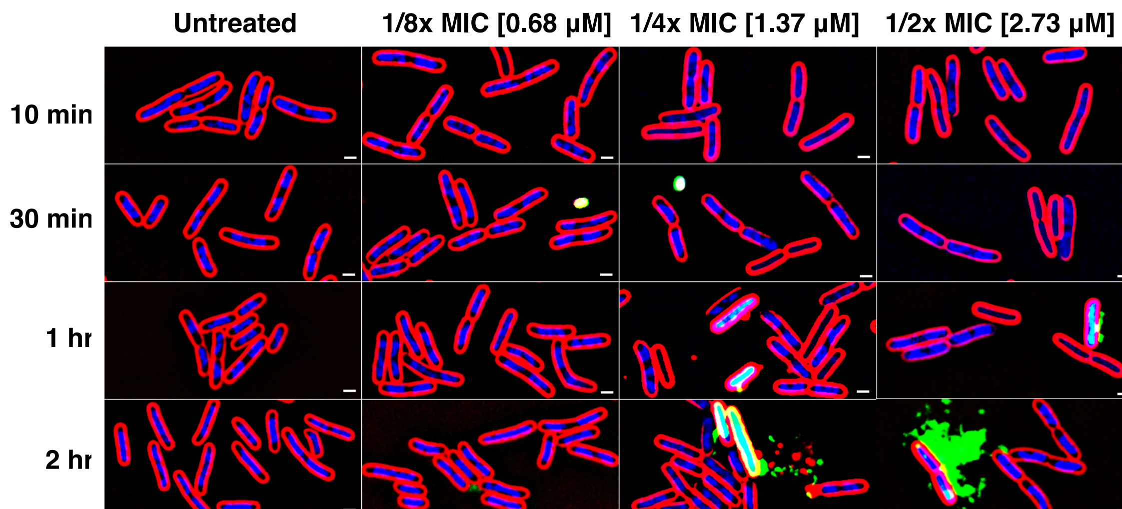


**Figure 3.3** Bacterial cytological profiling of *E. coli* ATCC 25922 treated with pentabromopseudilin at concentrations less than the MIC value.

**Table 3.1** SYTOX Green intensity cell counts

| Time | Untreated | 0.68 µM | 1.37 µM | 2.73 µM | 5.47 µM | 10.94 µM | 21.88 µM | 43.75 µM | Total |
|---|---|---|---|---|---|---|---|---|---|
| 10 min | 262 | 1275 | 234 | 926 | 833 | 756 | 1116 | 347 | 5749 |
| 30 min | 1443 | 1896 | 266 | 1272 | 880 | 603 | 428 | 316 | 7104 |
| 1 hr | 563 | 1377 | 1037 | 608 | 546 | 646 | 295 | 127 | 5199 |
| 2 hrs | 2212 | 2277 | 544 | 315 | 708 | 473 | 249 | 458 | 7236 |
| Total | 4480 | 6825 | 2081 | 3121 | 2967 | 2478 | 2088 | 1248 | 25288 |

**Table 3.2** Average SYTOX Green intensity values

| Time | Untreated | 0.68 µM | 1.37 µM | 2.73 µM | 5.47 µM | 10.94 µM | 21.88 µM | 43.75 µM |
|---|---|---|---|---|---|---|---|---|
| 10 min | 36.95 | 16.12 | 19.43 | 48.55 | 62.6 | 1875.58 | 4021.23 | 4514.18 |
| 30 min | 20.41 | 36.28 | 31.28 | 49.05 | 124.71 | 2343.63 | 7442.46 | 6859.10 |
| 1 hr | 17.81 | 17.81 | 41.50 | 105.41 | 158.80 | 971.90 | 3741.96 | 4073.03 |
| 2 hrs | 35.122 | 22.31 | 26.34 | 53.53 | 931.29 | 931.29 | 2233.19 | 4536.04 |

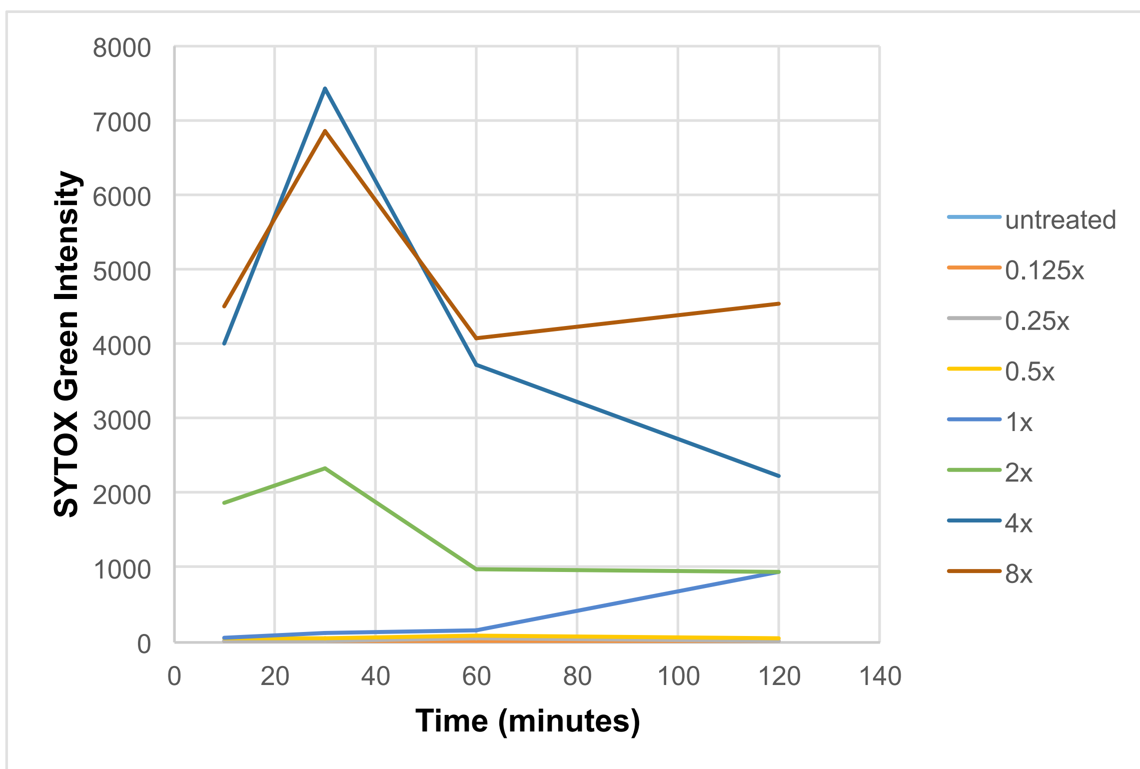


**Figure 3.4** SYTOX Green intensity of *E. coli* ATCC 25922 cells treated with pentabromopseudilin over time.

### 3.4.3 Pentabromopseudilin-treated cell viability

Cell viability assays were performed in parallel with the cytological profiling experiments. The amount of culture available for viability assays was limited due to the concurrent BCP experiments; therefore, the assays were not replicated. *E. coli* ATCC 25922 cells were harvested at the same time points as the cultures used for microscopy, diluted and plated on LB medium. Cell counts were normalized by calculating the ratio of CFUs/mL in the treated cultures to the untreated culture at time zero. The values were graphed as log(CFU/mL) over time (Figure 3.5). As expected, increasing the concentration of PBP caused a decrease in *E. coli* colony forming units (CFUs). Cell viabilities for the 1/2X and 1X PBP MIC concentrations were nearly identical. At the 4X and 8X MIC concentrations of PBP, a steep decrease in CFUs/mL was observed after only 10 minutes, indicating that cell viability is rapidly lost at these high concentrations.

The slight increase observed in the 4X MIC treated culture may be due to cell recovery, development of resistance, or error. To further explore these results, the experiments should be repeated including longer time points and replicate plate counts.

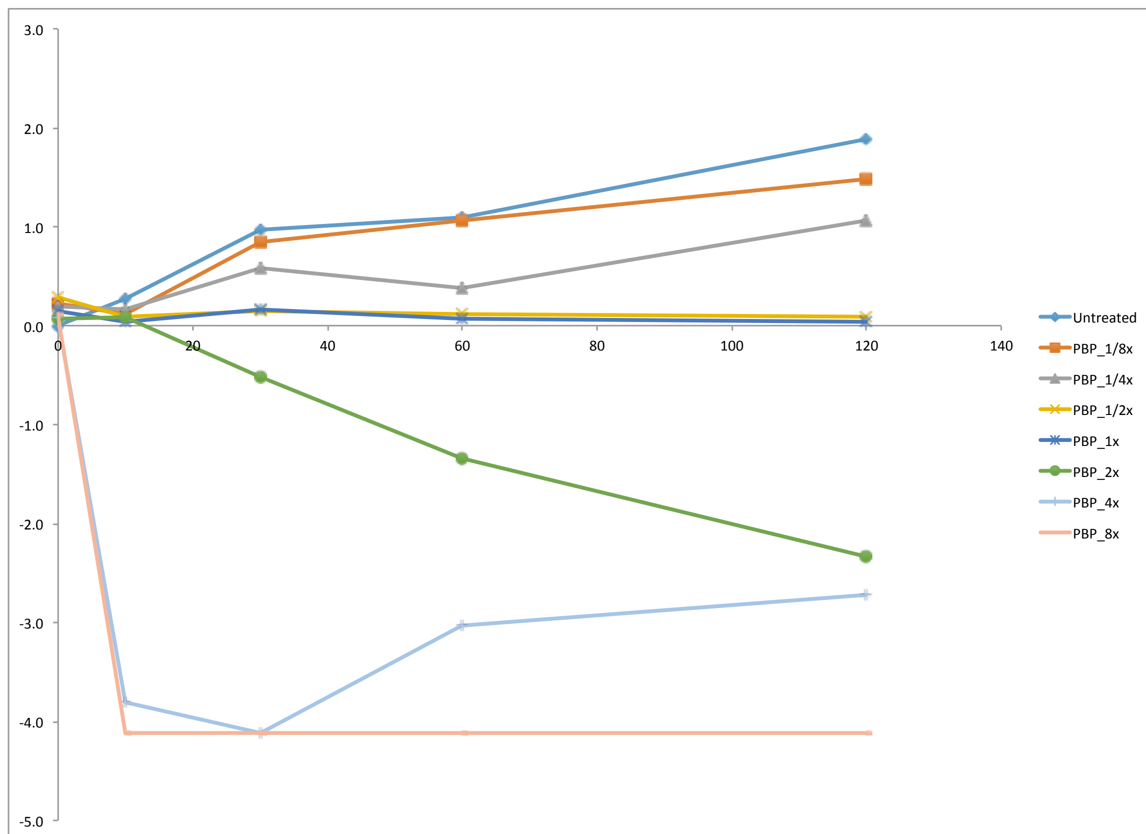

**Figure 3.5** The viability of PBP-treated cells represented as log(CFU/mL) vs. time in minutes.

### 3.4.4 The potentiation of pentabromopseudilin

To test the effects of pentabromopseudilin on membrane permeability, potentiation assays were performed with vancomycin and erythromycin. Most Gram-negative bacteria including *E. coli* are intrinsically resistant to these antibiotics because their outer membranes are not permeable to large glycopeptides. This mechanism of resistance presents a challenge for the treatment of infections caused by Gram-negative bacteria. Therefore, potentiator molecules are often used in conjunction with antibiotics to disrupt the outer membrane and enhance drug efficacy (Zabawa et al. 2016).

The MIC values of vancomycin and erythromycin were determined for *E. coli* ATCC 25922. The same strain was then incubated with the antibiotics and sub-MIC (>5.47 μM) concentrations of PBP to test for antibiotic potentiation. The MIC value for vancomycin was 250 μg/mL with no added PBP, 158.3 μg/mL with 1 μM PBP added, and 124 μg/mL with 2 μM PBP added (Figure 3.6). The MIC value for erythromycin was 91.7 μg/mL with no added PBP, 44.8 μg/mL with 1 μM PBP added, and 23.7 μg/mL with 2 μM PBP added (Figure 3.7). These results suggest that PBP potentiates the activity of PBP vs. *E. coli* ATCC 25922 and lowers the MIC of erythromycin by 74%. The potentiation effect of PBP on cells treated with vancomycin lowered the MIC by 50%. This is likely because the antibiotic is a large molecule (1449.3 g/ml) and nearly twice the size of erythromycin. To verify that the sub-MIC concentrations of PBP were not responsible for the decreased MIC values, the average $OD_{600}$ of the positive control wells (cells with PBP but no antibiotic) were calculated as a percentage of the values from cells with no PBP added (Figure 3.8). Minimal decreases in cell density of 7% for 1 μM PBP and 6% for 2 μM PBP were observed, supporting the conclusion that the lowered MIC values were largely due to the antibiotics, not the potentiator molecule.

Pentabromopseudilin was tested for cytotoxicity against a human lung cancer cell line (H460) and found to have an $IC_{50}$ value of 480 ±70 nM. Because of it's highly cytotoxic activity, PBP would not likely be an ideal candidate for therapeutic use. However, it may play an important ecological role in bacterial strains that produce the compound if they also produce other antibiotics. The BGC responsible for the production of PBP has been identified in several *Pseudoalteromonas* species, a very diverse and ubiquitous group of marine bacteria. Strains from this genus are often isolated from

biofilms on both living and abiotic surfaces in the ocean, implying that they live in close association with other microbes (Skovhus et al. 2004; Skovhus et al. 2007). It is possible that the bacterial strains capable of synthesizing PBP may also produce other, more potent antibacterial compounds, and a small amount of PBP production enhances the potency of other secondary metabolites. The potentiation concept has not been described for *Pseudoalteromonas* and very little research has been done in this area for environmental bacteria. In addition to their therapeutic applications, identifying potentiator molecules may mediate microbial population dynamics in biofilms.

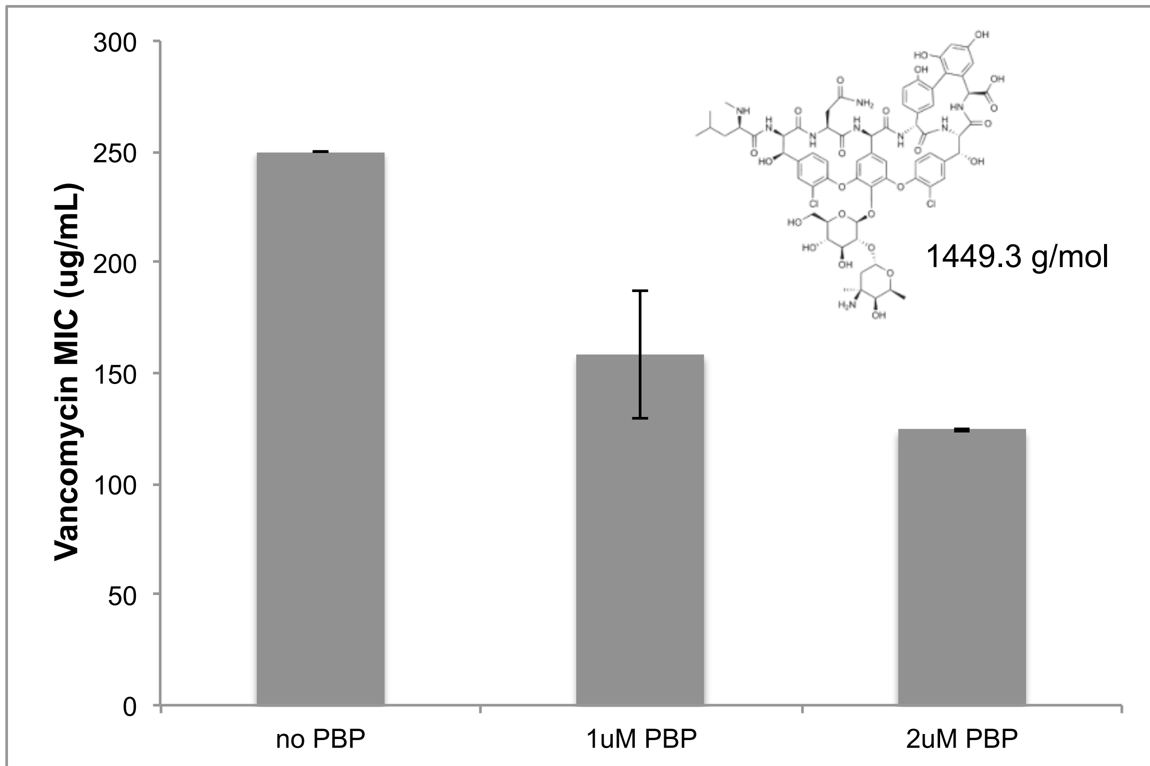


**Figure 3.6** Vancomycin potentiation assay of *E. coli* ATCC 25922.

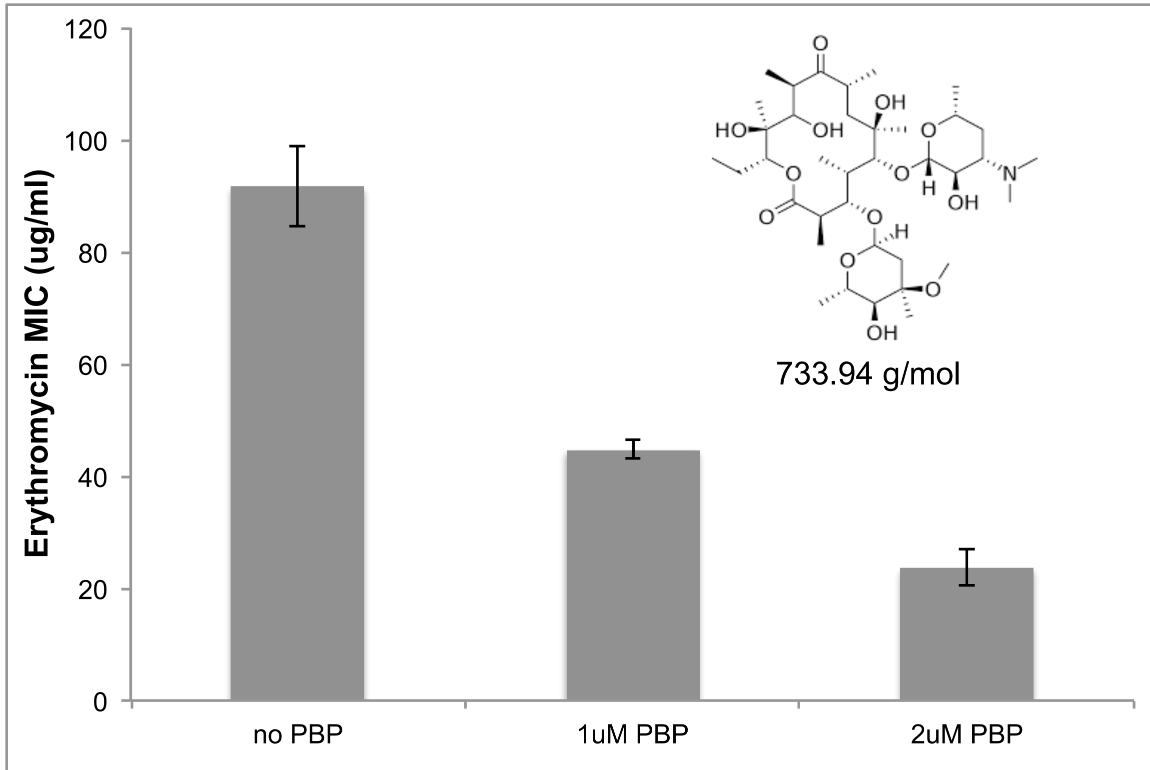

**Figure 3.7** Erythromycin potentiation assay of *E. coli* ATCC 25922.

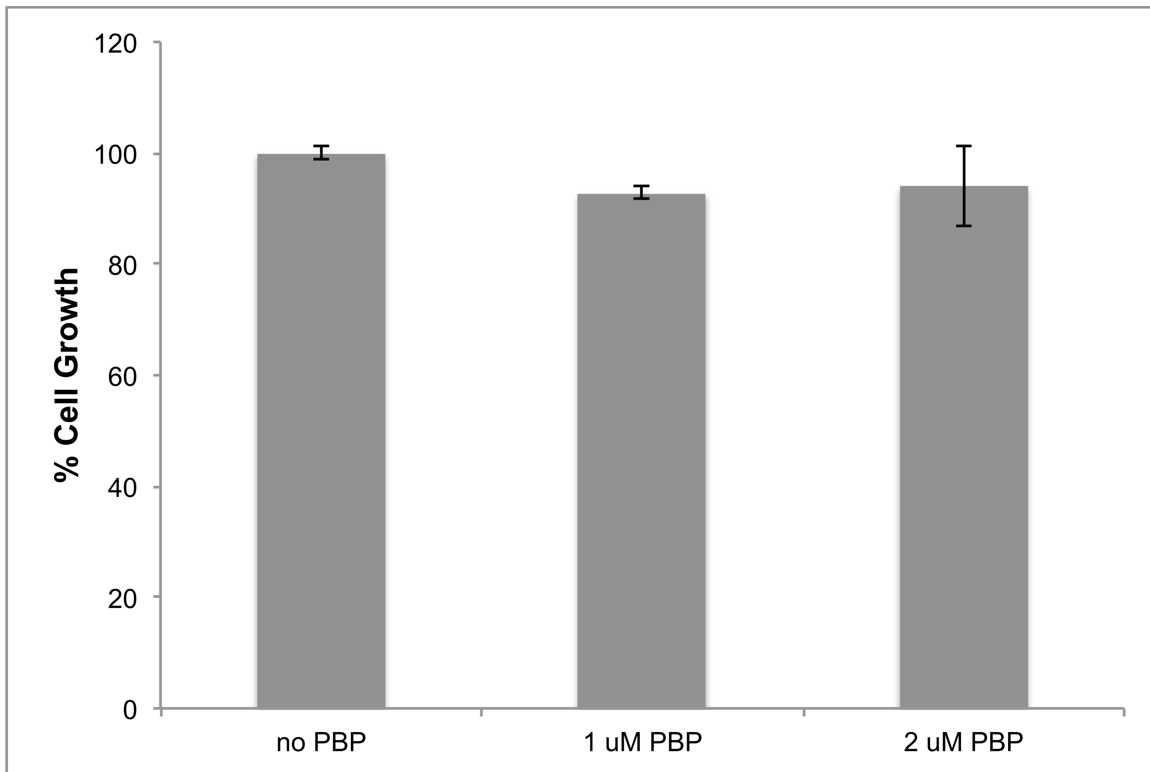

**Figure 3.8** Percent growth based on $OD_{600}$ ratio of untreated *E. coli* ATCC 25922 cells to cells treated with PBP. The sub-MIC concentrations had minimal effects on culture density after 24 hours.

### 3.5 Acknowledgements

Thanks to Joe Pogliano for allowing me to perform experiments in his lab and his advice and input on experimental design and data analysis. Elizabeth Montano was integral to the completion of this study, helping with the MIC assays and the cytological profiling work. Abdhesh Kumar synthesized the pentabromopseudilin, Joseph Sugie ran the SYTOX Green quantification analysis, and Evgenia Glukhov performed the cytotoxicity assay.

Chapter 3 is in prep and coauthored with Elizabeth Montano, Joseph Sugie, Abdhesh Kumar, William Fenical, Paul Jensen, and Joe Pogliano. The dissertation author was the primary investigator on these studies.

# Chapter 4: Genome mining and bioactive metabolite production in *Pseudoalteromonas*

## 4.1 Abstract

Species in the *Pseudoalteromonas* genus of marine bacteria have been well studied for their production of bioactive compounds but very few gene clusters have been linked to specific molecules. In this chapter, bioactivity assays of chemical extracts, genome mining for biosynthetic genes, and molecular networks were employed to explore the secondary metabolite potential of five *Pseudoalteromonas* species. Chemical extracts from all strains tested displayed some level of activity against *B. subtilis* PY70 with strains 2ta16 and PS5 displaying the most bioactivty. The genomes analyzed in this study were rich with biosynthetic gene clusters, only a very small number of which could be assigned predicted products. Similarly, the MS/MS molecular networks revealed numerous compounds that could not be identified. These results highlight the genus as a largely untapped resource of undiscovered bioactive secondary metabolites.

## 4.2 Introduction

*Pseudoalteromonas* is a genus of obligate marine bacteria that has been isolated from nearly every marine sample type including seawater, rock surfaces, and eukaryotic host organisms and multiple species have been reported to produce bioactive molecules compounds (Skovhus et al. 2004; Isnansetyo and Kamei 2003b; Offret et al. 2016). An interesting correlation has been observed between pigmentation and bioactivity in various species, and in the case of violacien, the pigment itself displays antibacterial activity (Egan et al. 2002; Huang et al. 2011; Thøgersen et al. 2016).

Despite the extensive research exploring the bioactive potential of *Pseudoalteromonas* species, very few molecules have been isolated and even fewer BGCs have been experimentally characterized. One BGC that has been investigated in

great detail both as a part of this thesis as well as previous studies is the *bmp* cluster. Each of the *bmp* genes has been experimentally characterized and the suite of compounds produced by the cluster is well known (Agarwal et al. 2014). Additionally, the bioactivity of some of the brominated metabolites was established prior to the discovery and description of the BGC (Isnansetyo and Kamei 2009). Another bioactive compound produced by a *Pseudoalteromonas* strain is YP1, a member of the tambjamine group for which the biosynthetic gene cluster has been proposed based on the results of a functional genomic study (Burke et al. 2007). Tambjamines have been isolated from nudibranchs, ascidians, and bryozoans, but *P. tunicata* was the first marine bacteria with confirmed production of these bioactive compounds (Blackman and Li 1994; Carbone et al. 2010; Lindquist and Fenical 1990).

*Pseudoalteromonas* is ubiquitous in the marine environment with species isolated from both poles and everywhere in between (Al Khudary et al. 2008; Bozal et al. 1997). The abundance and distribution of *Pseudoalteromonas* strains associated with eukaryotic hosts has been studied, but there is a very limited understanding of the specific ecological roles of these bacteria or the specific molecules they produce (Skovhus et al. 2004). In this regard, perhaps the most well studied metabolite produced by *Pseudoalteromonas* strains is tetrabromopyrrole, a product of the *bmp* gene cluster. The brominated pyrrole induces the settlement and development of coral larvae and is an example of a synergistic relationship between a microbial secondary metabolite and a eukaryotic host (Tebben et al. 2011; Sneed et al. 2014). It's also possible that *Pseudoalteromonas* produce compounds that function as feeding deterrents for seaweeds, filter feeders, and other invertebrates, but no studies have established these roles to date. Many of the bioactive

compounds produced by *Pseudoalteromonas* species may be used to ward off other bacteria that are competing for space on the surface of or nutrients from larger eukaryotic marine organisms. It has also been hypothesized that producing antimicrobial compounds in biofilms helps keep the community diversity high by ensuring that there isn't a single winning species of bacteria (Rao, Webb, and Kjelleberg 2005).

The aims of this study were to evaluate the metabolite production of five *Pseudoalteromonas* species and identify known bioactive compounds. Additionally, the genomes were mined for BGCs using antiSMASH v3.0 (Weber et al. 2015) to investigate the biosynthetic potential of the strains. The bioactive metabolite production of each strain was assessed by testing the crude extracts as well as fractions in disc diffusion assays and these results were mapped onto molecular networks. Although there were only a small number of dependable library hits for the chemical data, there are many candidate novel molecules, especially in strain 2ta16 worthy of further investigation. The number of biosynthetic gene clusters found in *Pseudoalteromonas* genomes vastly outnumbers the molecules reported. In the present study, bioactivity-guided assays in combination with chemical analysis and genome mining help identify strains and BGCs of interest.

## 4.3 Materials and Methods

### 4.3.1 Strain growth and chemical extractions

Five *Pseudoalteromonas* strains were used in this study: 2ta16, 2ta6, O-BC30, A757, and PS5. Each strain was grown in 1 L of marine broth (5 g peptone, 1 g yeast extract, 22 g/L instant ocean) for 24 hours at 30°C with agitation (230 rpm). All of the strains were grown with and without 1 g/L of KBr supplemented in the media. Adding 1 g/L of KBr to marine broth medium can help increase the production level of brominated

molecules, making them easier to detect by LC/MS. The liquid cultures were extracted twice with equal volumes of ethyl acetate (EtOAc) and the combined extracts were dried *in vacuo*. Media controls of the same volume (1 L) were also extracted using the same methods. The crude extracts were weighed and re-suspended in HPLC-grade MeOH to a concentration of 10 mg/mL for use in disc diffusion assays and LC-MS analysis.

**4.3.2 Fractionation of crude extracts**

Between 20-30 mg of crude extract generated from the five *Pseudoalteromonas* strains used in this study were fractionated using normal phase flash chromatography. Only crude extracts without 1 g/L of KBr added to media were fractionated because the bioactivity assay results showed no relationship between KBr-supplemented media and increased activity of extracts. A step-wise gradient of a series of solvent compositions were passed through a column packed with Silica sorbent (particle size 40-60 µm, pore size 60 Å, mesh 230-400, Agela Technologies) in order of increasing polarity in 10 mL volumes: 1) hexane, 2) 1:3 ethyl acetate (EtOAc):hexane, 3) EtOAC, 4) 5:95 methanol (MeOH):dichloromethane (DCM), 5) 1:4 MeOH:DCM, 6) 1:1 MeOH:DCM, 7) MeOH, 8) MeOH. The fractions were dried under a stream of nitrogen gas, weighed, and re-suspended in HPLC-grade MeOH at a concentration of 2 mg/mL for use in disc diffusion assays and LC-MS analysis.

**4.3.3 Disc diffusion assays**

The crude extracts and media controls (with and without KBr added) were serially diluted 1:10 in MeOH and 20 µL of each concentration (10 mg/mL, 1 mg/mL, 0.1 mg/mL, and 0.01 mg/mL) and solvent controls added to sterile paper discs and allowed to dry. 10 mL liquid cultures of the test strains, ATCC 25922 (*E. coli*) and PY79 (*Bacillus*

*subtilis*) were inoculated from overnight cultures into 10 mL of LB at and shaken at 230 rpm until an $OD_{600}$ between 0.2-0.4 (exponential phase) was reached. 100 μL of each strain was then spread onto three replicate LB plates and the discs placed directly on the media. The assay plates were incubated for 24 hours at 37°C and the radii of zones of inhibition were measured and reported. The fractions were tested at 2 mg/mL against the *Bacillus subtilis* PY79 strain.

**4.3.4 Chemical analyses of crude extracts and fractions**

Samples were analyzed on an Agilent 1260 Infinity LC system equipped with a diode array detector and coupled with a Bruker amaZon SL ion-trap mass spectrometer. Extracts were prepared in MeOH at a concentration of 2 mg/mL and 5 μL was injected onto a Phenomenex Kinetex $C_{18}$ column (5 μm, 100 Å, 150 mm x 4.6 mm) coupled to a $C_{18}$ guard cartridge and eluted using a gradient of acetonitrile (ACN) and water ($H_2O$). Solvents were prepared with 0.1% formic acid when acquiring data in positive mode. The flow rate was 0.75 mL/min beginning with an isocratic hold of 5% ACN for 3 min, followed by a gradient from 5-100% ACN over 10 min and a 4 min isocratic hold at 100% ACN. The column was re-equilibrated with a gradient from 100-5% ACN over 1 min before the next injection. Solvent control injections (10 μL of MeOH) were performed after every fourth sample for crudes and every eighth sample for fractions. The LC flow was sent to the source between minutes 5-17 and the remaining time was diverted to waste.

Mass spectra were collected using Ultrascan mode over a range of 70-2200 *m/z* and speed of 32500 *m/z* per second. Molecular ions were generated using electrospray ionization (ESI) with the following parameters: Capillary 3500 V, end plate offset 500 V,

nebulizer 2.0 bar, dry gas 9 L/min, and dry temp 250 °C. Ion charge control was implemented with the following settings: Target 200000, max accu time 100 ms, range 100-2000 *m/z*, and 3 averages. Tandem MS was collected using auto MS/MS mode with n = 2 and set to collect two precursor ions that are excluded after acquisition of two spectra with the exclusion released after 1.0 minutes. UV absorption was detected using ultraviolet and visible lamps over a range of 190-800 nm with a step of 2.0 nm.

4.3.5 Molecular Networking

A molecular network was created using the online workflow at GNPS (https://gnps.ucsd.edu/ProteoSAFe/static/gnps-splash.jsp). The data were filtered by removing all MS/MS peaks within +/- 17 Da of the precursor *m/z*. MS/MS spectra were window filtered by choosing only the top 6 peaks in the +/- 50Da window throughout the spectrum. The data was then clustered with MS-Cluster with a parent mass tolerance of 2.0 Da and a MS/MS fragment ion tolerance of 0.5 Da to create consensus spectra. Further, consensus spectra that contained less than 2 spectra were discarded. A network was then created where edges were filtered to have a cosine score above 0.7 and more than 6 matched peaks. Further edges between two nodes were kept in the network if and only if each of the nodes appeared in each other's respective top 10 most similar nodes. The spectra in the network were then searched against GNPS' spectral libraries. The library spectra were filtered in the same manner as the input data. All matches kept between network spectra and library spectra were required to have a score above 0.7 and at least 6 matched peaks

## 4.4 Results

### 4.4.1 Bioactivity of *Pseudoalteromonas* extracts

In this study, extract from *Pseudoalteromonas* strains 2ta16, 2ta6, O-BC30, A757, and PS5 were tested for bioactivity and their genomes were mined for BGCs (Table 4.1). These strains were selected for their accessibility for culture work as well as available genome sequences. Strain O-BC30 is the *P. phenolica* type strain and the other four strains used in this study were classified based on the results reported in Chapter 2 of this thesis. Strain 2ta16 and A757 are proposed novel species, PS5 has been classified as *P. peptidolytica*, and *P. elyakovii, P. piscicida*, and strain 2ta6 are the same species based on their ANI values (Figure 2.2, Figure 2.3).

**Table 4.1** Pseudoalteromonas strains used in study.

| Strain ID | Genome Accession number | Classification |
|---|---|---|
| 2ta16 | AUSV00000000 | *Pseudoalteromonas* sp. |
| 2ta6 | 2505119014 (JGI) | *Pseudoalteromonas elyakovii/piscicida* |
| O-BC30 | RCWG00000000 | *Pseudoalteromonas phenolica* |
| A757 | QNQN00000000 | *Pseudoalteromonas* sp. |
| PS5 | RCSQ00000000 | *Pseudoalteromonas peptidolytica* |

The crude extracts from each strain, grown with and without 1 g/L of KBr added to the media, were tested in disc diffusion assays using *E. coli* ATCC 25922 and *B. subtilis* PY79 as test strains. The only extracts displaying bioactivity against *E. coli* were generated from 2ta16 and PS5 (Table 4.2, Figure 4.1). *Pseudoalteromonas* strain PS5 displayed bioactivity against *E. coli* when grown with and without the bromine supplement, however, only the extract from 2ta16 grown without KBr added to media was active. The average zone of inhibition produced by strain 2ta16 was 0.83 mm while the crude extracts of PS5 had average zone of inhibition of 2.17 mm and 1.33 with and

without KBr added, respectively. None of the extracts were active against *E. coli* ATCC 25922 at concentrations lower than 10 mg/mL.

All of the *Pseudoalteromonas* crude extracts were active against *B. subtilis* PY79 (Figure 4.1). Both crude extracts from strain 2ta16 were again the most active resulting in a zone of inhibition of 6.3 mm for the culture grown without KBr and 5 mm for the culture with KBr added. PS5 was also highly bioactive against the Gram-positive strain, with the extract from the KBr-supplemented culture producing a larger ZOI, consistent with the assay results with the *E. coli* strain. There was no observed difference in the activity of extracts from A757 with and without KBr added, while both O-BC30 and 2ta6 were slightly more active when grown in KBr-supplemented media. The only strain with a bioactive extract against *B. subtilis* PY79 at concentrations lower than 10 mg/mL was 2ta16 (Table 4.3, Figure 4.2). At all of the lower concentrations tested, the 2ta16 extracts from the cultures grown without KBr added to the media were more active than those grown with added bromine.

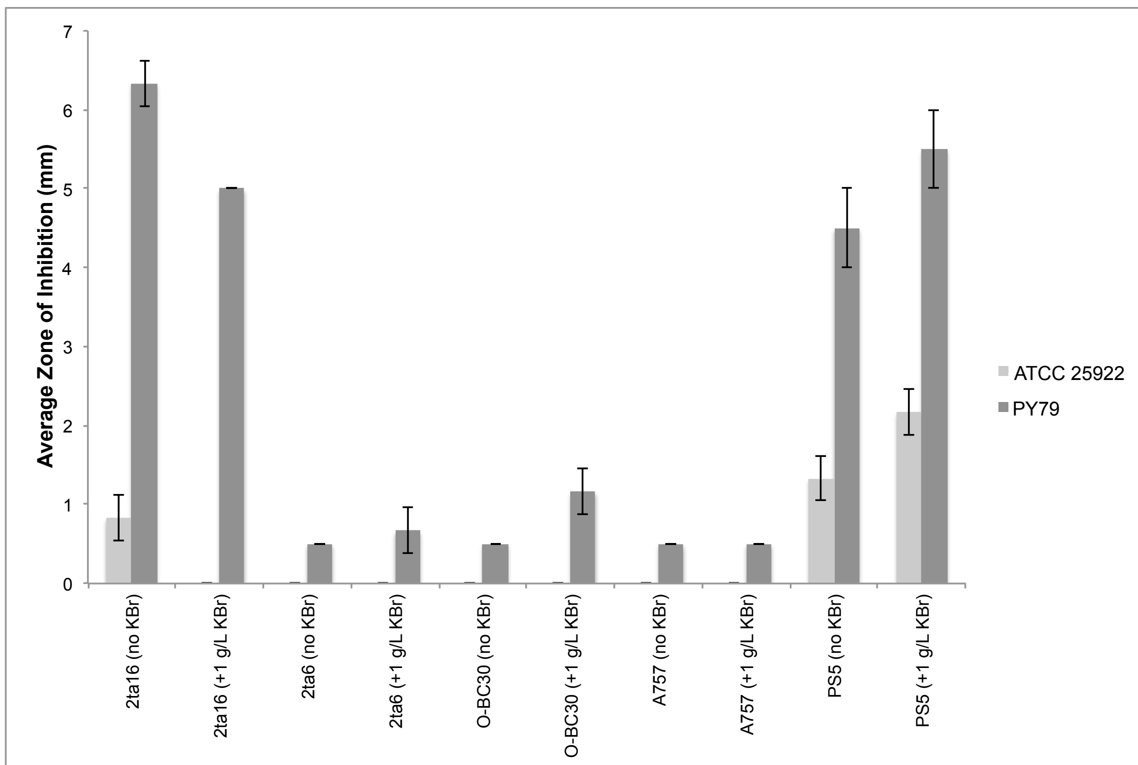

**Figure 4.1** Disc diffusion assay results for *Pseudoalteromonas* crude extracts at 10 mg/mL concentration. *E. coli* ATCC 25922 and *B. subtilis* PY79 were the test strains. Activities reported as the diameter of the region of no growth surrounding the disc.

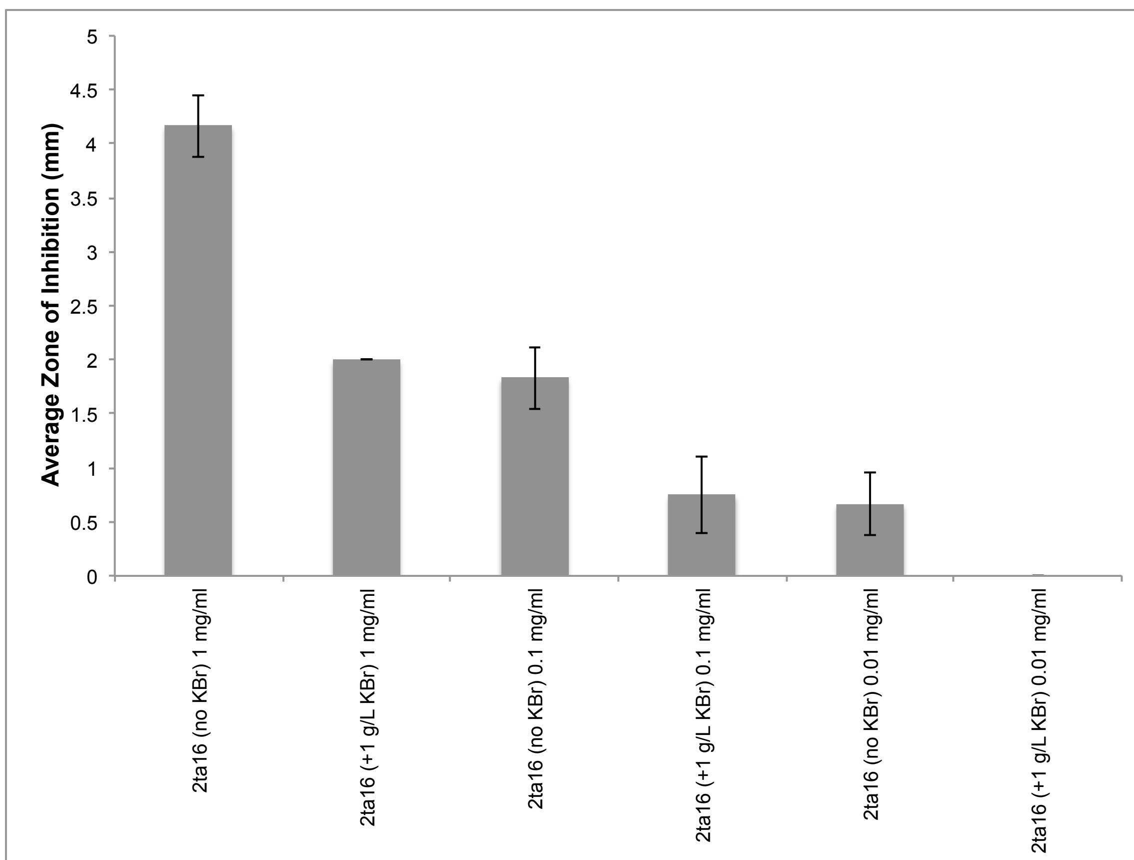


**Figure 4.2** Disc diffusion assay results for strain 2ta16 crude extracts at lower concentration. *B. subtilis* PY79 was the test strain.

### 4.4.2 LC-MS analysis of *Pseudoalteromonas* crude extracts

The crude extracts of all five strains grown with and without added KBr were analyzed by LC-MS with molecular networks generated from the MS/MS fragmentation data (Nguyen et al. 2013). Molecular ions detected in the blank (MeOH injection) and media samples were removed from the network and the remaining nodes labeled based on media type, bioactivity, and strain. The *Pseudoalteromonas* crude extracts were run in both positive and negative modes to ensure a comprehensive chemical profile for each strain.

There were no molecular families from the samples acquired in positive or negative mode that were specific to extracts from bacterial cultures grown with or

without KBr added to the media (Figure 4.3, Figure 4.4). Interestingly, in both the positive and negative mode data, there were several molecular families that were exclusive to certain strains; every strain except for 2ta6 has at least one molecular family comprised of strain-specific nodes (Figure 4.5, Figure 4.6). These strain-specific molecular families were also correlated with the bioactivity results (Figure 4.7, Figure 4.8).

A total of six library hits were identified for mass spectra acquired in negative mode and 16 in positive mode (Table 4.4). Bromoalterochromide A was detected in the crude extracts with 17 shared peaks and a cosine value of 0.87. There was a reasonable match for an Arenimycin-like compound, most likely an analogue of Arenimycin A.

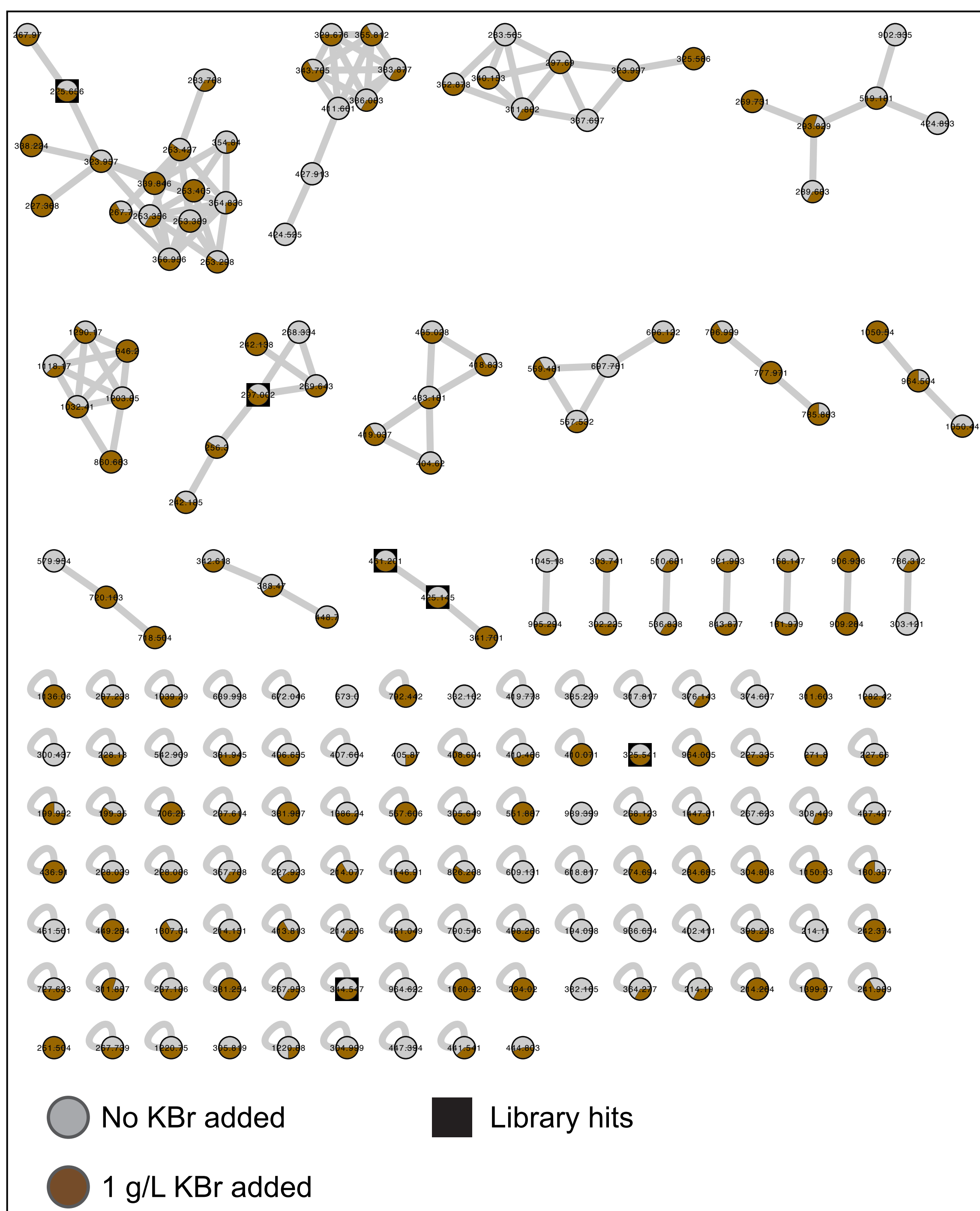


**Figure 4.3** Molecular network of crude extracts from strains 2ta16, O-BC30, PS5, A757, and 2ta6 acquired in negative mode. Each node is colored based on media type and GNPS library hits are indicated with a black square.

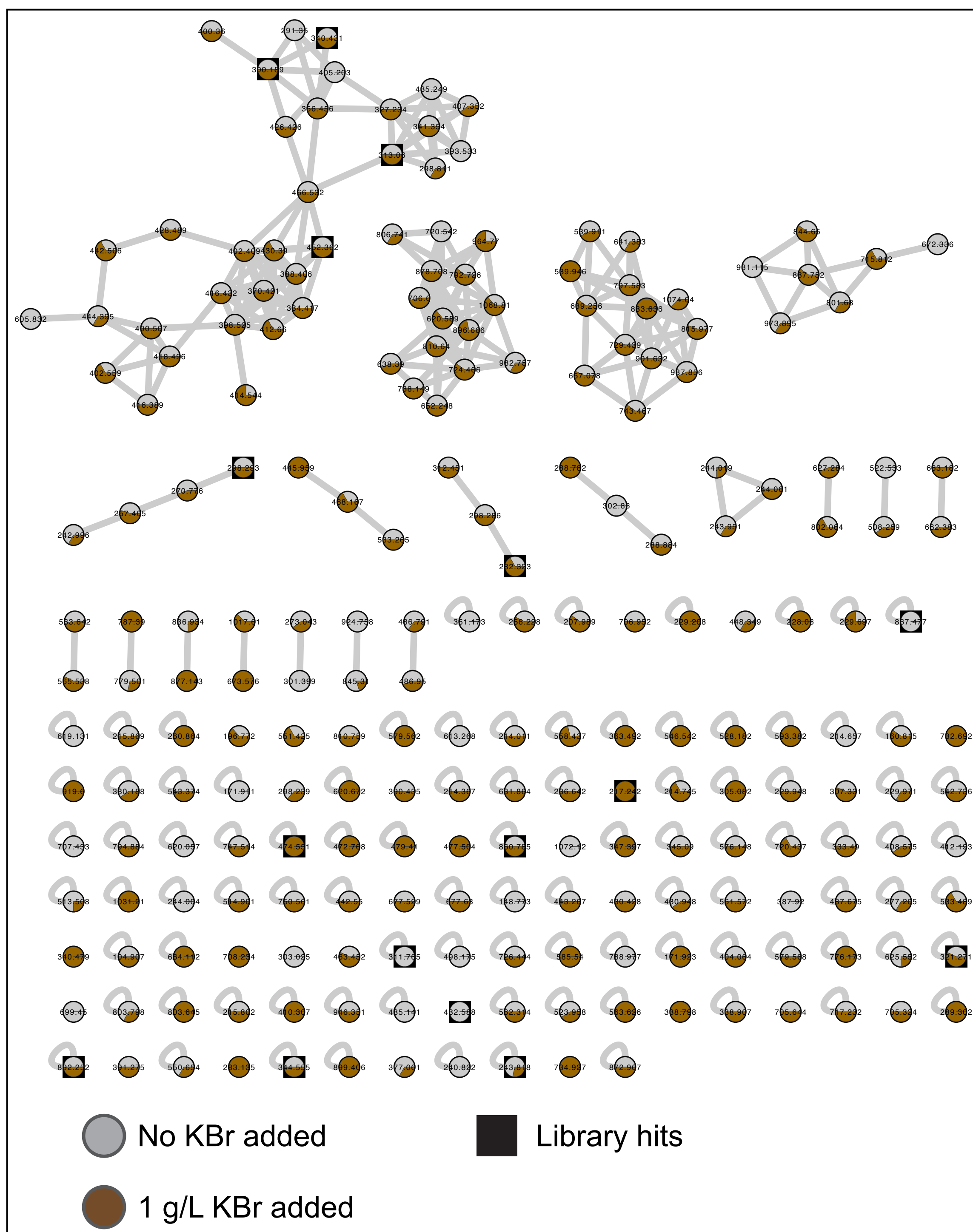


**Figure 4.4** Molecular network of crude extracts from strains 2ta16, O-BC30, PS5, A757, and 2ta6 acquired in positive mode. Each node is colored based on media type and GNPS library hits are indicated with a black square

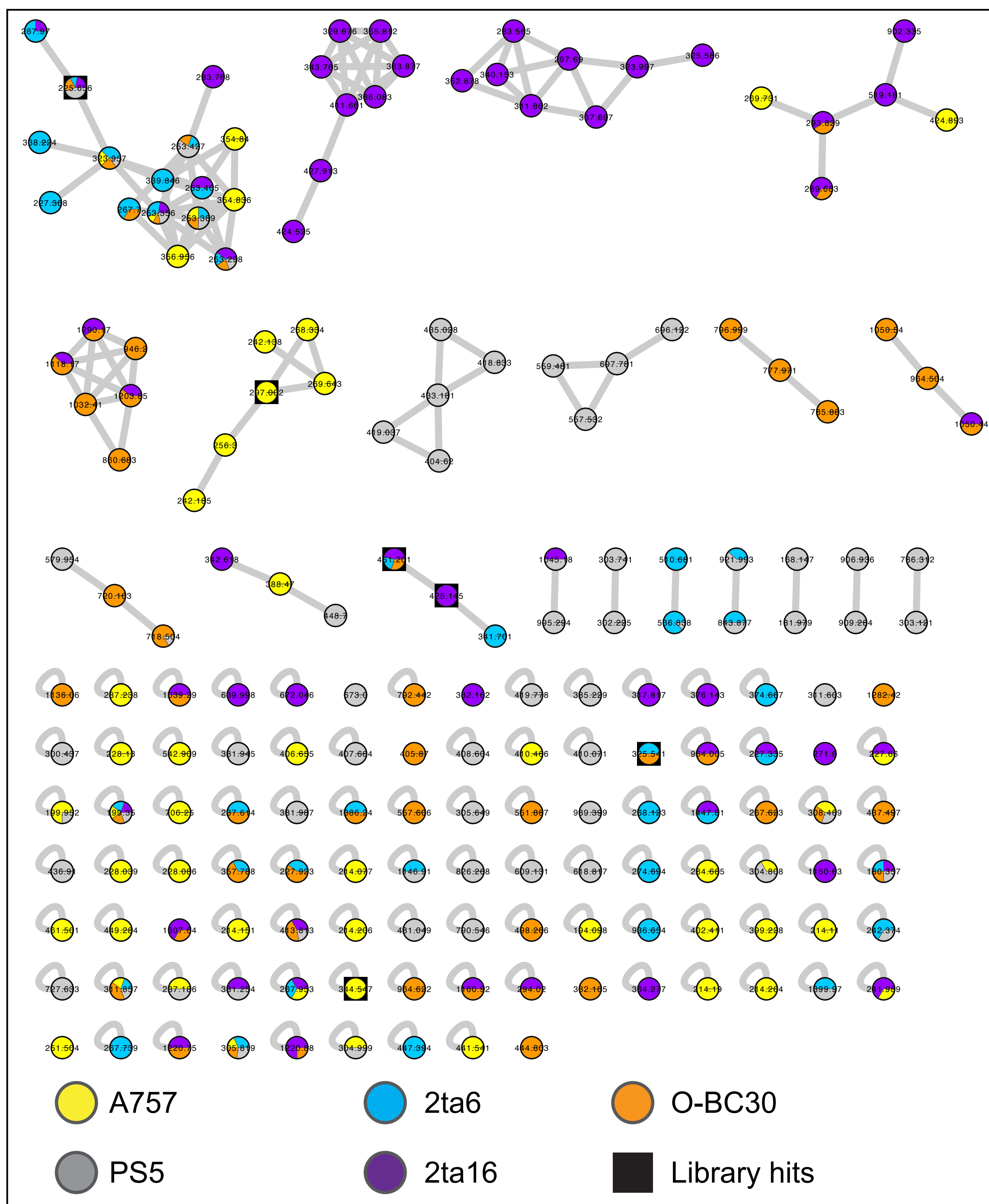


**Figure 4.5** Molecular network of crude extracts from strains 2ta16, O-BC30, PS5, A757, and 2ta6 acquired in negative mode. Each node is colored based on strain and GNPS library hits are indicated with a black square.

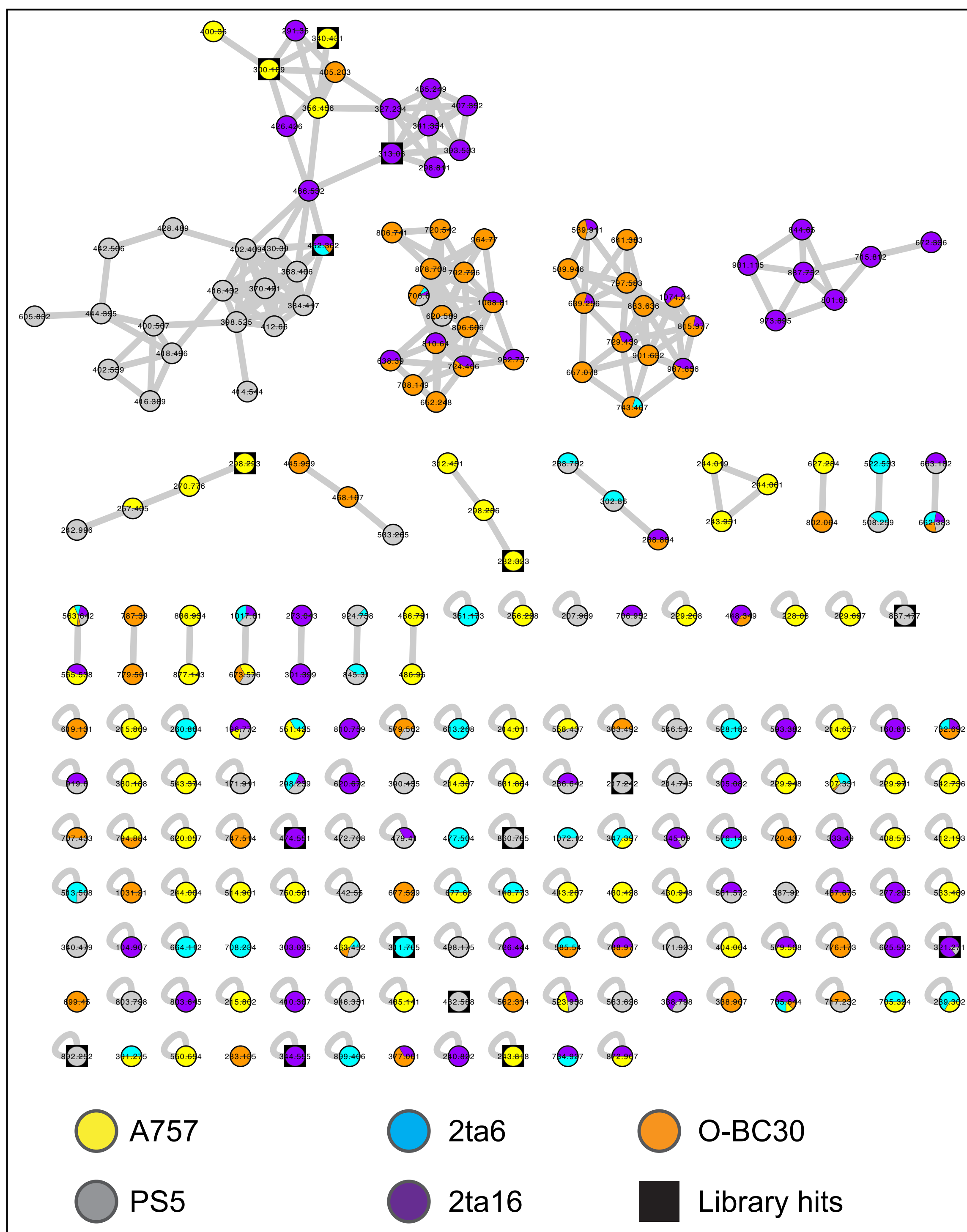


**Figure 4.6** Molecular network of crude extracts from strains 2ta16, O-BC30, PS5, A757, and 2ta6 acquired in positive mode. Each node is colored based on strain and GNPS library hits are indicated with a black square.

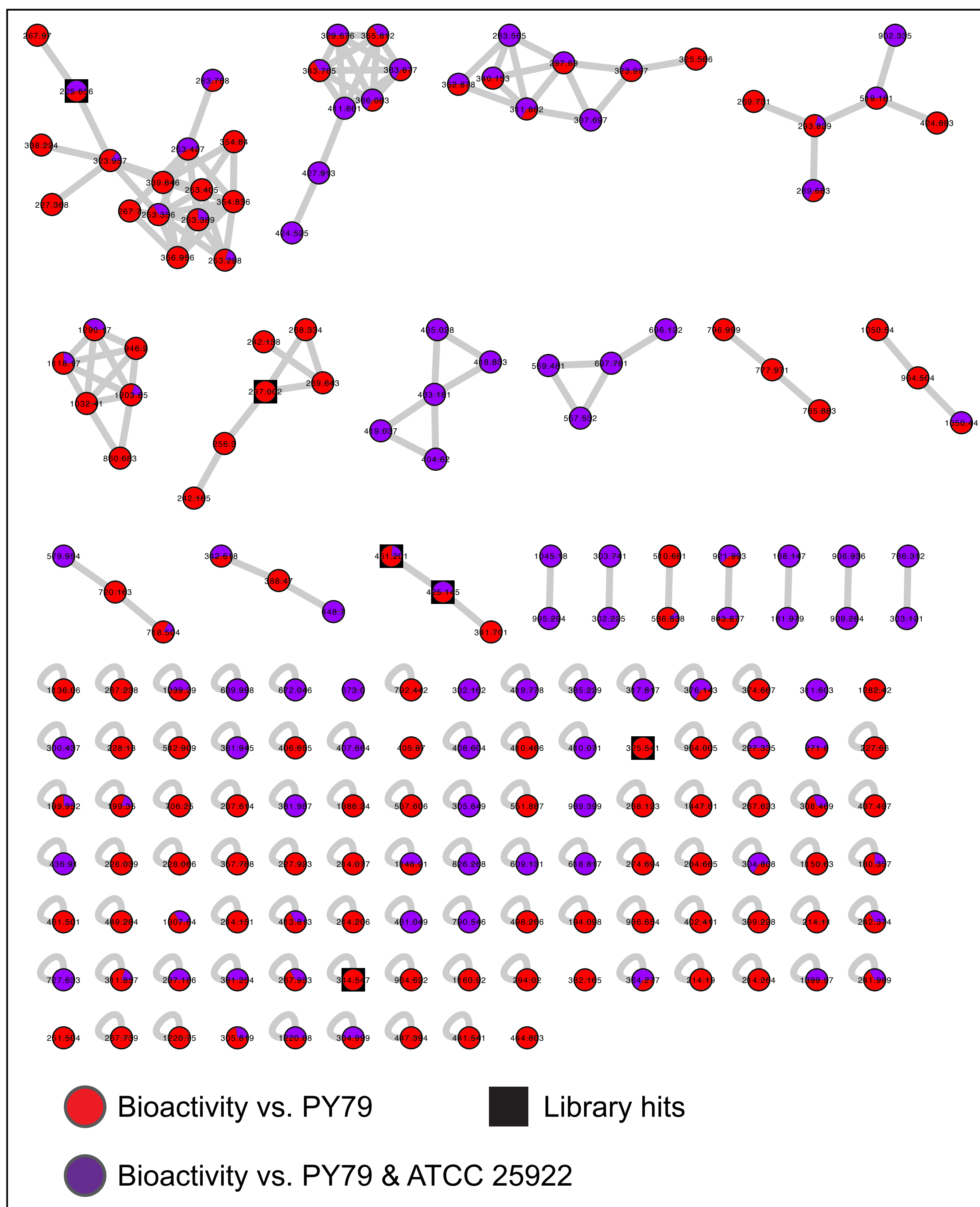


**Figure 4.7** Molecular network of crude extracts from strains 2ta16, O-BC30, PS5, A757, and 2ta6 acquired in negative mode. Each node is colored based on bioactivity and GNPS library hits are indicated with a black square. Red colored nodes represent fragments from a crude extract that was active against PY79 while those from extracts active against both strains are indicated in purple.

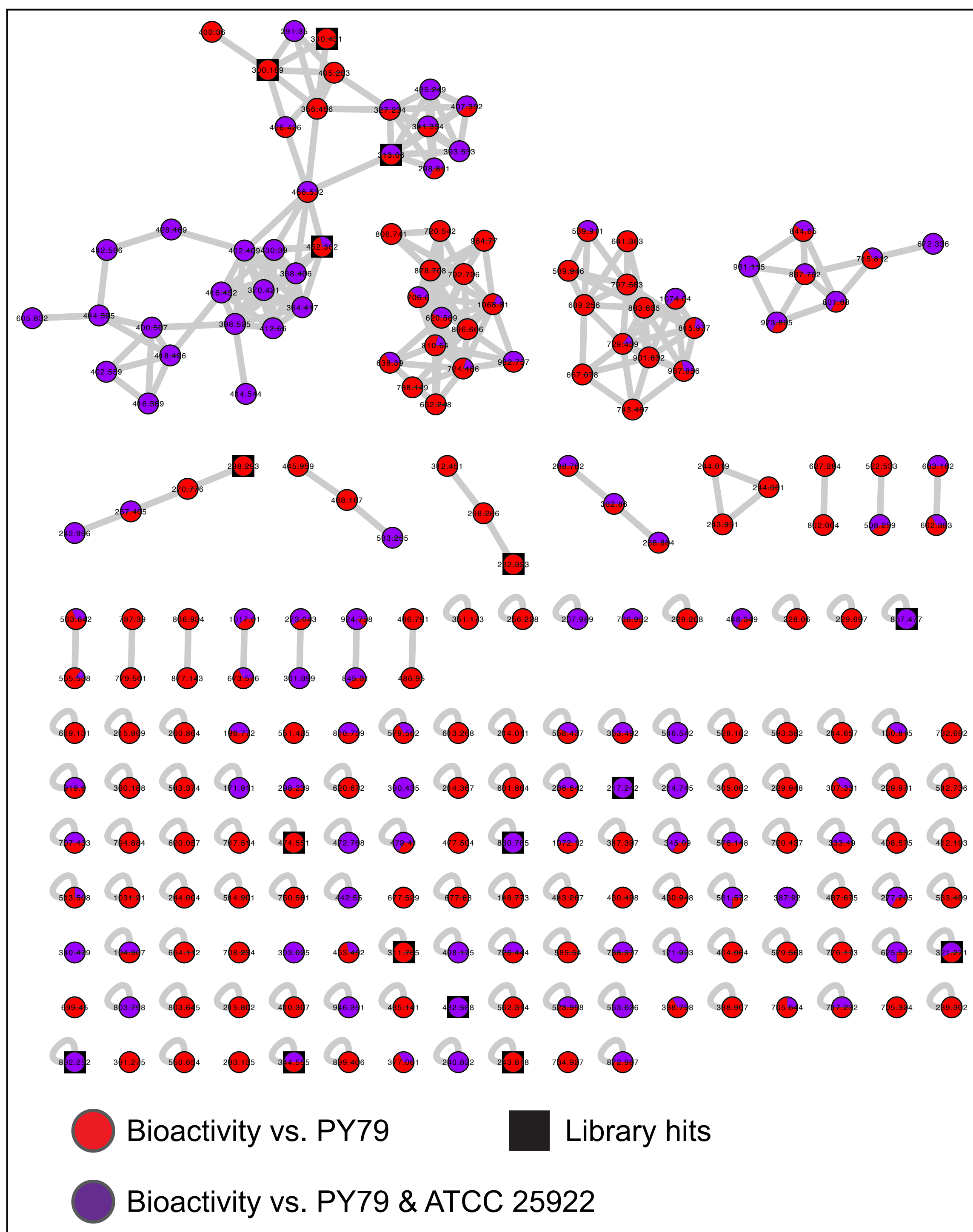


**Figure 4.8** Molecular network of crude extracts from strains 2ta16, O-BC30, PS5, A757, and 2ta6 acquired in positive mode. Each node is colored based on bioactivity and GNPS library hits are indicated with a black square. Red colored nodes represent fragments from a crude extract that was active against PY79 while those from extracts active against both strains are indicated in purple.

**Table 4.2** GNPS library hits found in *Pseudoalteromonas* crude extracts. The asterisk (*) refers to spectral matches, not direct library hits.

| Compound Name | Shared peaks | MassDiff | Mode | Cosine | Precursor mass |
|---|---|---|---|---|---|
| phenazine-1-carboxylic acid | 6 | 0.656006 | negative | 0.79 | 225.656 |
| *Spectral Match to 1-Myristoyl-2-hydroxy-sn-glycero-3-phosphoethanolamine from NIST14 | 6 | 0.895996 | negative | 0.82 | 425.145 |
| Pesticide3_Iprovalicarb Isomer 1_C18H28N2O3_Isopropyl [(2S)-3-methyl-1-{[1-(4-methylphenyl)ethyl]amino}-1-oxo-2-butanyl]carbamate | 6 | 1.34799 | negative | 0.79 | 344.547 |
| PE(16:1/0:0); [M-H]- C21H41N1O7P1 | 8 | 0.0690002 | negative | 0.84 | 451.201 |
| MLS001195659-01 sumatriptan | 8 | 0.864014 | negative | 0.81 | 297.002 |
| 2,4-dihydroxyheptadec-16-enyl acetate | 6 | 1.71301 | negative | 0.73 | 325.541 |
| xenorhabdin 1 | 6 | 0.0429993 | positive | 0.72 | 313.06 |
| phenylethylamide 343 | 8 | 0.260986 | positive | 0.74 | 344.555 |
| UHQ C11:1 aka 2-undecenyl-quinoloin-4(1H)-one position of double bond unknown | 7 | 0.0759888 | positive | 0.85 | 298.293 |
| TriamcinoloneAcetonide | 6 | 0.842041 | positive | 0.96 | 892.252 |
| * Spectral Match to Palmitoleoyl 3-carbacyclic phosphatidic acid from NIST14 | 6 | 1.968 | positive | 0.73 | 217.242 |
| * Spectral Match to Monopalmitolein (9c) from NIST14 | 11 | 0.505005 | positive | 0.82 | 311.765 |
| *Spectral Match to D-erythro-C18-Sphingosine from NIST14 | 6 | 0.0950012 | positive | 0.81 | 300.189 |
| *Spectral Match to 11-Deoxyprostaglandin F1.alpha. from NIST14 | 6 | 1.98502 | positive | 0.73 | 321.271 |
| * Spectral Match to 1-Palmitoyl-2-hydroxy-sn-glycero-3-phosphoethanolamine from NIST14 | 7 | 1.72601 | positive | 0.89 | 474.551 |
| * Spectral Match to 1-Palmitoyl-2-hydroxy-sn-glycero-3-phosphoethanolamine from NIST14 | 7 | 1.931 | positive | 0.73 | 452.362 |
| Simvastatin | 6 | 1.24896 | positive | 0.92 | 860.785 |
| PE(18:0/0:0); [M+H]+ C23H49N1O7P1 | 6 | 1.27103 | positive | 0.82 | 482.588 |
| NCGC00385643-01_C20H30O3_1-Phenanthrenecarboxylic acid, 7-ethenyl-1,2,3,4,4a,4b,5,6,7,9,10,10a-dodecahydro-9-hydroxy-1,4a,7-trimethyl- | 6 | 0.778015 | positive | 0.90 | 340.431 |

**Table 4.2** GNPS library hits found in *Pseudoalteromonas* crude extracts (continued).

| Compound Name | Shared peaks | MassDiff | Mode | Cosine | Precursor mass |
|---|---|---|---|---|---|
| HHQ aka 2-heptylquinolin-4(1H)-one | 6 | 0.352005 | positive | 0.86 | 243.818 |
| Bromoalterochromide A/A' | 17 | 0.80304 | positive | 0.87 | 867.477 |
| 35212-22-7 (Ipriflavone) | 6 | 1.20599 | positive | 0.75 | 282.323 |

### 4.4.3 Bioactivity of fractions vs. PY79

Crude extracts from the five *Pseudoalteromonas* strains were separated into eight fractions using flash chromatography. Because there was no consistent or substantial difference in the bioactivity between the extracts from strains cultured with and without KBr-supplemented media, only the extracts with no added KBr were fractionated. The fractions were re-suspended in MeOH to a final concentration of 2 mg/mL and tested for bioactivity in a disc diffusion assay with *B. subtilis* as the challenge strain.

The majority of the strains had no active fractions while nearly all of the 2ta16 fractions (Fractions 3-8) resulted in zones of inhibition (Figure 4.9). Fraction 5 of 2ta16 was the most active with an average ZOI of 4.83 mm. Additionally, PS5 had a single active fraction (fraction 2) with an average ZOI of 1.17 mm, and it was the only strain for which fraction 2 was active. Fraction 1 was not active for any of the strains at the tested concentration.

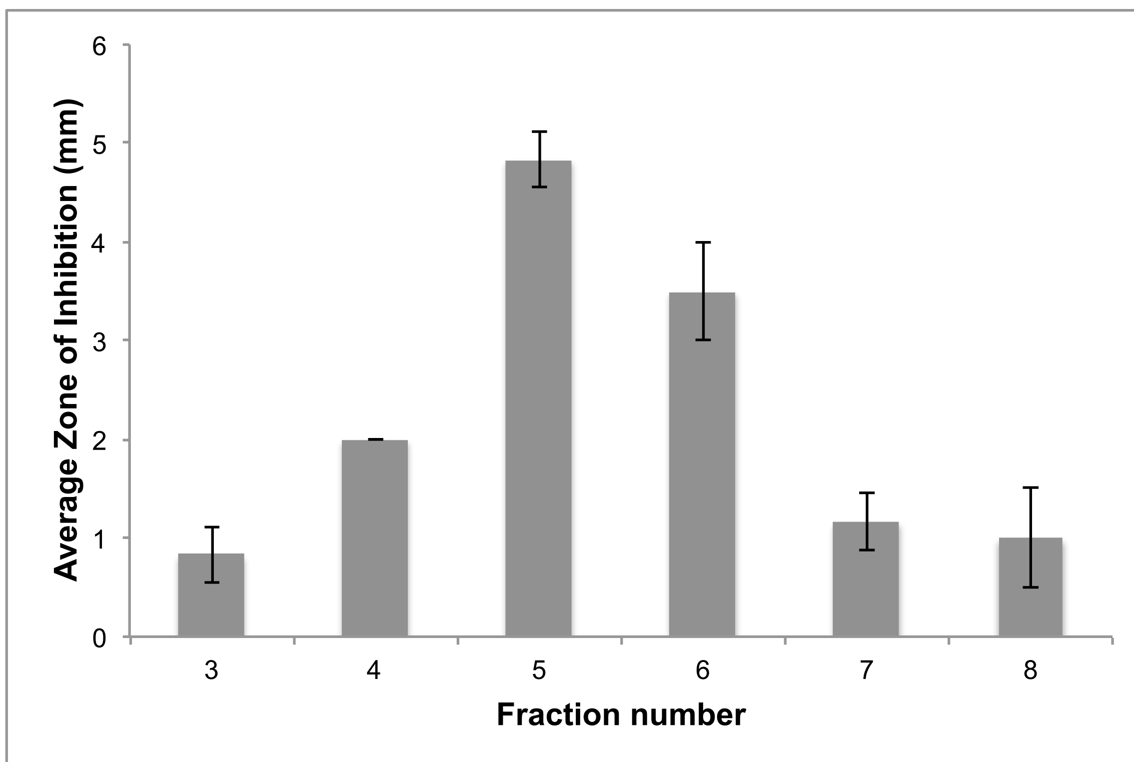

**Figure 4.9** Disc diffusion assay results for 2ta16 active fractions at 2 mg/mL concentration. *B. subtilis* PY79 was the challenge strain.

### 4.4.4 LC-MS analysis of *Pseudoalteromonas* fractions

To identify molecular families associated with observed activity, all eight fractions from strains 2ta16, PS5, and 2ta6 were analyzed using LC-MS and the data was used to construct molecular networks. The results include entire molecular families as well as subsets of larger families that are exclusive to strains 2ta16 or PS5 (Figure 4.10, Figure 4.11). No molecular families specific to strain 2ta6 were observed. These results suggest that strains 2ta16 and PS5 both produce distinct molecules, some of which are active against *B. subtilis* PY79.

The samples acquired in positive mode revealed a library hit for bromoalterochromide that displayed 19 shared peaks and a cosine value of 0.92 when compared with the library spectrum. There was also a reasonable match for the antibiotic

chloramphenicol (6 shared peaks, cosine = 0.81). Alteramide B, a compound previously isolated from *Pseudoalteromonas* (Shiroyama et al. 2017), was found with nine shared peaks and a cosine value of 0.73 with the library spectrum. The same fractions acquired in negative mode did not produce any reliable library hits.

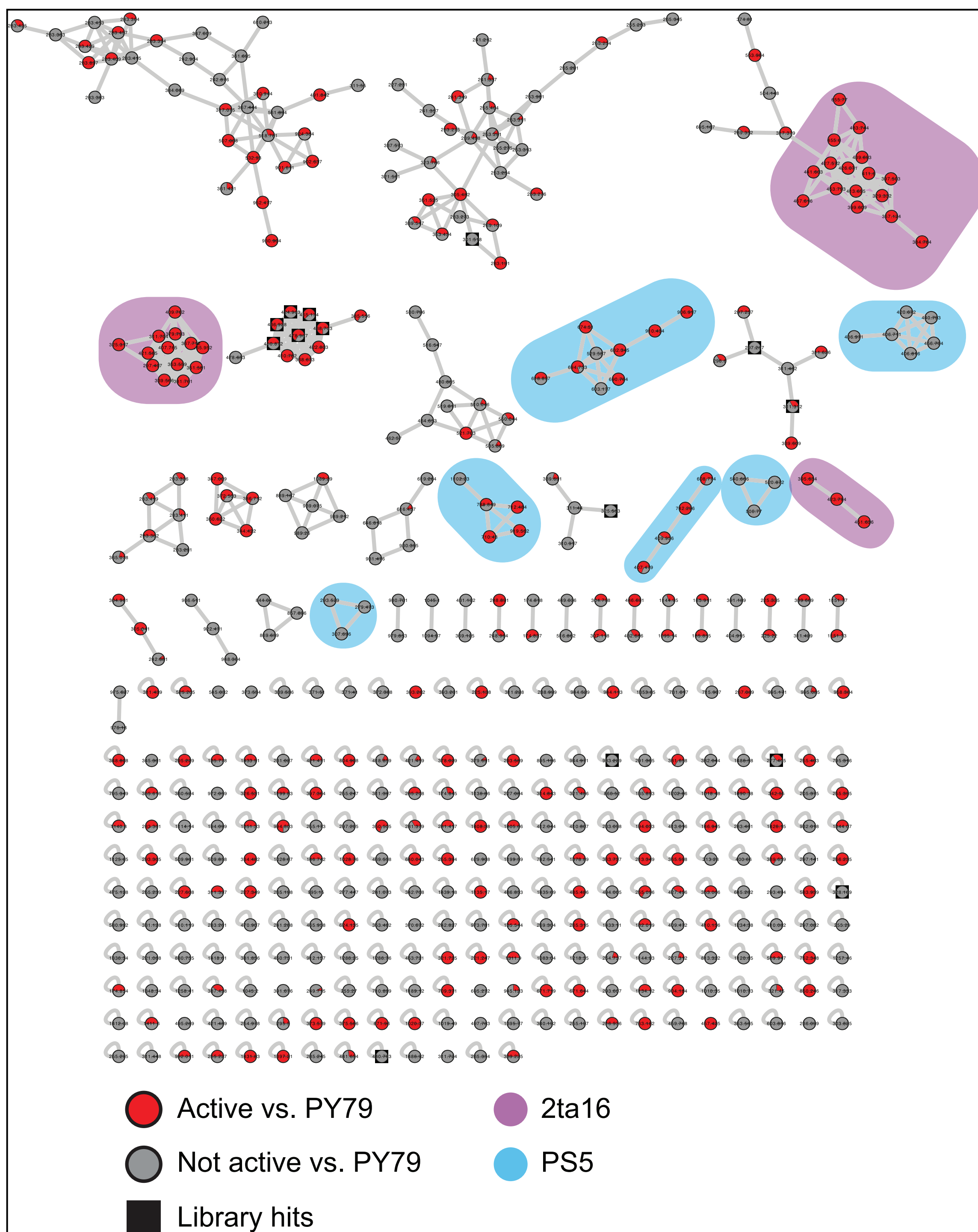


**Figure 4.10** Molecular network of fractions from strain 2ta16, PS5, 2ta6 acquired in negative mode. The red coloring on nodes indicates the proportion of spectra from active fractions. The molecular families (or sections of molecular families) highlighted in purple represent molecular ions found exclusively in strain 2ta16. Those in blue are restricted to strain PS5. GNPS library hits are indicated with a black square.

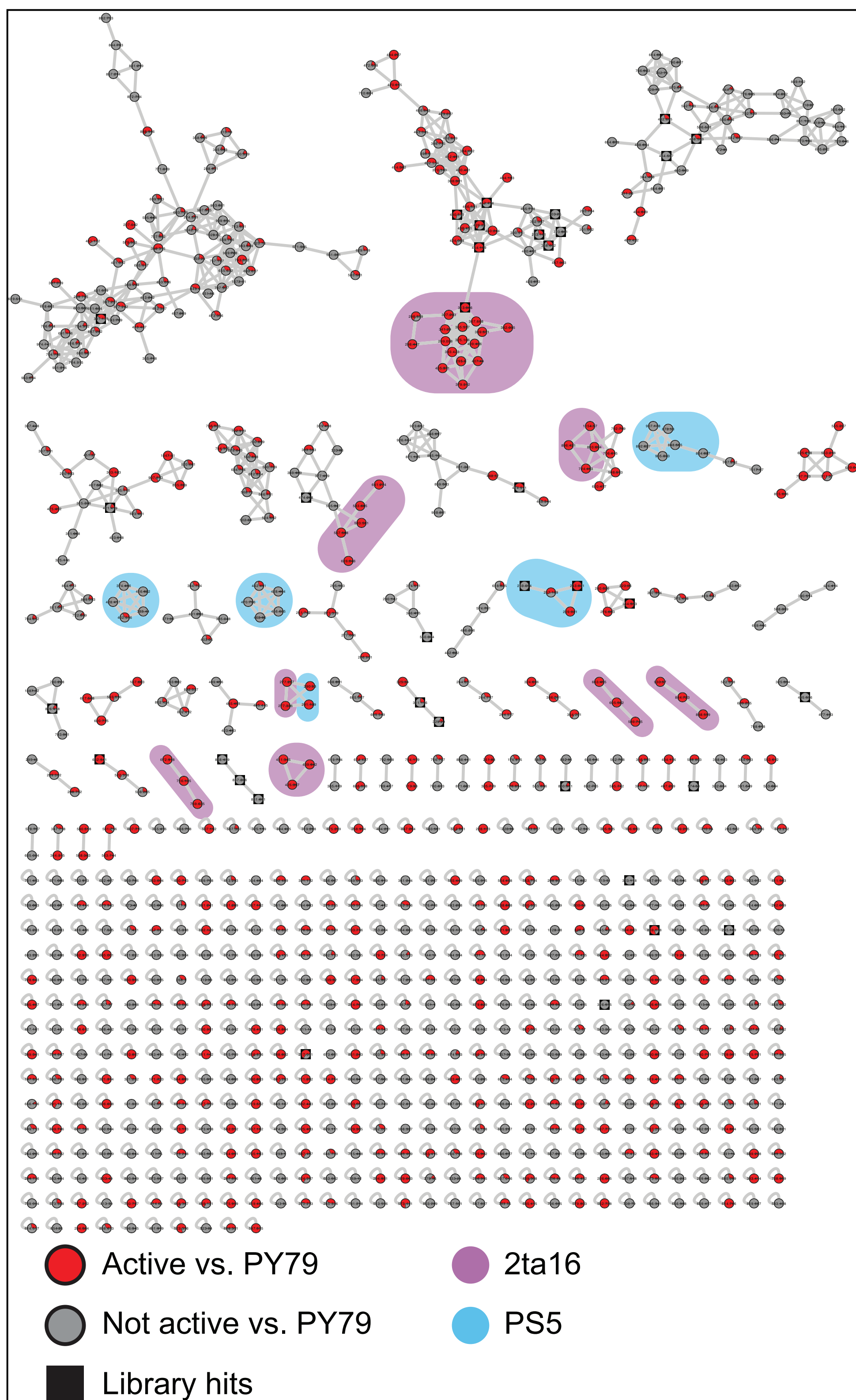


**Figure 4.11** Molecular network of fractions from strain 2ta16, PS5, 2ta6 acquired in positive mode. The red coloring on nodes indicates the proportion of spectra from active fractions. The molecular families (or sections of molecular families) highlighted in purple represent molecular ions found exclusively in strain 2ta16. Those in blue are restricted to from strain PS5. GNPS library hits are indicated with a black square.

### 4.4.5 Biosynthetic gene clusters in *Pseudoalteromonas* genomes

The antiSMASH results show that the five strains used in this study contain diverse and distinct biosynthetic gene clusters. In total, 147 BGCs belonging to 19 biosynthetic classes were identified in the five genomes. The analysis was run with ClusterFinder in order to detect all BGCs, including the *bmp* gene cluster in strains A757, PS5, O-BC30, and 2ta16. However, this resulted in the annotation of a large number of "putative/other" gene clusters (Table 4.6).

Strain 2ta16 contains the most gene clusters with a total of 36, followed by PS5 with 34, 2ta6 with 29, A757 with 25, and O-BC30 with 23. After the putative/other BGCs, the next most abundant were fatty acid gene clusters with 19 total found in the five strains. All of the strains except O-BC30 contain at least two type 1 PKS-NRPS cluster and NRPS gene clusters were found in all five *Pseudoalteromonas* strains. Many of the remaining classes of BGCs were distinct for each strain. For example, a single type 1 PKS was found in the PS5 genome and a type 3 PKS was found only in strain 2ta16. No type 2 PKS clusters were found in the genomes.

While the genome mining results reveal a substantial number of BGCs in the *Pseudoalteromonas* strains used in this study, the majority of do not have similar known gene clusters or predicted products. However, each strain had several gene clusters with various levels of gene similarity BGCs with known products. The only BGC with a high level of gene similarity in strain O-BC30 was the bromophenols/bromopyrroles (*bmp*) cluster (Table 4.7). As expected, 100% of the *bmp* genes are similar because the strain contains all 10 genes identified as part of the BGC. The same result is observed in 2ta16, another *Pseudoalteromonas* strain with the gene cluster (Table 4.10). The results from

Chapter 2 of this thesis indicated that strains A757 and PS5 are known to contain an abbreviated version of the *bmp* cluster with only six of the 10 genes present in the genome, which is consistent with the 60% gene similarity identified by the antiSMASH analysis (Table 4.8, Table 4.9).

**Table 4.3** Genome mining analysis summary for five *Pseudoalteromonas* genomes. The number of BGCS in each cluster type identified by antiSMASH v3.0 is listed for the five *Pseudoalteromonas* strains.

| BGC type | O-BC30 | A757 | 2ta6 | PS5 | 2ta16 | Total |
|---|---|---|---|---|---|---|
| Putative/other | 12 | 12 | 12 | 13 | 17 | 66 |
| Fatty acid | 4 | 4 | 4 | 3 | 4 | 19 |
| Bacteriocin | 2 | 2 | 3 | 2 | 2 | 11 |
| Saccharide | 2 | 1 | 1 | 4 | 2 | 10 |
| T1PKS-NRPS | 0 | 3 | 2 | 5 | 3 | 13 |
| NRPS | 1 | 1 | 3 | 4 | 2 | 11 |
| Fatty acid-saccharide | 0 | 1 | 0 | 0 | 0 | 1 |
| Thiopeptide | 0 | 0 | 1 | 0 | 0 | 1 |
| Arylpolyene-NRPS | 0 | 0 | 0 | 1 | 0 | 1 |
| Lantipeptide | 1 | 1 | 0 | 1 | 0 | 3 |
| TransAT PKS-Lantipeptide | 0 | 0 | 0 | 0 | 1 | 1 |
| Lantipeptide-NRPS | 0 | 0 | 1 | 0 | 0 | 1 |
| T1 PKS | 0 | 0 | 0 | 1 | 0 | 1 |
| H-Ser Lactone Acyl amino acids | 0 | 0 | 0 | 0 | 1 | 1 |
| Acyl amino acids | 0 | 0 | 1 | 0 | 0 | 1 |
| NRPS-Ladderane | 0 | 0 | 1 | 0 | 1 | 2 |
| TransAT PKS-NRPS | 1 | 0 | 0 | 0 | 1 | 2 |
| Indole-T3 PKS | 0 | 0 | 0 | 0 | 1 | 1 |
| TransAT PKS-Other KS-NRPS | 0 | 0 | 0 | 0 | 1 | 1 |
| Total | 23 | 25 | 29 | 34 | 36 | 147 |

PS5 and 2ta6 show 100% and 77% gene similarity respectively to the alterochromide cluster, which was previously identified and characterized in *Pseudoalteromonas* (Ross et al. 2015) (Table 4.9, Table 4.11). The genome for strain 2ta16 contains BGCs with 50% gene similarity to the bromoalterochromide cluster, 80% similarity to the violacien cluster, and 100% similarity to the thiomarinol cluster (Table 4.10). The gene cluster for the pigment Violacien was expected because the strain is a dark purple color. Thiomarinol is a known antibiotic and the BGC has previously been described in *Pseudoalteromonas* (Murphy et al. 2014).

**Table 4.4** Similar known biosynthetic gene clusters to those found in strain O-BC30. The cluster number is an arbitrary numbering system used by antiSMASH to distinguish between BGCs of the same type. The percentage of shared genes between the BGC and the most similar known cluster is reported.

| Cluster No. | Type | Most similar known cluster | % gene similarity | MIBiG BGC-ID |
|---|---|---|---|---|
| 7 | Putative | O-antigen | 10 | BGC0000788_c1 |
| 9 | NRPS | Vibriobactin | 18 | BGC0000945_c1 |
| 12 | Saccharide | O&K-antigen | 6 | BGC0000780_c1 |
| 15 | PBDE | Bromophenols/bromopyrroles | 100 | BGC0000890_c1 |
| 19 | Putative | Myxochelin | 16 | BGC0001345_c1 |
| 20 | Trans AT PKS-NRPS | Kalimantacin | 10 | BGC00001099_c1 |

**Table 4.5** Similar known biosynthetic gene clusters to those found in strain A757. The cluster number is an arbitrary numbering system used by antiSMASH to distinguish between BGCs of the same type. The percentage of shared genes between the BGC and the most similar known cluster is reported.

| Cluster No. | Type | Most similar known cluster | % gene similarity | MIBiG BGC-ID |
|---|---|---|---|---|
| 3 | Saccharide | O-antigen | 23 | BGC0000791_c1 |
| 14 | T1PKS-NRPS | Serobactins | 23 | BGC0000424_c1 |
| 17 | Putative | Bromophenols/bromopyrroles | 60 | BGC0000891_c1 |
| 20 | Putative | Fengycin | 13 | BGC0001095_c1 |
| 22 | Putative | Bacillomycin | 20 | BGC0001090_c1 |

**Table 4.6** Similar known biosynthetic gene clusters to those found in strain PS5. The cluster number is an arbitrary numbering system used by antiSMASH to distinguish between BGCs of the same type. The percentage of shared genes between the BGC and the most similar known cluster is reported.

| Cluster No. | Type | Most similar known cluster | % gene similarity | MIBiG BGC-ID |
|---|---|---|---|---|
| 2 | Putative | Fengycin | 13 | BGC0001095_c1 |
| 4 | T1PKS-NRPS | Turnerbactin | 23 | BGC0000451_c1 |
| 14 | Arylpolyene-NRPS | Alterochromides | 77 | BCG0000299_c1 |
| 18 | Putative | Bromophenols/bromopyrroles | 60 | BGC0000890_c1 |
| 19 | Saccharide | O&K-antigen | 8 | BGC0000780_c1 |
| 20 | T1PKS-NRPS | Pyoverdine | 2 | BGC0000413_c1 |
| 21 | T1PKS-NRPS | Syringomycin | 11 | BGC0000437_c1 |
| 25 | NRPS | Bromoalterochromides | 14 | BGC0000314_c1 |
| 26 | Other | Bromoalterochromides | 21 | BGC0000314_c1 |

**Table 4.7** Similar known biosynthetic gene clusters to those found in strain 2ta16. The cluster number is an arbitrary numbering system used by antiSMASH to distinguish between BGCs of the same type. The percentage of shared genes between the BGC and the most similar known cluster is reported.

| Cluster No. | Type | Most similar known cluster | % gene similarity | MIBiG BGC-ID |
|---|---|---|---|---|
| 2 | Saccharide | O&K-antigen | 13 | BGC0000780_c1 |
| 11 | TransAT PKS-Lantipeptide | Cystobactamide | 8 | BGC0001413_c1 |
| 17 | NRPS-Ladderane | Bromoalterochromides | 50 | BGC0000314_c1 |
| 18 | NRPS | Turnerbactin | 23 | BGC0000451_c1 |
| 23 | TransAT PKS-NRPS | Kalimantacin/batumin | 10 | BGC0001099_c1 |
| 25 | Putative | Bromophenols/bromopyrroles | 100 | BGC0000891_c1 |
| 28 | Fatty acid | Xenocyloins | 25 | BGC0000189_c1 |
| 34 | Indole-T3 PKS | Violacein | 80 | BGC0000831_c1 |
| 36 | TransAT PKS-Other KS-NRPS | Thiomarinol | 100 | BGC0001115_c1 |

**Table 4.8** Similar known biosynthetic gene clusters to those found in strain 2ta6. The cluster number is an arbitrary numbering system used by antiSMASH to distinguish between BGCs of the same type. The percentage of shared genes between the BGC and the most similar known cluster is reported.

| Cluster No. | Type | Most similar known cluster | % gene similarity | MIBiG BGC-ID |
|---|---|---|---|---|
| 11 | Ladderane-NRPS | Alterochromides | 100 | BGC0000299_c1 |
| 13 | NRPS | Bacillibactin | 60 | BGC0001185_c1 |
| 15 | Saccharide | O-antigen | 16 | BGC0000784_c1 |
| 29 | Putative | Bacillomycin | 20 | BGC0001090_c1 |

## 4.5 Discussion

The results of this study indicate that *Pseudoalteromonas* strains are rich in yet to be characterized biosynthetic gene clusters with no known products, suggesting they have the potential to produce novel bioactive molecules. A number of *Pseudoalteromonas* species are known to produce bioactive molecules, however, only a handful of specific structures have been elucidated and even fewer of the responsible gene clusters have been identified. Even when the gene cluster for a known bioactive molecule was identified in the genome, it was likely not the only active compound produced by that strain. For example, 2ta16 contains the BGC for thiomarinol, a known antibiotic (Murphy et al. 2014; Shiozawa et al. 1993; Murphy et al. 2014). However, six of the eight fractions tested from this strain were bioactive, suggesting that there are multiple compounds responsible for the observed activity. No molecular families were found only in strains grown with or without KBr added to the media, which indicates that while supplementing the media can increase the level of production for some brominated compounds, it most likely does not result in an increased diversity of brominated metabolites.

*Pseudoalteromonas* is an ideal organism to study because it is ubiquitous in the marine environment, thus facilitating the collection of environmental isolates. The strains can be readily cultured and most species thrive under laboratory conditions, allowing for the rapid generation of chemical extracts and other samples. This study has confirmed the

immense potential for *Pseudoalteromonas* as an important player in marine natural products research and identified strains and BGCs of interest. Generating more sequence data from different species distributed across the genus, mining genomes for BGCs, and performing subsequent chemical analyses, will help to further illuminate the biosynthetic capacity of this genus. An in-depth comparative study of annotated BGCs using a phylogenetic approach will help identify which clusters are homologous or distinct and will provide a greater understanding of the secondary metabolite diversity in *Pseudoalteromonas*. Additionally, applying techniques such as TAR-cloning to characterize interesting BGCs in addition to performing isolation studies, may lead to the identification of novel bioactive compounds from this genus of marine bacteria (Yamanaka et al. 2014).

**4.6 Acknowledgements**

A very big thanks to Leesa Klau for all of her help with the chemistry work and subsequent data analysis and interpretation. I also want to thank Henrique Ramalho Machado for helpful discussion about experimental design and the overall scope of this study. The dissertation author was the primary investigator on these studies.

Chapter 4 is coauthored with Leesa Klau, and Paul Jensen. The dissertation author was the primary investigator on these studies.

# Chapter 5: Concluding Remarks

Marine bacteria have proven to be a rich source of bioactive secondary metabolites. A group of specific interest and focus of this dissertation is *Pseudoalteromonas*, a genus of marine bacteria found throughout the ocean environment. The work presented in this dissertation explores the diversity and distribution of the *bmp* gene cluster and is specifically focused on one of its products, pentabromopseudilin. The bioactivity and mechanism of action in bacteria was tested for this toxic metabolite as well as testing a diverse sample of *Pseudoalteromonas* strains for their potential to produce interesting molecules.

Defining the species phylogeny of bacteria is crucial to understanding the distribution and evolution of BGCs. In chapter 2 of this dissertation, the most complete and well-supported species tree for *Pseudoalteromonas* was generated and used as a powerful tool to infer the evolution of a biosynthetic gene cluster. Over 100 *Pseudoalteromonas* genomes were mined for the *bmp* cluster and the results were mapped onto the species phylogeny to identify groups within the genus having the potential to produce the suite of brominated molecules encoded by the BGC. Chapter 3 looks closely at the cellular target of pentabromopseudilin in an *E. coli* strain and the viability of cells treated with different concentrations of the molecule over time. Finally, in chapter 4, the broad bioactivity of metabolites from five *Pseudoalteromonas* species was assessed. There still remains a great deal of research to be done on *Pseudoalteromonas* and the biologically active compounds they produce, but this work includes a foundation on which to build future studies of this genus and the interesting molecules they make.

The accessibility and affordability of genome sequencing has played a significant role in facilitating comparative genomics studies including the exploration of secondary metabolite gene cluster diversity and evolution. Additionally, genome mining tools including antiSMASH are constantly being improved to enable the investigation of bacterial biosynthetic potential. While sequence data is critical for genome mining and the identification of BGCs and a comparative study could be conducted using all currently available genome sequences, having access to the strains themselves for experimental use is equally important for natural product research. Only by increasing the number of genomes sequenced for *Pseudoalteromonas* available strains or alternatively, acquiring strains with published genomes from their respective collections, the secondary metabolite profile of the genus can be established. Expanding on the research presented in this dissertation, it would be interesting to sequence the genomes of more *Pseudoalteromonas* genomes to explore the diversity of their BGCs across the genus. While this can already be done with the large number of publically available genomes, it's challenging to form hypotheses from genomic data without the organisms being experimentally available and vice versa. Having access to strains with genome sequences presents endless possibilities for future studies.

With an increase in available genome sequence data and taxonomic certainty comes an overwhelming number of interesting biosynthetic gene clusters to explore. In this area of research, there is a substantial bottleneck between the identification of BGCs and identifying the molecules they encode. While success with methods such as TAR-cloning can be experimentally difficult, tools such as this are absolutely critical and with

time, perseverance, and perhaps a little luck, we can slowly start to assign orphan gene clusters their respective natural products.

## References


Agarwal, Vinayak, Jessica M. Blanton, Sheila Podell, Arnaud Taton, Michelle A. Schorn, Julia Busch, Zhenjian Lin, Eric W. Schmidt, Paul R. Jensen, Valerie J. Paul, Jason S. Biggs, James W. Golden, Eric E. Allen, Bradley S. Moore. 2017. "Metagenomic Discovery of Polybrominated Diphenyl Ether Biosynthesis by Marine Sponges." *Nature Chemical Biology*, 537–43. doi:10.1038/nature15540.Genetic.

Agarwal, Vinayak, Abrahim El Gamal, Kazuya Yamanaka, Dennis Poth, Roland D. Kersten, Michelle Schorn, Eric E. Allen, and Bradley S. Moore. 2014. "Biosynthesis of Polybrominated Aromatic Organic Compounds by Marine Bacteria." *Nature Chemical Biology* 10 (8). Nature Publishing Group: 640–47. doi:10.1038/nchembio.1564.

Ahmed, Lina, Paul R. Jensen, Kelle C. Freel, Ros Brown, Amanda L. Jones, Byung Yong Kim, and Michael Goodfellow. 2013. "Salinispora Pacifica Sp. Nov., an Actinomycete from Marine Sediments." *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology* 103 (5): 1069–78. doi:10.1007/s10482-013-9886-4.

Akagawa-Matsushita, Masayo (University of Occupational and Environmental Health), Masaru (The University of Tsukuba) Matsuo, Yosuke (University of Occupational and Environmental Health) Koga, and Kazuhide (The University of Tokyo) Yamasato. 1992. "Alteromonas Atlantica Sp. Nov. and Alteromonas Carrageenovora Sp. Nov., Bacteria That Decompose Algal Polysaccharides." *International Journal of Systematic Bacteriology* 42 (4): 621–27.

Al Khudary, Rami, Nele Isabelle Stösser, Farah Qoura, and Garabed Antranikian. 2008. "Pseudoalteromonas Arctica Sp. Nov., an Aerobic, Psychrotolerant, Marine Bacterium Isolated from Spitzbergen." *International Journal of Systematic and Evolutionary Microbiology* 58 (Pt 9): 2018–24. doi:10.1099/ijs.0.64963-0.

Alley, M.C., Scudiero, D.A., Monks, P.A., Hursey, M.L., Czerwinski, R.H. & Boyd M.J., Fine, D.L., Abbott, B.J., Mayo, J.G., Shoemaker, and M.R. 1988. "Feasibility of Drug Screening with Panels of Human Tumor Cell Lines Using a Mocroculture Microculture Tetrazolium Assay." *Cancer Research* 48: 589–601.

Amos, Gregory C. A., Takayoshi Awakawa, Robert N. Tuttle, Anne-Catrin Letzel, Min Cheol Kim, Yuta Kudo, William Fenical, Bradley S. Moore, and Paul R. Jensen. 2017. "Comparative Transcriptomics as a Guide to Natural Product Discovery and Biosynthetic Gene Cluster Functionality." *Proceedings of the National Academy of Sciences* 114 (52): E11121–30. doi:10.1073/pnas.1714381115.

Andersen, R. J., M. S. Wolfe, and D. J. Faulkner. 1974. "Autotoxic Antibiotic Production by a Marine Chromobacterium." *Marine Biology* 27 (4): 281–85. doi:10.1007/BF00394363.

Azam, Farooq, and Francesca Malfatti. 2007. "Microbial Structuring of Marine Ecosystems." *Nature Reviews Microbiology* 5 (10): 782–91. doi:10.1038/nrmicro1747.

Aziz, Ramy K., Daniela Bartels, Aaron Best, Matthew DeJongh, Terrence Disz, Robert A. Edwards, Kevin Formsma, Svetlana Gerdes, Elizabeth M. Glass, Michael Kubal, Folker Meyer, Gary J. Olsen, Robert Olson, Andrei L. Osterman, Ross A. Overbeek, Leslie K. McNeil, Daniel Paarmann, Tobias Paczian, Bruce Parrello, Gordon D. Pusch, Claudia Reich, Rick Stevens, Olga Vassieva, Veronika Vonstein, Andreas Wilke and Olga Zagnitko. 2008. "The RAST Server: Rapid Annotations Using Subsystems Technology." *BMC Genomics* 9: 1–15. doi:10.1186/1471-2164-9-75.

Bein, Selwyn Jack. 1954. "A Study of Certain Chromogenic Bacteria Isolated from 'Red Tide' Water with a Description of a New Species." *Bulletin of Marine Science* 4 (10): 110–19.

Beurmann, Silvia, Blake Ushijima, Christina M. Svoboda, Patrick Videau, Ashley M. Smith, Stuart P. Donachie, Greta S. Aeby, and Sean M. Callahan. 2017. "Pseudoalteromonas Piratica Sp. Nov., a Budding, Prosthecate Bacterium from Diseased Montipora Capitata, and Emended Description of the Genus Pseudoalteromonas." *International Journal of Systematic and Evolutionary Microbiology* 67 (8): 2683–88. doi:10.1099/ijsem.0.001995.

Blackman, Adrian J., and Caiping Li. 1994. "New Tambjamine Alkaloids from the Marine Bryozoan Bugula Dentata." *Australian Journal of Chemistry* 47: 1625–29.

Bowman, John, and John P. 2007. "Bioactive Compound Synthetic Capacity and Ecological Significance of Marine Bacterial Genus Pseudoalteromonas." *Marine Drugs* 5 (4): 220–41. doi:10.3390/md504220.

Bowman, John P. 1998. "Pseudoalteromonas Prydzensis Sp. Nov., a Psychrotrophic, Halotolerant Bacterium from Antarctic Sea Ice." *International Journal of Systematic Bacteriology* 48: 1037–41.

Bozal, N, E Tudela, R Rosselló-Mora, J Lalucat, and J Guinea. 1997. "Pseudoalteromonas Antarctica Sp. Nov., Isolated from an Antarctic Coastal Environment." *International Journal of Systematic Bacteriology* 47 (2): 345–51. http://www.ncbi.nlm.nih.gov/pubmed/9103620.

Bruns, Hilke, Max Crüsemann, Anne-Catrin Letzel, Mohammad Alanjary, James O McInerney, Paul R Jensen, Stefan Schulz, Bradley S Moore, and Nadine Ziemert. 2017. "Function-Related Replacement of Bacterial Siderophore Pathways." *The ISME Journal* 12 (2). Nature Publishing Group: 320–29. doi:10.1038/ismej.2017.137.

Bull, Alan T., and James E M Stach. 2007. “Marine Actinobacteria: New Opportunities for Natural Product Search and Discovery.” *Trends in Microbiology* 15 (11): 491–99. doi:10.1016/j.tim.2007.10.004.

Bundy, Jacob G., Tara L. Willey, Rachel S. Castell, David J. Ellar, and Kevin M. Brindle. 2005. “Discrimination of Pathogenic Clinical Isolates and Laboratory Strains of Bacillus Cereus by NMR-Based Metabolomic Profiling.” *FEMS Microbiology Letters* 242 (1): 127–36. doi:10.1016/j.femsle.2004.10.048.

Burja, Adam M, Bernard Banaigs, Eliane Abou-mansour, J Grant, and Phillip C Wright. 2001. “Marine Cyanobacteria — A Prolific Source of Natural Products Marine CyanobacteriaĐa Proli ® c Source of Natural Products” 4020 (November 2015): 9377. doi:10.1016/S0040-4020(01)00931-0.

Burke, Catherine, Torsten Thomas, Suhelen Egan, and Staffan Kjelleberg. 2007. “The Use of Functional Genomics for the Identification of a Gene Cluster Encoding for the Biosynthesis of an Antifungal Tambjamine in the Marine Bacterium Pseudoalteromonas Tunicata: Brief Report.” *Environmental Microbiology* 9 (3): 814–18. doi:10.1111/j.1462-2920.2006.01177.x.

Burkholder, P R, R M Pfister, and F H Leitz. 1966. “Production of a Pyrrole Antibiotic by a Marine Bacterium.” *Applied Microbiology* 14 (4): 649–53.

Carbone, Marianna, Carlo Irace, Francesca Costagliola, Francesco Castelluccio, Guido Villani, Goncalo Calado, Vinicius Padula, Guido Cimino, J. Lucas Cervera, Rita Santamaria, Margherita Gavagnin. 2010. “A New Cytotoxic Tambjamine Alkaloid from the Azorean Nudibranch Tambja Ceutae.” *Bioorganic and Medicinal Chemistry*. Elsevier Ltd, 2668–70. doi:10.1016/j.bmcl.2010.02.020.

Carte, Brad, and D John Faulkner. 1980. “POLYBROMINATED DIPHENYL ETHERS FROM DYSIDEA HERBACEA, DYSIDEA CHLOREA AND PHYLLOSPONGIA FOLIASCENS” 17 (3): 88–90.

Carté, Brad, Michael R. Kernan, Ellen B. Barrabee, D. John Faulkner, Gayle K. Matsumoto, and Jon Clardy. 1986. “Metabolites of the Nudibranch Chromodoris Funerea and the Singlet Oxygen Oxidation Products of Furodysin and Furodysinin.” *Journal of Organic Chemistry* 51 (18): 3528–32. doi:10.1021/jo00368a025.

Carver, Tim J., Kim M. Rutherford, Matthew Berriman, Marie Adele Rajandream, Barclay G. Barrell, and Julian Parkhill. 2005. “ACT: The Artemis Comparison Tool.” *Bioinformatics* 21 (16): 3422–23. doi:10.1093/bioinformatics/bti553.

Chan, K. Y., L. Baumann, M. M. Garza, and Davis) Baumann, P. (University of California. 1978. “Two New Species of Alteromonas: Alteromonas Espejiana and Alteromonas Undina.” *International Journal of Systematic Bacteriology* 28 (2): 217–22.

Chengkui, Zeng, and Zhang Junfu. 1984. "Chinese Seaweeds in Herbal Medicine." *Hydrobiologia*, 152–54. www.mdpi.net/marinedrugs/.

Dang, Hongyue, and Charles R. Lovell. 2016. "Microbial Surface Colonization and Biofilm Development in Marine Environments." *Microbiology and Molecular Biology Reviews* 80 (1): 91–138. doi:10.1128/MMBR.00037-15.Address.

Dias, Daniel A., Sylvia Urban, and Ute Roessner. 2012. "A Historical Overview of Natural Products in Drug Discovery." *Metabolites* 2 (2): 303–36. doi:10.3390/metabo2020303.

Dillehay, Tom D, C Ramírez, M Pino, M B Collins, J Rossen, and J.D. Pino-Navarro. 2008. "Monte Verde: Seaweed, Food, Medicine, and the Peopling of South America." *Science* 320 (May): 784–86.

Dinsdale, Elizabeth A., Olga Pantos, Steven Smriga, Robert A. Edwards, Florent Angly, Linda Wegley, Mark Hatay, Dana Hall, Elysa Brown, Matthew Haynes, Lutz Krause, Enric Sala, Stuart A. Sandin, Rebecca Vega Thurber, Bette L. Willis, Farooq Azam, Nancy Knowlton, Forest Rohwer. 2008. "Microbial Ecology of Four Coral Atolls in the Northern Line Islands." *PLoS ONE* 3 (2). doi:10.1371/journal.pone.0001584.

Dorrestein, P. C., E. Yeh, S. Garneau-Tsodikova, N. L. Kelleher, and C. T. Walsh. 2005. "Dichlorination of a Pyrrolyl-S-Carrier Protein by FADH2-Dependent Halogenase PltA during Pyoluteorin Biosynthesis." *Proceedings of the National Academy of Sciences* 102 (39): 13843–48. doi:10.1073/pnas.0506964102.

Duarte, Carlos M., Josep M. Gasol, and Dolors Vaqué. 1997. "Role of Experimental Approaches in Marine Microbial Ecology." *Aquatic Microbial Ecology* 13 (1): 101–11. doi:10.3354/ame013101.

Edgar, Robert C, Roque Moraes Drive, and Mill Valley. 2004. "MUSCLE : Multiple Sequence Alignment with High Accuracy and High Throughput" 32 (5): 1792–97. doi:10.1093/nar/gkh340.

Egan, Suhelen, Carola Holmström, and Staffan Kjelleberg. 2001. "Pseudoalteromonas Ulvae Sp. Nov., a Bacterium with Antifouling Activities Isolated from the Surface of a Marine Alga." *International Journal of Systematic and Evolutionary Microbiology* 51 (4): 1499–1504. doi:10.1099/00207713-51-4-1499.

Egan, Suhelen, Sally James, Carola Holmström, and Staffan Kjelleberg. 2002. "Correlation between Pigmentation and Antifouling Compounds Produced by Pseudoalteromonas Tunicata." *Environmental Microbiology* 4 (8): 433–42. doi:10.1046/j.1462-2920.2002.00322.x.

El Gamal, Abrahim, Vinayak Agarwal, Stefan Diethelm, Imran Rahman, Michelle A. Schorn, Jennifer M. Sneed, Gordon V. Louie, Kristen E. Whalen, Tracy J. Mincere, Joseph P. Noel, Valerie J. Paul, and Bradley S. Moore. 2016. "Biosynthesis of Coral Settlement Cue Tetrabromopyrrole in Marine Bacteria by a Uniquely Adapted Brominase–Thioesterase Enzyme Pair." *Proceedings of the National Academy of Sciences* 113 (14): 3797–3802. doi:10.1073/pnas.1519695113.

El Gamal, Abrahim, Vinayak Agarwal, Imran Rahman, and Bradley S. Moore. 2016. "Enzymatic Reductive Dehalogenation Controls the Biosynthesis of Marine Bacterial Pyrroles." *Journal of the American Chemical Society*, jacs.6b08512. doi:10.1021/jacs.6b08512.

Enger, Oivind. 1987. "Characterization of Alteromonas Denitrificans Sp. Nov." *International Journal of Systematic Bacteriology* 37 (4): 416–21.

Faulkner, D John, Mia D Unson, and Carole A Bewley. 1994. "The Chemistry of Some Sponges and Their Symbionts" 66 (1011): 1983–90. doi:10.1351/pac199466101983.

Fedorov, Roman, Markus Böhl, Georgios Tsiavaliaris, Falk K. Hartmann, Manuel H. Taft, Petra Baruch, Bernhard Brenner, Rene´ Martin, Hans-Joachim Knölker, Herwig O. Gutzei, Dietmar J. Manstein. 2009. "The Mechanism of Pentabromopseudilin Inhibition of Myosin Motor Activity." *Nature Structural and Molecular Biology* 16 (1): 80–88. doi:10.1038/nsmb.1542.

Fehér, Domonkos, Russell Barlow, Jesse McAtee, and Thomas K Hemscheidt. 2010. "Highly Brominated Antimicrobial Metabolites from a Marine Pseudoalteromonas Sp." *Journal of Natural Products* 73 (11): 1963–66. doi:10.1021/np100506z.

Feling, Robert H., Greg O. Buchanan, Tracy J. Mincer, Christopher A. Kauffman, Paul R. Jensen, and William Fenical. 2003. "Salinosporamide A: A Highly Cytotoxic Proteasome Inhibitor from a Novel Microbial Source, a Marine Bacterium of the New Genus Salinospora." *Angewandte Chemie - International Edition* 42 (3): 355–57. doi:10.1002/anie.200390115.

Fernie, Kim J, J Laird Shutt, Greg Mayne, David Hoffman, Robert J Letcher, Ken G Drouillard, and Ian J Ritchie. 2005. "Exposure to Polybrominated Diphenyl Ethers (PBDEs): Changes in Thyroid, Vitamin A, Glutathione Homeostasis, and Oxidative Stress in American Kestrels (Falco Sparverius)." *Toxicological Sciences : An Official Journal of the Society of Toxicology* 88 (2): 375–83. doi:10.1093/toxsci/kfi295.

Fischbach, Michael A., Christopher T. Walsh, and Jon Clardy. 2009. "The Evolution of Gene Collectives: How Natural Selection Drives Chemical Innovation." *PNAS* 106 (5): 4601–8.

Fischbach, Michael A, Christopher T Walsh, and Jon Clardy. 2008. "The Evolution of

Gene Collectives: How Natural Selection Drives Chemical Innovation." *PNAS* 106 (33): 11587–88.

Freel, Kelle C., Sang Jip Nam, William Fenical, and Paul R. Jensen. 2011. "Evolution of Secondary Metabolite Genes in Three Closely Related Marine Actinomycete Species." *Applied and Environmental Microbiology* 77 (20): 7261–70. doi:10.1128/AEM.05943-11.

Fuhrman, Jed A., Jacob A. Cram, and David M. Needham. 2015. "Marine Microbial Community Dynamics and Their Ecological Interpretation." *Nature Reviews Microbiology* 13 (3). Nature Publishing Group: 133–46. doi:10.1038/nrmicro3417.

Gadberry, Michael D., Simon T. Malcomber, Andrew N. Doust, and Elizabeth a. Kellogg. 2005. "Primaclade - A Flexible Tool to Find Conserved PCR Primers across Multiple Species." *Bioinformatics* 21 (7): 1263–64. doi:10.1093/bioinformatics/bti134.

Gauthier, G, M Gauthier, and R Christen. 1995. "Phylogenetic Analysis of the Genera *Alteromonas*, *Shewanella*, and *Moritella* Using Genes Coding for Small-Subunit RRNA Sequences and Division of the Genus *Alteromonas* into Two Genera , *Alteromonas* ( Emended ) and Pseudoalt," no. 32: 755–61.

Gauthier, M. J. 1982. "Validation of the Name Alteromonas Luteoviolacea." *International Journal of Systematic Bacteriology* 32 (1): 82–86. doi:10.1099/00207713-32-1-82.

Gauthier, M. J. 1977. "Alteromonas Citrea, a New Gram-Negative, Yellow-Pigmented Species from Seawater." *International Journal of Systematic Bacteriology* 27 (4): 349–54.

Gauthier, M. J., and V. a. Breittmayer. 1979. "A New Antibiotic-Producing Bacterium from Seawater: Alteromonas Aurantia Sp. Nov." *International Journal of Systematic Bacteriology* 29 (4): 366–72. doi:10.1099/00207713-29-4-366.

Gauthier, M. J., and G. N. Flatau. 1976. "Antibacterial Activity of Marine Violet-Pigmented Alteromonas with Special Reference to the Production of Brominated Compounds." *Canadian Journal of Microbiology*.

Gauthier, M J, Centre Etudes, De Recherches, and De Biologie. 1976. "Alteromonas Rubra Sp . Nov ., a New Marine Antibiotic-Producing Bacterium." *International Journal of Systematic Bacteriology* 26 (4): 459–66.

Gopal, Radhika, M Vijayakumaran, R Venkatesan, and S Kathiroli. 2008. "Marine Organisms in Indian Medicine and Their Future Prospects." *NPR Vol.7(2) [March-April 2008]* 7 (2): 139–45.

Goris, Johan, Konstantinos T. Konstantinidis, Joel A. Klappenbach, Tom Coenye, Peter Vandamme, and James M. Tiedje. 2007. "DNA-DNA Hybridization Values and Their Relationship to Whole-Genome Sequence Similarities." *International Journal of Systematic and Evolutionary Microbiology* 57 (1): 81–91. doi:10.1099/ijs.0.64483-0.

Gribble, Gordon W. 2003. "The Diversity of Naturally Produced Organohalogens." *Chemosphere* 52 (2): 289–97. doi:10.1016/S0045-6535(03)00207-8.

Hallgren, Sara, Taha Sinjari, Helen Håkansson, and Per Darnerud. 2001. "Effects of Polybrominated Diphenyl Ethers (PBDEs) and Polychlorinated Biphenyls (PCBs) on Thyroid Hormone and Vitamin A Levels in Rats and Mice." *Archives of Toxicology* 75 (4): 200–208. doi:10.1007/s002040000208.

Hoffmann, Thomas, Daniel Krug, Nisa Bozkurt, Srikanth Duddela, Rolf Jansen, Ronald Garcia, Klaus Gerth, Heinrich Steinmetz, and Rolf Müller. 2018. "Correlating Chemical Diversity with Taxonomic Distance for Discovery of Natural Products in Myxobacteria." *Nature Communications* 9 (1). Springer US: 1–10. doi:10.1038/s41467-018-03184-1.

Holmstromfl, Carola, Sally James, Brett A Neilan, and David C White. 1998. "Pseudoalteromonas Tunicata Sp. Nov., a Bacterium That Produces Antifouling Agents." *International Journal of Systematic Bacteriology* 48: 1205–12.

Huang, Yi Li, M. Li, Zhiliang Yu, and Pei Yuan Qian. 2011. "Correlation between Pigmentation and Larval Settlement Deterrence by Pseudoalteromonas Sp. Sf57." *Biofouling* 27 (3): 287–93. doi:10.1080/08927014.2011.562978.

Isnansetyo, Alim, and Yuto Kamei. 2003a. "MC21-A , a Bactericidal Antibiotic Produced by a New Marine Bacterium, Pseudoalteromonas Phenolica Sp. Nov. O-BC30T, against Methicillin-Resistant Staphylococcus Aureus." *Antimicrobial Agents and Chemotherapy* 47 (2): 480–88. doi:10.1128/AAC.47.2.480.

Isnansetyo, Alim, Yuto Kamei. 2003. "Pseudoalteromonas phenolica sp. nov., a Novel Marine Bacterium That Produces Phenolic Anti-Methicillin-Resistant Staphylococcus Aureus Substances." *International Journal of Systematic and Evolutionary Microbiology* 53 (2): 583–88. doi:10.1099/ijs.0.02431-0.

Isnansetyo, Alim, Yuto Kamei. 2009. "Anti-Methicillin-Resistant Staphylococcus Aureus (MRSA) Activity of MC21-B, an Antibacterial Compound Produced by the Marine Bacterium Pseudoalteromonas Phenolica O-BC30T." *International Journal of Antimicrobial Agents* 34 (2): 131–35. doi:10.1016/j.ijantimicag.2009.02.009.

Ivanova, E P, J Chun, L a Romanenko, M E Matte, V V Mikhailov, G M Frolova, A Huq, and R R Colwell. 2000. "Reclassification of Alteromonas Distincta Romanenko et Al. 1995 as Pseudoalteromonas Distincta Comb. Nov." *International Journal of*

*Systematic and Evolutionary Microbiology* 50 Pt 1 (January): 141–44. http://www.ncbi.nlm.nih.gov/pubmed/10826797.

Ivanova, Elena P., Tomoo Sawabe, Yuliya V. Alexeeva, Anatolii M. Lysenko, Nataliya M. Gorshkova, Karin Hayashi, Natalia V. Zukova, Richard Christen, and Valery V. Mikhailov. 2002. "Pseudoalteromonas Issachenkonii Sp. Nov., a Bacterium That Degrades the Thallus of the Brown Alga Fucus Evanescens." *International Journal of Systematic and Evolutionary Microbiology* 52 (1): 229–34. doi:10.1099/00207713-52-1-229.

Ivanova, Elena P., Natalia V. Zhukova, Vassilii I. Svetashev, Nataliya M. Gorshkova, Valerie V. Kurilenko, Galina M. Frolova, and Valery V. Mikhailov. 2000. "Evaluation of Phospholipid and Fatty Acid Compositions as Chemotaxonomic Markers of Alteromonas-like Proteobacteria." *Current Microbiology* 41 (5): 341–45. doi:10.1007/s002840010146.

Ivanova, Elena P, Nataliya M Gorshkova, Natalia V Zhukova, Anatolii M Lysenko, Elena a Zelepuga, Nina G Prokof'eva, Valery V Mikhailov, Dan V Nicolau, and Richard Christen. 2004. "Characterization of Pseudoalteromonas Distincta-like Sea-Water Isolates and Description of Pseudoalteromonas Aliena Sp. Nov." *International Journal of Systematic and Evolutionary Microbiology* 54 (Pt 5): 1431–37. doi:10.1099/ijs.0.03053-0.

Ivanova, Elena P, Tomoo Sawabe, Anatolii M Lysenko, Nataliya M Gorshkova, K Hayashi, Natalia V Zhukova, Dan V Nicolau, Richard Christen, Valery V Mikhailov, and T Atcc. 2002. "Pseudoalteromonas Translucida Sp. Nov. and Pseudoalteromonas Paragorgicola Sp. Nov., and Emended Description of the Genus." *International Journal of Systematic and Evolutionary Microbiology* 52: 1759–66.

Ivanova, Elena P, Tomoo Sawabe, Anatolii M Lysenko, Nataliya M Gorshkova, Vassilii I Svetashev, Dan V Nicolau, Noboru Yumoto, Takahisa Taguchi, Susumu Yoshikawa, Richard Christen, Valery V. Mikhailov. 2002. "Pseudoalteromonas ruthenica sp. nov., isolated from marine invertebrates." *International Journal of Systematic and Evolutionary Microbiology* 52: 235–40.

Ivanova, Elena P, Ludmila S Shevchenko, Tomoo Sawabe, Anatolii M Lysenko, Vasilii I Svetashev, Nataliya M Gorshkova, Masataka Satomi, Richard Christen, and Valery V Mikhailov. 2002. "Pseudoalteromonas Maricaloris Sp. Nov., Isolated from an Australian Sponge, and Reclassification of [Pseudoalteromonas Aurantia] NCIMB 2033 as Pseudoalteromonas Flavipulchra Sp. Nov." *International Journal of Systematic and Evolutionary Microbiology* 52 (Pt 1): 263–71. http://www.ncbi.nlm.nih.gov/pubmed/11837311.

Jensen, Paul R., Philip G. Williams, Dong Chan Oh, Lisa Zeigler, and William Fenical. 2007. "Species-Specific Secondary Metabolite Production in Marine Actinomycetes

of the Genus Salinispora." *Applied and Environmental Microbiology* 73 (4): 1146–52. doi:10.1128/AEM.01891-06.

Jensen, Paul R. 2010. "Linking Species Concepts to Natural Product Discovery in the Post-Genomic Era." *Journal of Industrial Microbiology & Biotechnology* 37 (3): 219–24. doi:10.1007/s10295-009-0683-z.

Johnson-Restrepo, Boris, and Kurunthachalam Kannan. 2009. "An Assessment of Sources and Pathways of Human Exposure to Polybrominated Diphenyl Ethers in the United States." *Chemosphere* 76 (4). Elsevier Ltd: 542–48. doi:10.1016/j.chemosphere.2009.02.068.

Katoh, Kazutaka, Kazuharu Misawa, Kei-ichi Kuma, and Takashi Miyata. 2002. "MAFFT : A Novel Method for Rapid Multiple Sequence Alignment Based on Fast Fourier Transform" 30 (14): 3059–66.

Lamsa, Anne, Javier Lopez-Garrido, Diana Quach, Eammon P. Riley, Joe Pogliano, and Kit Pogliano. 2016. "Rapid Inhibition Profiling in Bacillus Subtilis to Identify the Mechanism of Action of New Antimicrobials." *ACS Chemical Biology* 11 (8): 2222–31. doi:10.1021/acschembio.5b01050.

Lau, Stanley C K, Mandy M Y Tsoi, Xiancui Li, Sergey Dobretsov, Yulia Plakhotnikova, Po Keung Wong, and Pei Yuan Qian. 2005. "Pseudoalteromonas Spongiae Sp. Nov., a Novel Member of the Gamma-Proteobacteria Isolated from the Sponge Mycale Adhaerens in Hong Kong Waters." *International Journal of Systematic and Evolutionary Microbiology* 55 (4): 1593–96. doi:10.1099/ijs.0.63638-0.

Letzel, Anne Catrin, Jing Li, Gregory C.A. Amos, Natalie Millán-Aguiñaga, Joape Ginigini, Usama R. Abdelmohsen, Susana P. Gaudêncio, Nadine Ziemert, Bradley S. Moore, and Paul R. Jensen. 2017. "Genomic Insights into Specialized Metabolism in the Marine Actinomycete Salinispora." *Environmental Microbiology* 19 (9): 3660–73. doi:10.1111/1462-2920.13867.

Lind, Abigail L., Jennifer H. Wisecaver, Catarina Lameiras, Philipp Wiemann, Jonathan M. Palmer, Nancy P. Keller, Fernando Rodrigues, Gustavo H. Goldman, and Antonis Rokas. 2017. "Drivers of Genetic Diversity in Secondary Metabolic Gene Clusters within a Fungal Species." *PLoS Biology* 15 (11): 1–26. doi:10.1371/journal.pbio.2003583.

Lindquist, N., and W. Fenical. 1990. "New Tambjamine Class Alkaloids from the Marin Ascidian Atapozoa Sp. and Its Nudibranch Predators. Origin of the Tambjamines in Atapozoa," 504–6.

Lu, Liang, Jijie Wang, Ying Xu, Kailing Wang, Yingwei Hu, Renmao Tian, Bo Yang, Qiliang Lai, Yongxin Li, Weipeng Zhang, Zongze Shao, Henry Lam, Pei-Yuan Qian. 2014. "A High-Resolution LC-MS-Based Secondary Metabolite Fingerprint

Database of Marine Bacteria.” *Scientific Reports* 4: 1–7. doi:10.1038/srep06537.

Luo, Peng, Wei Wang, Yanhong Wang, Chaoqun Hu, and Xiangyan He. 2013. “Pseudoalteromonas Xishaensis Sp. Nov., Isolated from Acanthaster Planci in the Xisha Islands.” *Antonie van Leeuwenhoek* 104 (5): 779–85. doi:10.1007/s10482-013-9987-0.

Machado, Henrique, Nikolaj G Vynne, Gunna Christiansen, and Lone Gram. 2017. “Reclassification of Alteromonas Fuliginea (Romanenko et al. 1995) as Pseudoalteromonas Fuliginea Comb. Nov. and an Emended Description.” *International Journal of Systematic and Evolutionary Microbiology* 66: 3737–42. doi:10.1099/ijsem.0.001259.

Maldonado, Luis A., William Fenical, Paul R. Jensen, Christopher A. Kauffman, Tracy J. Mincer, Alan C. Ward, Alan T. Bull, and Michael Goodfellow. 2005. “Salinispora Arenicola Gen. Nov., Sp. Nov. and Salinispora Tropica Sp. Nov., Obligate Marine Actinomycetes Belonging to the Family Micromonosporaceae.” *International Journal of Systematic and Evolutionary Microbiology* 55 (5): 1759–66. doi:10.1099/ijs.0.63625-0.

Malmvärn, Anna, Göran Marsh, Lena Kautsky, Maria Athanasiadou, Ake Bergman, and Lillemor Asplund. 2005. “Hydroxylated and Methoxylated Brominated Diphenyl Ethers in the Red Algae Ceramium Tenuicorne and Blue Mussels from the Baltic Sea.” *Environmental Science & Technology* 39 (9): 2990–97. http://www.ncbi.nlm.nih.gov/pubmed/15926543.

Marx, Léo B., and Jonathan W. Burton. 2018. “A Total Synthesis of Salinosporamide A.” *Chemistry - A European Journal* 24 (26): 6747–54. doi:10.1002/chem.201800046.

Matsuyama, Hidetoshi, Hideki Minami, Hirokazu Kasahara, Yoshihisa Kato, Masafumi Murayama, and Isao Yumoto. 2013. “Pseudoalteromonas Arabiensis Sp. Nov., a Marine Polysaccharide-Producing Bacterium.” *International Journal of Systematic and Evolutionary Microbiology* 63 (Pt 5): 1805–9. doi:10.1099/ijs.0.043604-0.

Matsuyama, Hidetoshi, Kazuya Sawazaki, Hideki Minami, Hirokazu Kasahara, Keiji Horikawa, and Isao Yumoto. 2014. “Pseudoalteromonas Shioyasakiensis Sp. Nov., a Marine Polysaccharide-Producing Bacterium.” *International Journal of Systematic and Evolutionary Microbiology* 64 (Pt 1): 101–6. doi:10.1099/ijs.0.055558-0.

McConnell, Oliver J., and William Fenical. 1980. “Halogen Chemistry of the Red Alga Bonnem Aisonia.” *Phytochemistry* 19: 233–47.

Medema, Marnix H., Peter Cimermancic, Andrej Sali, Eriko Takano, and Michael A. Fischbach. 2014. “A Systematic Computational Analysis of Biosynthetic Gene Cluster Evolution: Lessons for Engineering Biosynthesis.” *PLoS Computational Biology* 10 (12). doi:10.1371/journal.pcbi.1004016.

Mohammad, Haroon, Waleed Younis, Lu Chen, Christine E. Peters, Joe Pogliano, Kit Pogliano, Bruce Cooper, Jianan Zhang, Abdelrahman Mayhoub, Eric Oldfield, Mark Cushman, Mohamed N. Seleem. 2017. "Phenylthiazole Antibacterial Agents Targeting Cell Wall Synthesis Exhibit Potent Activity in Vitro and in Vivo against Vancomycin-Resistant Enterococci." *Journal of Medicinal Chemistry* 60 (6): 2425–38. doi:10.1021/acs.jmedchem.6b01780.

Molinski, Tadeusz F., Doralyn S. Dalisay, Sarah L. Lievens, and Jonel P. Saludes. 2009. "Drug Development from Marine Natural Products." *Nature Reviews Drug Discovery* 8 (1): 69–85. doi:10.1038/nrd2487.

Montaser, Rana, and Hendrik Luesch. 2011. "Marine Natural Products: A New Wave of Drugs?" *Future Medical Chemistry* 3 (12): 1475–89.

Murphy, Annabel C., Shu Shan Gao, Li Chen Han, Simon Carobene, Daisuke Fukuda, Zhongshu Song, Joanne Hothersall, Russell J. Cox, John Crosby, Matthew P. Crump, Christopher M. Thomas, Christine L. Willis, Thomas J. Simpson. 2014. "Biosynthesis of Thiomarinol A and Related Metabolites of Pseudoalteromonas Sp. SANK 73390." *Chemical Science* 5 (1): 397–402. doi:10.1039/c3sc52281d.

Nam, Young-Do, Ho-Won Chang, Ja Ryeong Park, Hyuk-Yong Kwon, Zhe-Xue Quan, Yong-Ha Park, Jung-Sook Lee, Jung-Hoon Yoon, and Jin-Woo Bae. 2007. "Pseudoalteromonas Marina Sp. Nov., a Marine Bacterium Isolated from Tidal Flats of the Yellow Sea, and Reclassification of Pseudoalteromonas Sagamiensis as Algicola Sagamiensis Comb. Nov." *International Journal of Systematic and Evolutionary Microbiology* 57 (Pt 1): 12–18. doi:10.1099/ijs.0.64523-0.

Nguyen, D. D., C.-H. Wu, W. J. Moree, A. Lamsa, M. H. Medema, X. Zhao, R. G. Gavilan, Marystella Aparicio, Librada Atencio, Chanaye Jackson, Javier Ballesteros, Joel Sanchez, Jeramie D. Watrous, Vanessa V. Phelan, Corine van de Wiel, Roland D. Kersten, Samina Mehnaz, René De Mot, Elizabeth A. Shank, Pep Charusanti, Harish Nagarajan, Brendan M. Duggan, Bradley S. Moore, Nuno Bandeira, Bernhard Ø. Palsson, Kit Pogliano, Marcelino Gutiérrez, and Pieter C. Dorrestein. 2013. "MS/MS Networking Guided Analysis of Molecule and Gene Cluster Families." *Proceedings of the National Academy of Sciences* 110 (28): E2611–20. doi:10.1073/pnas.1303471110.

Nonejuie, P., M. Burkart, K. Pogliano, and J. Pogliano. 2013. "Bacterial Cytological Profiling Rapidly Identifies the Cellular Pathways Targeted by Antibacterial Molecules." *Proceedings of the National Academy of Sciences* 110 (40): 16169–74. doi:10.1073/pnas.1311066110.

Norton, Raymond S., Kevin D. Croft, and Robert J. Wells. 1981. "Polybrominated Oxydiphenol Derivatives from the Sponge Dysidea Herbacea. Structure Determination by Analysis Of13C Spin-Lattice Relaxation Data for Quaternary

Carbons And13C-1H Coupling Constants." *Tetrahedron* 37 (13): 2341–49. doi:10.1016/S0040-4020(01)88887-6.

Offret, Clément, Florie Desriac, Patrick Le Chevalier, Jérôme Mounier, and Camille Jégou. 2016. "Spotlight on Antimicrobial Metabolites from the Marine Bacteria Pseudoalteromonas : Chemodiversity and Ecological Significance." doi:10.3390/md14070129.

Oh, Yong-Sik, A-Rum Park, Je-Kwan Lee, Chae-Sung Lim, Jae-Soo Yoo, and Dong-Hyun Roh. 2011. "Pseudoalteromonas Donghaensis Sp. Nov., Isolated from Seawater." *International Journal of Systematic and Evolutionary Microbiology* 61 (Pt 2): 351–55. doi:10.1099/ijs.0.022541-0.

Park, Yoon-Dong, Keun Sik Baik, Hana Yi, Kyung Sook Bae, and Jongsik Chun. 2005. "Pseudoalteromonas Byunsanensis Sp. Nov., Isolated from Tidal Flat Sediment in Korea." *International Journal of Systematic and Evolutionary Microbiology* 55 (Pt 6): 2519–23. doi:10.1099/ijs.0.63750-0.

Pogliano, Joe, Nick Osborne, Marc D. Sharp, Angelica Abanes De Mello, Ana Perez, Ya Lin Sun, and Kit Pogliano. 1999. "A Vital Stain for Studying Membrane Dynamics in Bacteria: A Novel Mechanism Controlling Septation during Bacillus Subtilis Sporulation." *Molecular Microbiology* 31 (4): 1149–59. doi:10.1046/j.1365-2958.1999.01255.x.

Rao, Dhana, Jeremy S. Webb, and Staffan Kjelleberg. 2005. "Competitive Interactions in Mixed-Species Biofilms Containing the Marine Bacterium Pseudoalteromonas Tunicata." *Applied and Environmental Microbiology* 71 (4): 1729–36. doi:10.1128/AEM.71.4.1729-1736.2005.

Reddy, Christopher M, Li Xu, Gregory W O'Neil, Robert K Nelson, Timothy I Eglinton, D John Faulkner, Ross Norstrom, Peter S Ross, and Sheryl a Tittlemier. 2004. "Radiocarbon Evidence for a Naturally Produced, Bioaccumulating Halogenated Organic Compound." *Environmental Science & Technology* 38 (7): 1992–97. http://www.ncbi.nlm.nih.gov/pubmed/15112798.

Richardson, Vicki M, Daniele F Staskal, David G Ross, Janet J Diliberto, Michael J DeVito, and Linda S Birnbaum. 2008. "Possible Mechanisms of Thyroid Hormone Disruption in Mice by BDE 47, a Major Polybrominated Diphenyl Ether Congener." *Toxicology and Applied Pharmacology* 226 (3): 244–50. doi:10.1016/j.taap.2007.09.015.

Richter, Taylor K.S., Chambers C. Hughes, and Bradley S. Moore. 2015. "Sioxanthin, a Novel Glycosylated Carotenoid, Reveals an Unusual Subclustered Biosynthetic Pathway." *Environmental Microbiology* 17 (6): 2158–71. doi:10.1111/1462-2920.12669.

Romanenko, L. a. 2003a. “Pseudoalteromonas Agarivorans Sp. Nov., a Novel Marine Agarolytic Bacterium.” *International Journal of Systematic and Evolutionary Microbiology* 53 (1): 125–31. doi:10.1099/ijs.0.02234-0.

Romanenko, Lyudmila A., Natalia V. Zhukova, Anatoly M. Lysenko, Valery V. Mikhailov, Erko Stackebrandt. 2003. “Assignment of ‘Alteromonas Marinoglutinosa’ NCIMB 1770 to Pseudoalteromonas Mariniglutinosa Sp. Nov., Nom. Rev., Comb. Nov.” *International Journal of Systematic and Evolutionary Microbiology* 53 (4): 1105–9. doi:10.1099/ijs.0.02564-0.

Ross, Avena C., Lauren E.S. Gulland, Pieter C. Dorrestein, and Bradley S. Moore. 2015. “Targeted Capture and Heterologous Expression of the Pseudoalteromonas Alterochromide Gene Cluster in Escherichia Coli Represents a Promising Natural Product Exploratory Platform.” *ACS Synthetic Biology* 4 (4): 414–20. doi:10.1021/sb500280q.

Ruzzini, Antonio C., and Jon Clardy. 2016. “Gene Flow and Molecular Innovation in Bacteria.” *Current Biology* 26 (18). Elsevier Ltd: R859–64. doi:10.1016/j.cub.2016.08.004.

Sailler, Birgit, and Karl-Werner Glombitza. 1999. “Haolgenated Phlorethols and Fucophlorethols from the Brown Alga Cystophora Retroflexa.” *Natural Toxins* 7: 57–62. doi:10.1016/S0031-9422(98)00643-8.

Sawabe, T, R Tanaka, M M Iqbal, K Tajima, Y Ezura, E P Ivanova, and R Christen. 2000. “Assignment of Alteromonas Elyakovii KMM 162T and Five Strains Isolated from Spot-Wounded Fronds of Laminaria Japonica to Pseudoalteromonas Elyakovii Comb. Nov. and the Extended Description of the Species.” *International Journal of Systematic and Evolutionary Microbiology* 50 Pt 1 (January): 265–71. http://www.ncbi.nlm.nih.gov/pubmed/10826813.

Shiozawa, Hideyuki, Takeshi Kagasaki, Takeshi Kinoshita, Hideyuki Haruyama, Haruki Domon, Yukio Utsui, Kentaro Kodama, and Shuji Takahashi. 1993. “Thiomarinol, a New Hybrid Antimicrobial Antibiotic Produced by a Marine Bacterium.” *The Journal of Antibiotics* 2216: 1834–42.

Shiroyama, Taisuke, Casareto E Beatriz, Yoshimi Suzuki, and Shinya Kodani. 2017. “Isolation of Antagonistic Bacteria Associated with the Stony Coral Montipora Digitata in Okinawa , Japan.” *Galaxea, Journal of Coral Reef Studies* 19 (2006): 7–13.

Simidu, Usio, Kumiko Kita-Tsukamoto, Takeshi Yasumoto, and Mari Yotsu. 1990. “Taxonomy of Four Marine Bacterial Strains That Produce Tetrodotoxin.” *International Journal of Systematic Bacteriology* 40 (4): 331–36. doi:10.1099/00207713-40-4-331.

Skovhus, Torben L., Carola Holmström, Staffan Kjelleberg, and Ingela Dahllöf. 2007. "Molecular Investigation of the Distribution, Abundance and Diversity of the Genus Pseudoalteromonas in Marine Samples." *FEMS Microbiology Ecology* 61 (2): 348–61. doi:10.1111/j.1574-6941.2007.00339.x.

Skovhus, Torben L, Niels B Ramsing, Carola Holmström, Carola Holmstro, and Staffan Kjelleberg. 2004. "Real-Time Quantitative PCR for Assessment of Abundance of Pseudoalteromonas Species in Marine Samples Real-Time Quantitative PCR for Assessment of Abundance of Pseudoalteromonas Species in Marine Samples." *Applied and Environmental Microbiology* 70 (4): 2373–82. doi:10.1128/AEM.70.4.2373.

Sneed, Jennifer M., Koty H. Sharp, Kimberly B. Ritchie, and Valerie J. Paul. 2014. "The Chemical Cue Tetrabromopyrrole from a Biofilm Bacterium Induces Settlement of Multiple Caribbean Corals." *Proceedings of the Royal Society B: Biological Sciences* 281 (1786): 1–9. doi:10.1098/rspb.2013.3086.

Stamatakis, Alexandros. 2014. "RAxML Version 8: A Tool for Phylogenetic Analysis and Post-Analysis of Large Phylogenies." *Bioinformatics (Oxford, England)* 30 (9): 1312–13. doi:10.1093/bioinformatics/btu033.

Suita, Kenji, Takayuki Fujita, Wenqian Cai, Yuko Hidaka, Huiling Jin, Rajesh Prajapati, Masanari Umemura, Utako Yokoyama, Motohiko Sato, Björn C. Knollmann, Satoshi Okumura, Yoshihiro Ishikawa. 2018. "Vidarabine, an Anti-Herpesvirus Agent, Prevents Catecholamine-Induced Arrhythmias without Adverse Effect on Heart Function in Mice." *Pflugers Archiv European Journal of Physiology* 470 (6). Pflügers Archiv - European Journal of Physiology: 923–35. doi:10.1007/s00424-018-2121-4.

Tebben, Jan, Dianne M. Tapiolas, Cherie A. Motti, David Abrego, Andrew P. Negri, Linda L. Blackall, Peter D. Steinberg, and Tilmann Harder. 2011. "Induction of Larval Metamorphosis of the Coral Acropora Millepora by Tetrabromopyrrole Isolated from a Pseudoalteromonas Bacterium." *PLoS ONE* 6 (4): 1–8. doi:10.1371/journal.pone.0019082.

Teuten, Emma L, and Christopher M Reddy. 2007. "Halogenated Organic Compounds in Archived Whale Oil: A Pre-Industrial Record." *Environmental Pollution (Barking, Essex : 1987)* 145 (3): 668–71. doi:10.1016/j.envpol.2006.08.022.

Teuten, Emma L, Li Xu, and Christopher M Reddy. 2005. "Two Abundant Bioaccumulated Halogenated Compounds Are Natural Products." *Science (New York, N.Y.)* 307 (5711): 917–20. doi:10.1126/science.1106882.

Thøgersen, Mariane S., Marina W. Delpin, Jette Melchiorsen, Mogens Kilstrup, Maria Månsson, Boyke Bunk, Cathrin Spröer, Jörg Overmann, Kristian F. Nielsen, and Lone Gram. 2016. "Production of the Bioactive Compounds Violacein and

Indolmycin Is Conditional in a MaeA Mutant of Pseudoalteromonas Luteoviolacea S4054 Lacking the Malic Enzyme." *Frontiers in Microbiology* 7 (SEP): 1–11. doi:10.3389/fmicb.2016.01461.

Utkina, N. K., V. A. Denisenko, O. V. Scholokova, M. V. Virovaya, A. V. Gerasimenko, D. Yu Popov, V. B. Krasokhin, and A. M. Popov. 2001. "Spongiadioxins A and B, Two New Polybrominated Dibenzo-p-Dioxins from an Australian Marine Sponge Dysidea Dendyi." *Journal of Natural Products* 64 (2): 151–53. doi:10.1021/np0003544.

Van Pée, Karl Heinz. 2001. "Microbial Biosynthesis of Halometabolites." *Archives of Microbiology* 175 (4): 250–58. doi:10.1007/s002030100263.

Venkateswaran, Kasthuri, and Nobuhiko Dohmoto. 2000. "Pseudoalteromonas Peptidolytica Sp. Nov., a Novel Marine Mussel-Thread-Degrading Bacterium Isolated from the Sea of Japan." *International Journal of Systematic and Evolutionary Microbiology* 50: 565–74.

Voultsiadou Eleni, E. 2010. "Therapeutic Properties and Uses of Marine Invertebrates in the Ancient Greek World and Early Byzantium." *Journal of Ethnopharmacology* 130 (2): 237–47. doi:10.1016/j.jep.2010.04.041.

Vynne, Nikolaj G., Maria Mansson, and Lone Gram. 2012. "Gene Sequence Based Clustering Assists in Dereplication of Pseudoalteromonas Luteoviolacea Strains with Identical Inhibitory Activity and Antibiotic Production." *Marine Drugs* 10 (8): 1729–40. doi:10.3390/md10081729.

Vynne, Nikolaj G, Maria Månsson, Kristian F Nielsen, and Lone Gram. 2011. "Bioactivity, Chemical Profiling, and 16S RRNA-Based Phylogeny of Pseudoalteromonas Strains Collected on a Global Research Cruise." *Marine Biotechnology (New York, N.Y.)* 13 (6): 1062–73. doi:10.1007/s10126-011-9369-4.

Weber, Tilmann, Kai Blin, Srikanth Duddela, Daniel Krug, Hyun Uk Kim, Robert Bruccoleri, Sang Yup Lee, Michael A. Fischbach, Rolf Müller, Wolfgang Wohlleben, Rainer Breitling, Eriko Takano, Marnix H. Medema. 2015. "AntiSMASH 3.0-A Comprehensive Resource for the Genome Mining of Biosynthetic Gene Clusters." *Nucleic Acids Research* 43 (W1): W237–43. doi:10.1093/nar/gkv437.

Wender, Paul A., Clayton T. Hardman, Stephen Ho, Matthew S. Jeffreys, Jana K. Maclaren, Ryan V. Quiroz, Steven M. Ryckbosch, Akira J. Shimizu, Jack L. Sloane, and Matthew C. Stevens. 2017. "Scalable Synthesis of Bryostatin 1 and Analogs, Adjuvant Leads against Latent HIV." *Science* 358 (6360): 218–23. doi:10.1126/science.aan7969.

Whalen, Kristen E, Kelsey L Poulson-Ellestad, Robert W Deering, David C Rowley, and

Tracy J Mincer. 2015. "Enhancement of Antibiotic Activity against Multidrug-Resistant Bacteria by the Efflux Pump Inhibitor 3,4-Dibromopyrrole-2,5-Dione Isolated from a Pseudoalteromonas Sp." *Journal of Natural Products* 78 (3): 402–12. doi:10.1021/np500775e.

Winter, Jaclyn M, Swantje Behnken, and Christian Hertweck. 2011. "Genomics-Inspired Discovery of Natural Products." *Current Opinion in Chemical Biology* 15 (1). Elsevier Ltd: 22–31. doi:10.1016/j.cbpa.2010.10.020.

Xing, Haibo, Mengting Tong, Nanyu Jiang, Xiaomin Zhang, Hong Hu, Hongming Pan, and Da Li. 2017. "Antitumour Bioactive Peptides Isolated from Marine Organisms." *Clinical and Experimental Pharmacology and Physiology* 44 (11): 1077–82. doi:10.1111/1440-1681.12808.

Xu, Xue-Wei, Yue-Hong Wu, Chun-Sheng Wang, Xiao-Hui Gao, Xiao-Gu Wang, and Min Wu. 2010. "Pseudoalteromonas Lipolytica Sp. Nov., Isolated from the Yangtze River Estuary." *International Journal of Systematic and Evolutionary Microbiology* 60 (Pt 9): 2176–81. doi:10.1099/ijs.0.017673-0.

Yamanaka, Kazuya, Kirk A. Reynolds, Roland D. Kersten, Katherine S. Ryan, David J. Gonzalez, Victor Nizet, Pieter C. Dorrestein, and Bradley S. Moore. 2014. "Direct Cloning and Refactoring of a Silent Lipopeptide Biosynthetic Gene Cluster Yields the Antibiotic Taromycin A." *Proceedings of the National Academy of Sciences* 111 (5): 1957–62. doi:10.1073/pnas.1319584111.

Zabawa, Thomas P., Michael J. Pucci, Thomas R. Parr, and Troy Lister. 2016. "Treatment of Gram-Negative Bacterial Infections by Potentiation of Antibiotics." *Current Opinion in Microbiology* 33 (Figure 1). Elsevier Ltd: 7–12. doi:10.1016/j.mib.2016.05.005.

Zhao, Chang-Hui, Jing-Jing Luo, Ting Gong, Xiang-Ling Huang, De-Zan Ye, and Zhu-Hua Luo. 2014. "Pseudoalteromonas Xiamenensis Sp. Nov., a Marine Bacterium Isolated from Coastal Surface Seawater." *International Journal of Systematic and Evolutionary Microbiology* 64 (Pt 2): 444–48. doi:10.1099/ijs.0.050229-0.

Zhou, Tong, Michele M Taylor, Michael J DevVito, and Kevin M Crofton. 2002. "Developmental Exposure to Brominated Diphenyl Ethers Results in Thyroid Hormone Disruption" 116: 105–16.