Natural Product Discovery & Development of a Novel Method to Quantify Bacteria from Marine Habitats in Remote Locations

Marie J. Charles
Department of Biology
University of Prince Edward Island
Charlottetown, Prince Edward Island, Canada

A thesis submitted in partial fulfilment of the requirements of the
Honours Programme in the Department of Biology

This Thesis is Accepted

_____

Dean of Science
University of Prince Edward Island

May 2018

Abstract

Bacteria are an important source of natural products; however, only 1% of all bacterial species have been cultivated through the use of standard laboratory methods. To isolate new bacteria and potentially discover untapped resources of natural products, new isolation methods are required. The Kerr lab modified one such device, the isolation chip (ichip), for use in marine sponges. A representative sample of microbes isolated from marine sponges using the Kerr lab's ichip was fermented in various media to induce natural product biosynthesis. This resulted in the production of four putatively novel metabolites. One of these metabolites, referred to as metabolite 1, was confirmed to be a new amino acid, and seemed to have selective activity against *Mycobacterium tuberculosis*. Based on full 16S rRNA gene sequencing, it appears that the strain RKMC9, the bacterium responsible for the production of metabolite 1, is a novel species of the genus *Alteromonas*. Preliminary field research revealed that, to use the isolation chip efficiently, it is necessary to quantify the bacterial densities of the environmental samples. In the laboratory, this can be achieved via fluorescence microscopy; however, this technique is generally not applicable to field studies. Alternatively, adenosine triphosphate (ATP) can be used as a measure of numbers of living cells. Due to the availability of relatively low-cost portable luminometers and the commercial availability of ATP test kits, this approach is an appealing method for estimating bacterial abundance for ichip studies conducted in the field. In this project, the Hyigena EnSURE luminometers system was evaluated and preliminary data suggest that the luminometer provides linear readings for pure ATP, DAPI stained *E. coli* cells, as well as for viable cell counts.

Acknowledgments

It is my pleasure to acknowledge the following individuals. Their support has not only made this Honours project possible but has also contributed to my success as an undergraduate student at the University of Prince Edward Island.

Firstly, I would like to express my gratitude to my supervisor Dr. Russell Kerr for his support and insight. I would like to thank you for so willingly providing undergraduate students enriching and rewarding experiences.

Many thanks to Brad Haltli; your guidance has been most meaningful to me and has taught me so much. To the Nautilus staff and Kerr lab students, thank you for always lending a hand. A special thanks to Logan Macintyre, your work with the compound purification and structural elucidation is certainly appreciated.

I would also like to express my gratitude towards my thesis advisor, Dr. Marva Sweeney-Nixon, the Biology Honours Coordinator, Dr. Lawrence Hale, and all of the faculty and staff in the UPEI Biology Department. You have made my time at UPEI memorable, and have pushed me to achieve to the best of my capabilities.

Finally, I would like to thank my parents, Jean and Patrick, as well as my sister, Lise. Your endless support and encouragement are undoubtedly the most significant contributors to all of my endeavours.

Table of Contents

List of Tables

List of Figures

Abbreviations

A	Adenine
ATP	Adenosine triphosphate
BFM	Bacterial Fermentation Media
bp	Base pairs
C	Cytosine
CV	Coefficients of variation
COSY	Correlation Spectroscopy
DAPI	4', 6-Diamidino-2-phenylindole dihydrochloride
DMSO	Dimethyl sulfoxide
ELSD	Evaporative light scattering detector
ESI-MS	Electrospray ionization mass spectrometry
G	Guanine
HMBC	Heteronuclear Multiple Bond Correlation
HSQC	Heteronuclear Single Quantum Correlation
ichip	Isolation chip
m/z	Mass/charge
NOESY	Nuclear Overhauser Effect Spectroscopy
NMR	Nuclear Magnetic Resonance
PCR	Polymerase Chain Reaction
PDA	Photodiode array
RLU	Relative Light Unit
rRNA	Ribosomal ribonucleic acid
T	Thymine
TB	Tuberculosis
UHPLC-HRMS	Ultra High Performance Liquid Chromatography-High Resolution Mass Spectrometry

INTRODUCTION AND LITERATURE REVIEW

Introduction

Natural products are biological molecules that are not required for the basic sustenance of life, but whose production presents an adaptive advantage to the organisms that produce them. These compounds, also known as secondary metabolites, have broad implications for pharmaceutical and healthcare industries; however, the rate of discovery of these novel compounds has been diminishing since the 1970s (Watve et al., 2001). Ultimately, the discovery of these compounds relies on the organisms that synthesize them, such as bacteria, fungi and plants. Bacteria are an important source of natural products; however, approximately 99% of all bacterial species are not readily cultivated through the use of standard laboratory methods and are coined "uncultivable" (Amann et al., 1995). Evidence for the presence of uncultivable microbes is the fact that the number of cells observed microscopically far outnumbers the number of colonies growing on a petri plate inoculated from the same sample. These microorganisms have not been viable in the laboratory settings due to an imperfect imitation of the natural environment and possibly other factors that are not well understood.

Altering synthetic media to mimic suspected environmental conditions have resulted in the cultivation of thousands of bacteria; however, extensive modification of all of these conditions to satisfy these poorly understood requirements would entail excessively large efforts (Stewart, 2012). A promising strategy to overcome these

challenges is to enhance the rate at which different strains are isolated through the development of novel isolation methodologies.

Developments of innovative isolation methods are required to increase bacterial diversity. The isolation chip (ichip) serves to culture and purify bacterial species within their natural environment, or *in situ* (Nichols et al*.,* 2010). The Kerr lab modified the isolation chip in an attempt to culture new strains of bacteria from marine sponges and sediment. Field collections of bacteria were conducted in the Bahamas using this ichip in two marine sponges: *Xestospongia muta* and *Verongula rigida*. This project aims to investigate the metabolites produced by the bacterial library collected from these sponges and to develop a field method to quantify the microbial densities of environmental samples, an issue that would improve both the success of the Kerr lab's isolation chip and future developments.

Literature Review

Secondary metabolites are organic compounds produced by, but inessential for the growth of, organisms such as plants, vertebrates, invertebrates, and microorganisms. These compounds, known more commonly as natural products, have been the active ingredients of medicines since antiquity (Newman et al., 2000). Terrestrial plants have been known to produce the majority of natural products; however, the 1928 discovery of the antibiotic penicillin produced by the fungus *Penicillium rubens* and the 1943 isolation of the antibiotic streptomycin from the bacterium *Streptomyces grieus*, resulted in microorganisms becoming of larger interest to the chemical and pharmaceutical industries (Chin et al., 2006). Since 1981, roughly 50% of all US Food and Drug

Administration (FDA) approved drugs, ranging from antibacterial to anticancer agents, have been natural products or natural product-derived chemicals (Newman and Cragg, 2016).

Microorganisms, more specifically bacteria and fungi, can be found in both terrestrial and marine environments. Due to the limited access to marine environments, land microbes were the primary source of drugs until the first marine compounds were isolated from *Tethya crypta*, a marine sponge, in the 1950s (Bergman and Feeney, 1951). Not surprisingly, the investigation of marine environments had proliferated by the mid-1970s yielding a historical timeline similar to that of the development and popularization of the self-contained underwater breathing apparatus (SCUBA).

Natural products discovered from marine invertebrates, such as sponges, corals, tunicates, bryozoans, and mollusks, have a large significance in natural product chemistry and have provided the majority of marine natural products tested in clinical trials (Alonso et al., 2003). Marine sponges, sessile invertebrates belonging to the phylum Porifera, are the primary source of marine natural products (Koopmans et al., 2006). The sponges' anatomy explains this spectacle: the middle gelatinous matrix, mesohyl, serves as the habitat for many microbial species (Selvin et al., 2009). Interestingly, some of the compounds previously thought to have been produced by the sponges, are in reality manufactured by the bacteria and fungi living within their tissues (Abdel-Lateff, 2006). It is hypothesized that such products helped to drive symbiotic relationships between the sponges and microbes; a relationship in which sponges provide a habitat and microbial metabolites serve to protect against predation, infection, and competition (Armstrong et al., 2001). Of the dominant bacterial phyla found within sponges, Actinobacteria is the

most important source of natural products (Manivasagan et al., 2014). Actinobacteria, more specifically bacteria from the genus *Streptomyces*, are the largest producers of both terrestrial and marine drugs including but not limited to, antibacterial, antifungal, anticancer, antitumor, anti-inflammatory, antiparasitic, antimalarial, antiviral, and antioxidant drugs (Baltz, 2005). Natural product discovery is increasingly important due to the emergence of resistance; many pathogens are no longer susceptible to the drugs used to treat them (Fischbach and Walsh, 2009).

The rate of novel natural product discovery peaked in the 1970s and has since been declining (Watve et al., 2001). This decline can be explained by a constant re-isolation of previously discovered species (Osburne et al., 2000). Since natural product discoveries rely ultimately on the organisms that synthesize them, a lack of access to novel microbes consequently impedes the development of novel chemistry. Recent advances in genetic sequencing have uncovered the fact that the microbial diversity of earth's biosphere is not represented in current laboratory libraries (Amann et al., 1995). It is estimated that 99% of all accessible microbial species cannot be successfully cultured within laboratories (Amann et al., 1995). This phenomenon has been suspected since Amann (1911) noticed that the number of bacteria growing in laboratory Petri dishes did not correlate with the amount observed in environmental samples; this was coined the "Great Plate Count Anomaly" (Staley and Konopka, 1985). The presumed explanation for this significant gap is the fact that laboratory settings do not successfully mimic essential properties of natural environments: chemical signalling, temperature, pH, and nutrients (Alain and Querellou, 2009).

As most traditional cultivation techniques favoured dominant species, most culturing methodologies aimed at culturing "uncultivable" microbes have been focused on modifying laboratory incubation conditions in favour of slower-growing species (Ferrari et al., 2005). Approaches focused on varying nutrient media concentration, incubation time, inoculum size temperature, pH and atmospheric gas levels have all resulted in the isolation of novel bacteria (Davis et al., 2004 and Stevenson et al., 2004). Bacterial communication is equally important; in some cases, bacteria did not grow unless alongside other bacteria (Bollmann et al., 2007). This discovery has led to recent advancements in co-culturing techniques, where bacteria are cultured with others to promote growth.

To increase laboratory collections of bacterial diversity, recent developments have focused on utilizing the environment itself as an isolation strategy (Ferrari et al., 2005). One such development is known as the 'diffusion chamber'; this device is inoculated with bacteria and sealed with a 0.3 μm pore-sized membrane, which allows for environmental nutrients and signalling molecules to diffuse into the chamber without having to add them to the agar (Bollmann et al., 2007). Once assembled, the chamber can be returned to the environment from which the organisms were sourced or kept within a block of sediment within the laboratory. Numerous unusual species were successfully grown, or domesticated, in the diffusion chambers (Bollmann et al., 2007).

The advances of the diffusion chambers allowed for the development of other *in situ* incubation devices. One such device, the isolation chip (ichip), was designed to address one of the diffusion chamber's main issues: colony purification (Nichols et al., 2010). The ichip consists of hundreds of small diffusion chambers, and each chamber be

loaded with approximately one cell (Figure 1). The ichip allows for *in situ* incubation, the purification of hundreds of colonies, and the retrieval of new species (Nichols et al., 2010). In 2015, the ichip was successful in isolating a novel bacterial species from a terrestrial sediment sample. The bacterium, *Eleftheria terrae*, produces a unique natural product, known now as teixobactin that appears to be a potent antimicrobial agent (Ling et al., 2015). This workflow demonstrates perfect proof-of-principle for the discovery of new antibiotics: a new method, new bacteria, new product, and potentially, a new drug.

Due to the attractiveness of the ichip, the Kerr lab miniaturized this device in an attempt to isolate new strains of bacteria from marine sponges (Figure 2). In the summer of 2016, field collections were conducted in the Bahamas to test the modified ichip via bacterial collections from two marine sponges: *Xestospongia muta* and *Verongula rigida*. The isolated bacteria were identified by 16S rRNA gene sequencing, and a preliminary phylogenetic analysis was conducted to compare the relationship between the isolated strains and previously reported bacteria (Figure 3). This project firstly aims to investigate the metabolites produced by this bacterial library.

In efforts to isolate bacteria from sponges, it was revealed that to use the ichip effectively, it is necessary to estimate the bacterial concentration of the environmental sample. These concentrations must then be adjusted to concentrations of approximately one cell per ichip well. In the laboratory, bacterial density can be measured by fluorescence microscopy in combination with stains such as 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI). However, due to expense and portability issues, this technique is not applicable to field studies. Alternatively, adenosine triphosphate (ATP) can be used as a measure of numbers of living cells.

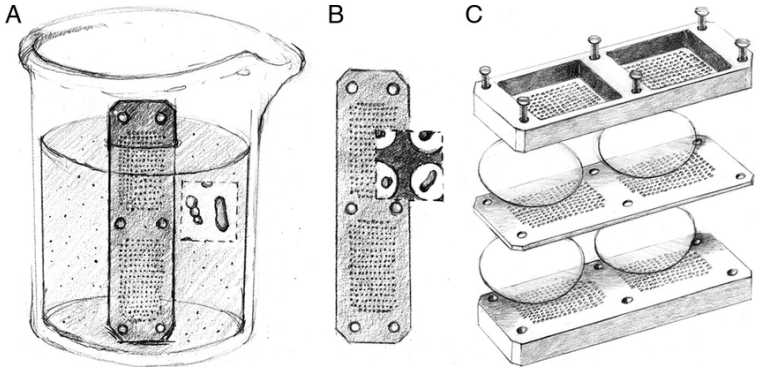


Figure 1. Isolation chip (ichip). Artwork by Stacie Bumgarner, Whitehead Institute for Biomedical Sciences, Cambridge, MA. (Nichols et al*.,* 2010).

All living cells use ATP; the amount of ATP per bacterial cell is estimated at one femtogram (Crombrugge and Waes, 1991), and studies have shown that ATP in environmental samples can be correlated with microbial counts (Egeberg et al., 2000). Due to the availability of relatively low-cost portable luminometers and the commercial availability of ATP test kits, this approach is an attractive method for estimating bacterial abundance for ichip studies conducted in the field. The Hygiena EnSURE luminometer tests surfaces and water; this luminometer is used in conjunction with sampling devices, which contain luciferin and luciferase. Luciferase catalyzes the reaction between luciferin, oxygen, and ATP, producing light, which can be then detected by the luminometer. The most sensitive detection device manufactured by Hygenia is the SuperSnap. For these reasons, the second goal of this project was to evaluate this instrument and create a protocol to quantify bacteria within the field.

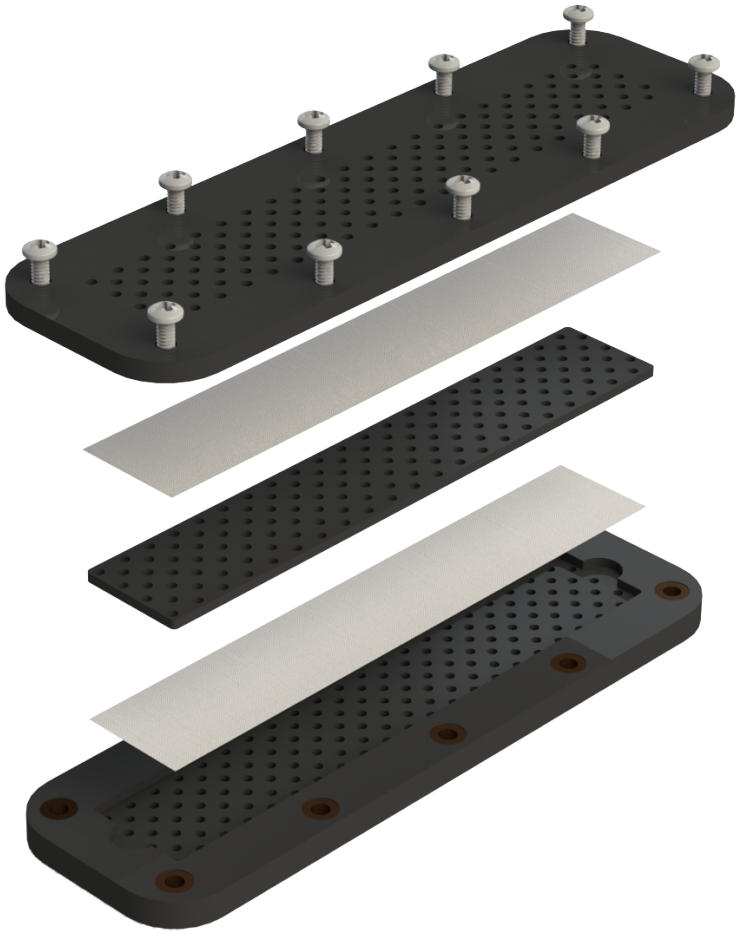


Figure 2. Kerr laboratory isolation chip (ichip). Designed in collaboration with the UPEI Engineering Department's 3D Printing lab, 2016.

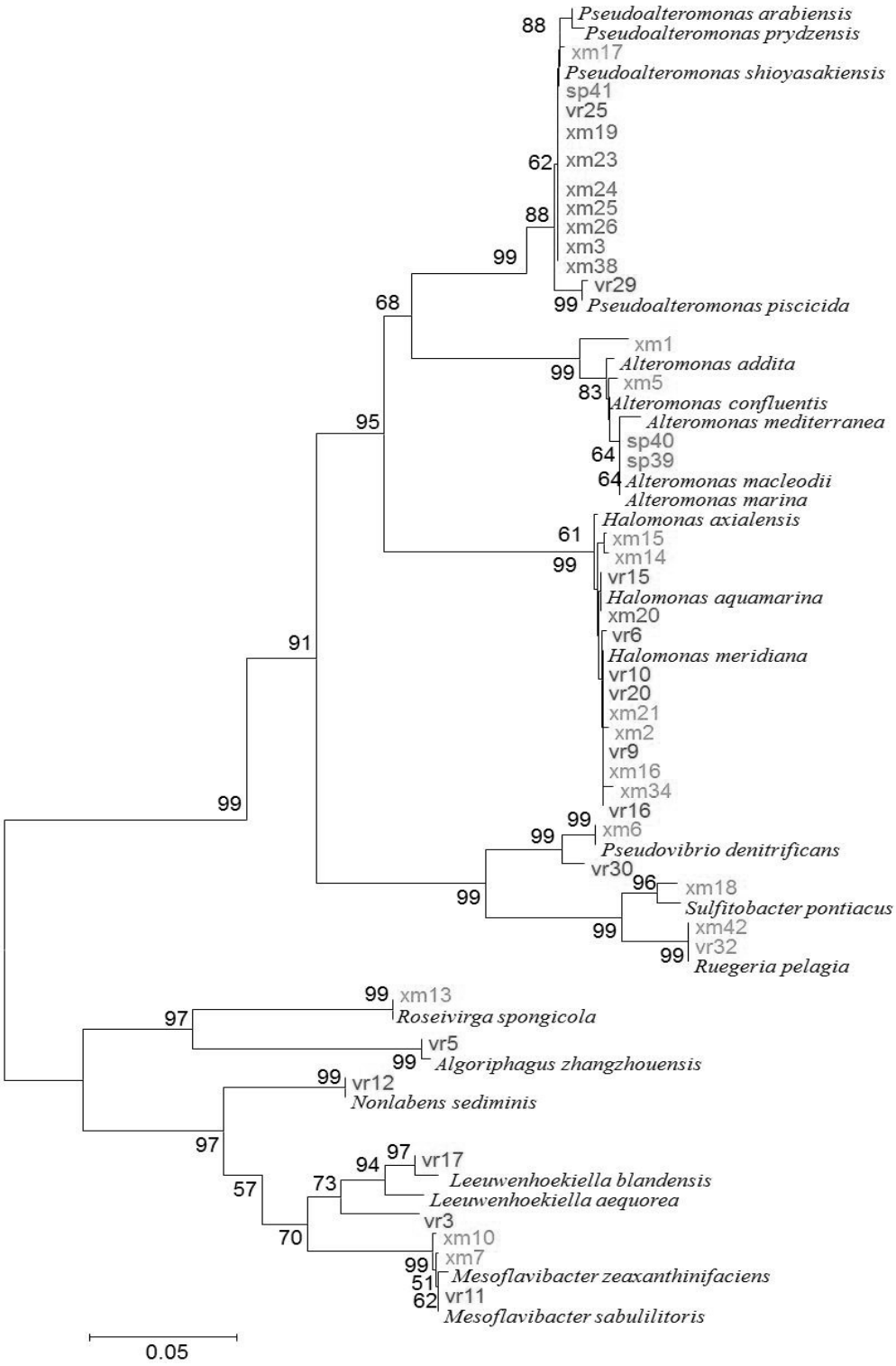


Figure 3. Neighbour-joining tree of bacterial isolates obtained from two marine sponges. Four strains from this experiment may constitute novel species (<98.65% 16S rRNA gene sequence ID to validly described species): *Altermonas* sp. RKMC1, *Altermonas sp.* RKMC9 *Leeuwenhoekiella* sp. RKMC21 and *Leeuwenhoekiella* sp. RKMC100.

METHODS

Investigating Natural Product Biosynthesis From A Library Of Bacteria Isolated From Two Sponges Using An ichip

Natural Product Discovery

1. Bacterial Strains:

In 2016, bacterial strains were isolated using the Kerr Lab Isolation Chip (ichip), adapted from Nichols *et al*., 2010, from two marine sponges *Xestospongia muta* and *Verongula rigida.* This collection was done off the west coast of San Salvador, The Bahamas at a depth of 10-12 m.

2. Small-Scale Fermentations and Media Study:

From this bacterial library, a group of 22 representative bacteria was selected, grown and purified on Difco™ Marine Agar at 30°C for 3 days (Table 1). Difco™ Marine Broth (7 mL) was inoculated with 3 pure colonies of each bacterium, and these seed tubes were incubated for 2 days at 30°C on a rotary shaker set at 200 rpm. Fermentation media (7 mL), marine versions of bacterial fermentation media 1, 2, 3, 4, 5, 8 and 11 (BFM#m), were selected based on their varying ingredients and properties (Appendix 1). Fermentation media were then inoculated with 210 µL of well-grown seed media (3% inoculum) and incubated for 3 days at 30°C on a rotary shaker set at 200 rpm.

Table 1. Representative bacteria chosen for fermentation and natural product discovery.

Strain Name	Seq ID[1]	Isol Meth[2]	Isol Source[3]	Closest related Species in GenBank	% Sim[4]	Put. Novel[5]
RKMC1	XM1	ichip	*X. muta*	*Alteromonas macleoidii*	94.24	Yes
RKMC3	XM6	ichip	*X. muta*	*Pseudovibrio denitrificans*	100	No
RKMC6	XM7	ichip	*X. muta*	*Mesoflavibacter sabulilitoris*	99.29	No
RKMC9	XM5	ichip	*X. muta*	*Alteromonas confluentis*	98.24	Yes
RKMC10	XM3	ichip	*X. muta*	P*seudoalteromonas shioyasakiensis*	99.42	No
RKMC14	XM13	ichip	*X. muta*	*Roseivirga spongicola*	99.86	No
RKMC16	XM14	ichip	*X. muta*	*Halomonas meridiana*	99.53	No
RKMC21	XM17	ichip	*X. muta*	*Pseudoalteromonas shioyasakiensis*	98.05	Yes
RKMC22	XM24	ichip	*X. muta*	*Pseudoalteromonas shioyasakiensis*	98.64	No
RKMC25	XM21	ichip	*X. muta*	*Halomonas meridiana*	99.41	No
RKMC59	VR5	ichip	*V. rigida*	*Algoriphagus zhangzhouensis*	99.86	No
RKMC65	VR9	ichip	*V. rigida*	*Halomonas meridiana*	99.54	No
RKMC66	VR12	ichip	*V. rigida*	*Nonlabens sediminis*	100	No
RKMC68	VR15	ichip	*V. rigida*	*Halomonas meridiana*	99.88	No
RKMC71	VR11	ichip	*V. rigida*	Mesoflavibacter sabulilitoris	99.88	No
RKMC73	VR16	ichip	*V. rigida*	*Halomonas meridiana*	99.88	No
RKMC76	VR25	ichip	*V. rigida*	*Pseudoalteromonas mariniglutinosa*	99.49	No
RKMC83	VR30 (orange)	ichip	*V. rigida*	*Pseudoalteromonas piscicida*	99.86	No
RKMC85	VR29	ichip	*V. rigida*	*Pseudoalteromonas piscicida*	99.07	No
RKMC97	SP40	Control Plate	*V. rigida*	*Alteromonas macleodii*	99.25	No
RKMC98	VR30 (white)	ichip	*V. rigida*	*Pseudoalteromonas shioyasakiensis*	99.89	No
RKMC99	VR11 (white)	ichip	*V. rigida*	/	?	No

[1]Sequence ID, [2]Isolation Method, [3]Isolation Source, [4]% Similarity to closest related species in GenBank, [5]Putatively Novel Species

3. Extractions:

Natural products were extracted by addition of 7 mL of ethyl acetate to the fermentation tube followed by shaking at 300 rpm for one hour. Tubes were removed from the shaker and phases were left to separate for 1 hour, the organic layer was removed and placed into a clean tube; this extraction process was repeated. The final extract was washed using 5 mL of deionized water. Extracts were dried under air to remove all the solvent, brought up in 1 mL of methanol and subsequently analyzed by Ultra High Performance Liquid Chromatography-High Resolution Mass Spectrometry (UHPLC-HRMS)

4. Ultra-High Performance Liquid Chromatography-High Resolution Mass Spectrometry:

UHPLC-HRMS analysis was conducted with an ESI-HRMS Exactive (Thermo Scientific) with a resolution of 30 000, using a Core-Shell 100 Å C18 column (Kinetex, 1.7 μm 50 × 2.1 mm). A linear solvent gradient from 95% H_2O/0.1% formic acid (solvent A) and 5% acetonitrile (CH_3CN)/0.1% formic acid (solvent B) to 100% solvent B over 4.8 min followed by a hold for 3.2 min with a flow rate of 500 μL/min was used. Eluent was detected by Mass Spectrometry (ESI-MS) monitoring m/z 190−2000 in positive mode, evaporative light scattering detector (ELSD) and photodiode array (PDA) (200–600 nm).

5. Metabolomics: Automatic Data Analysis:

MZmine Software was used for peak picking of UHPLC-HRMS profiles, set with an intensity threshold of 5E5 units, followed by deisotoping, bucketing alignment,

standardization and artifact suppression (Forner et al., 2013). The end result from the Metabolomic workflow was an Excel spreadsheet containing metabolite mass charge and retention time, their relative abundance, and the samples in which they were produced. This spreadsheet was then used to generate a heatmap highlighting metabolite abundance by sample using the Rstudio extension ggplot.

6. Manual Data Analysis:

Based on the heatmap, chromatograms generated from UHPLC-HRMS were viewed using XCalibur Software. Metabolite presence and abundance was compared between samples to generate a list of the metabolites produced.

7. Metabolite Identification:

The mass/charge, retention time and sample name were recorded for metabolites that appeared abundantly produced in samples other than media blanks. Metabolite identification was achieved by exact mass comparison to previously reported microbial metabolites within the AntiBase: Natural Compound Identifier (2017) database. Compounds that did not generate a hit within 5 ppm from a bacterial source were recorded as putatively novel. The identity of the compounds that generated hits within 5 ppm from a bacterial source was also recorded.

8. Scale-up Fermentations and Extractions:

Fermentations that yielded the putatively novel metabolites **1**, **2**, **3**, and **4** were subjected to scale-up fermentation (2 x 7 mL, 2 x 50 mL, 1 x 600 mL) in order to

determine the optimal scale for subsequent product production. A sample (7 mL) of each fermentation was extracted using the methods previously described and subsequently analyzed by UHPLC-HRMS.

Metabolite 1

1. Extraction: Metabolite 1:

From the 600 mL fermentation, natural products were extracted in a separatory funnel using 3 x 300 mL portions of ethyl acetate. The organic layer was washed using 3 x 300 mL portions of deionized water, the organic phase was collected, and the solvent was removed under reduced pressure using a rotovap.

2 Analysis in Negative and Positive Mode: Metabolite 1:

Four small-scale fermentations and extractions of RKMC9 in BF4Mm were conducted as described above. Using UHPLC-HRMS eluent was detected by Mass Spectrometry (ESI-MS) monitoring m/z 190−2000 in positive and negative mode, evaporative light scattering detector (ELSD) and photodiode array (PDA) (200–600 nm).

3. Bioactivity Assays:

A crude extract containing metabolite 1, RKMC9 grown in BFM4m, was tested for biological activity. All microbroth antibiotic susceptibility testing was carried out in 96-well plates in accordance with Clinical Laboratory Standards Institute testing standards (NCCLS, 2003) using the following pathogens: methicillin-resistant *Staphylococcus aureus* ATCC 33591 (MRSA), *Staphylococcus warneri* ATCC 17917,

vancomycin-resistant *Enterococcus faecium* EF379 (VRE), *Pseudomonas aeruginosa* ATCC 14210, *Proteus vulgaris* ATCC 12454, and *Candida albicans* ATCC 14035. Extract fractions were tested in triplicate against each organism. Extract fractions were resuspended in sterile 20% DMSO(aq) and assayed at 250 µg/mL with a final well volume concentration of 2% DMSO(aq). Each plate contained eight uninoculated positive controls (media + 20% DMSO(aq)), eight untreated negative controls (media + 20% DMSO(aq) + organism), and one column containing a concentration range of a control antibiotic (vancomycin for MRSA, and *S. warneri,* rifampicin for VRE, gentamycin for *P. aeruginosa*, ciprofloxacin for *P. vulgaris*, or nystatin for *C. albicans*). The optical density of the plate was recorded using a Thermo Scientific Varioskan Flash plate reader at 600 nm at time zero and then again after incubation of the plates for 22 h at 37°C. After subtracting the time zero OD600 from the final reading, the percentages of microorganism survival relative to vehicle control wells were calculated.

4. Cytotoxicity Assays:

A crude extract containing metabolite 1, RKMC9 grown in BFM4m, was tested for antimicrobial activity against the following human cell lines.

Human colon colorectal carcinoma cancer cells (ATCC HCT-116) were grown in 15 mL of Eagle's minimal essential medium (Sigma M5650) supplemented with 10% fetal bovine serum (VWR#CA95043-976), 0.01 mg/ml human recombinant insulin (Sigma #I9278) and 100 µU penicillin and 0.1 mg/mL streptomycin (VWR#CA12001-692) in at 37°C in a humidified atmosphere of 5% CO_2. Culture medium was refreshed every two to three days and cells were not allowed to exceed 80% confluency.

Human breast invasive breast ductal carcinoma cells (ATCC MCF-7) were grown

and maintained in 15 mL of Eagle's minimal essential medium (Sigma M5650) supplemented with 10% fetal bovine serum (VWR#CA95043-976), 0.01 mg/ml human recombinant insulin (Sigma #I9278) and 100 μU penicillin and 0.1 mg/mL streptomycin (VWR#CA12001-692) at 37°C in a humidified atmosphere of 5% CO_2. Culture medium was refreshed every two to three days and cells were not allowed to exceed 80% confluency.

Human breast adenocarcinoma cells (ATCC HTB-26) were grown and maintained in 15 mL of Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (Sigma D6421-500ML) supplemented with 10% fetal bovine serum (VWR#CA95043-976) and 100 μU penicillin and 0.1 mg/mL streptomycin (VWR#CA12001-692) at 37°C in a humidified atmosphere of 5% CO_2. Culture medium was refreshed every two to three days and cells were not allowed to exceed 80% confluency.

Cercopithecus aethiops kidney epithelial cells (Vero, ATCC CCL-81) were grown and maintained in 15 mL of Eagle's minimal essential medium (Sigma M5650) supplemented with 10% fetal bovine serum (VWR#CA95043-976) and 100 μU penicillin and 0.1 mg/mL streptomycin (VWR#CA12001-692) at 37°C in a humidified atmosphere of 5% CO_2. Culture medium was refreshed every two to three days and cells were not allowed to exceed 80% confluency.

At 80% confluency, the cells were counted, diluted and plated into 96-well treated cell culture plates. The HCT-116, MCF-7, HTB-26 cells were plated at cell density of 5000 cells per well and the vero cells were plated at cell density of 10,000 cells in 90 μL of respective growth medium. The plates were incubated at 37 °C in a humidified atmosphere of 5% CO_2 to allow cells to adhere to the plates for 24 hours

before treatment. DMSO was used as the vehicle at a final concentration of 1% in the wells. All compounds tested were resolublized in sterile DMSO and a dilution series was prepared for each cell line using the respective cell culture growth medium of which 10 µL were added to the respective assay plate well yielding eight final concentrations ranging from 128 µg/mL to 1 µg/mL per well (final well volume of 100 µL). The HCT-116, MCF-7, and HTB-26 cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 for 72 hours and the vero cells were incubated at 37°C in a humidified atmosphere of 5% CO2 for 24 hours. All samples were tested in triplicate. Each plate contained four uninoculated positive controls (media + 10% DMSO(aq)), four untreated negative controls (Media + 10% DMSO(aq) + cells), and one column containing a concentration range of doxorubicin. Alamar blue (Invitrogen#Dal1100) was added, 24 hrs after treatment, to each well at 10% of the culture volume (11µL in 100 µL). Fluorescence was monitored using a Thermo Scientific Varioskan Flash plate reader at 560/12 excitation, 590 nm emission 4 hrs after Alamar blue was added. The inferred percentage of cell viability relative to vehicle control wells was calculated and the IC_{50} was determined.

5. Antimycobacterial susceptibility screening assay against Mycobacterium tuberculosis:

A crude extract containing metabolite 1, RKMC9 grown in BFM4m, was tested for biological activity against *Mycobacterium tuberculosis* strain H37Ra (ATCC 25177). This screening was performed by a collaborator at the University of New Brunswick, using a microplate resazurin assay. Stock solutions of test compounds (6.4 mg/mL) and positive control rifampin (10 µg/mL) were prepared with sterile filtered DMSO and stored at 4°C. Immediately prior to use, stock solutions of test compounds and rifampin

(40 μL) were diluted with modified Middlebrook 7H9 broth (960 μL) and the resulting rifampin or test compounds (100 μL) were transferred to nonperipheral wells of a 96-well microtitre plate and inoculated with suspensions of *M. tuberculosis* H37Ra (100 μL) of cell density 2.0 × 106 cells/mL. To reduce evaporation from the plates, sterile water (200 μL) was added to perimeter wells. In addition to test compounds and the rifampin positive controls, negative controls [4% DMSO in modified Middlebrook 7H9 broth (100 μL) inoculated with suspensions of *M. tuberculosis* H37Ra (100 μL)], and blanks [2% DMSO in modified Middlebrook 7H9 broth (200 μL), test solutions (100 μL) with modified Middlebrook 7H9 broth (100 μL), and antibiotic (100μL) with modified Middlebrook 7H9 broth (100 μL)] were included in each plate. All controls and test compounds were tested in triplicate. Plates were incubated (37°C; 5% CO_2) for 3 days in a humid environment before a solution of resazurin 0.0625 mg/mL) in 5% aqueous Tween 80 (50 μL) was added to all wells. Plates were then incubated for a further 24 hours, sealed with an adhesive polyester film, and mycobacterial growth was assessed fluorometrically at 37°C. Fluorescence values were corrected for any background fluorescence of the media and test compounds by subtracting the mean fluorescence readings of the appropriate blanks from the mean fluorescence readings of the control and test compound wells. The percentage inhibition of mycobacterial growth was then defined as: [1 − (mean test or positive control fluorescence/mean negative control fluorescence)] × 100.

The crude extract submitted contained a large peak eluting at 0.5 minutes (Figure 4). Upon further purification, a second extract containing metabolite 1 was tested for activity against *Mycobacterium tuberculosis* (Figure 5).

6. Purification: Metabolite 1:

The crude extract of RKMC9 grown in BFM4m was fractionated using reverse-phase flash chromatography (CombiFlash Rf system) with a 15.5 g C18 column and a linear gradient from 10% methanol/water to 100% methanol over 20 minutes. Based on ultra-violet (UV) absorption properties, fractions were pooled and analyzed by UPLC-HRMS at a concentration of 500 μg/mL. The fermentation process was repeated on a larger scale (4 x 600 mL) and in order to defat the sample, the extraction procedure was changed, and a hexanes and water/methanol (20%/80%) partition wash was used prior to chromatographic purification. The fermentation extract was then fractionated using reverse-phase flash chromatography (CombiFlash Rf system) with a 15.5 g C18 column and a linear gradient from 5% methanol/water to 100% methanol over 40 minutes. Based on the generated UV peaks, tubes were pooled and analyzed by UPLC-HRMS at a concentration of 500 μg/mL.

(i)

(ii)

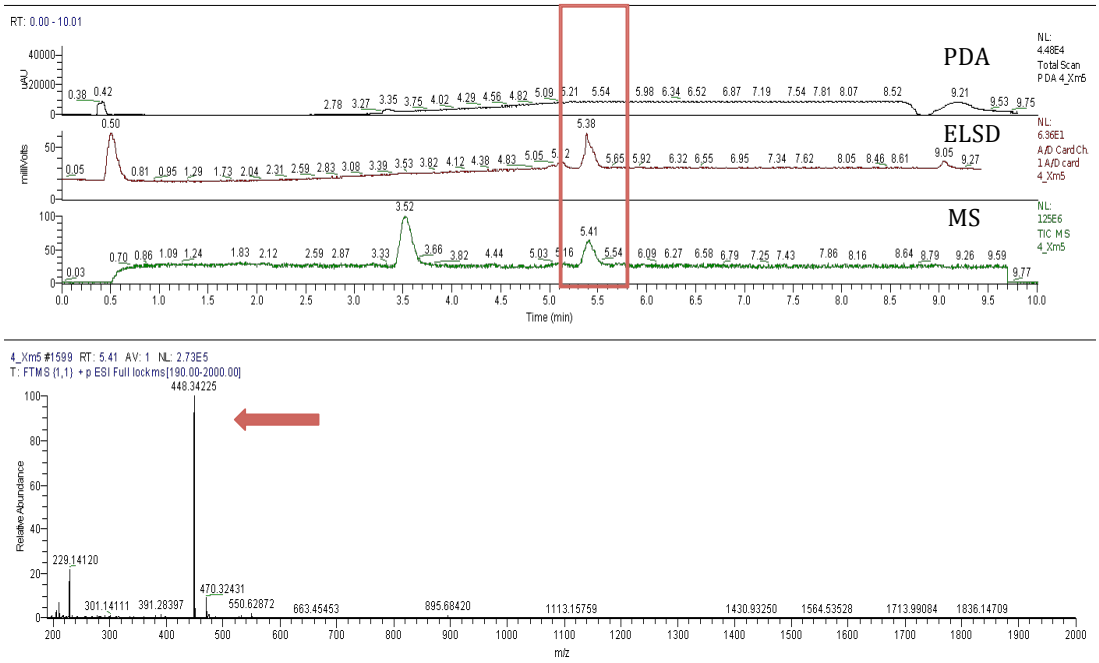


Figure 4. Chromatogram of the crude extract containing metabolite 1.

(i) Photodiode array (PDA) chromatograms measuring ultra-violet (UV) absorbance; Evaporative light scattering detector (ELSD) chromatograms illustrating compound abundance; Mass spectrometer (MS) chromatograms indicating compound ionization. (ii) Mass spectrum at the highlighted retention time, arrow indicates the mass/charge (m/z) value of metabolite 1.

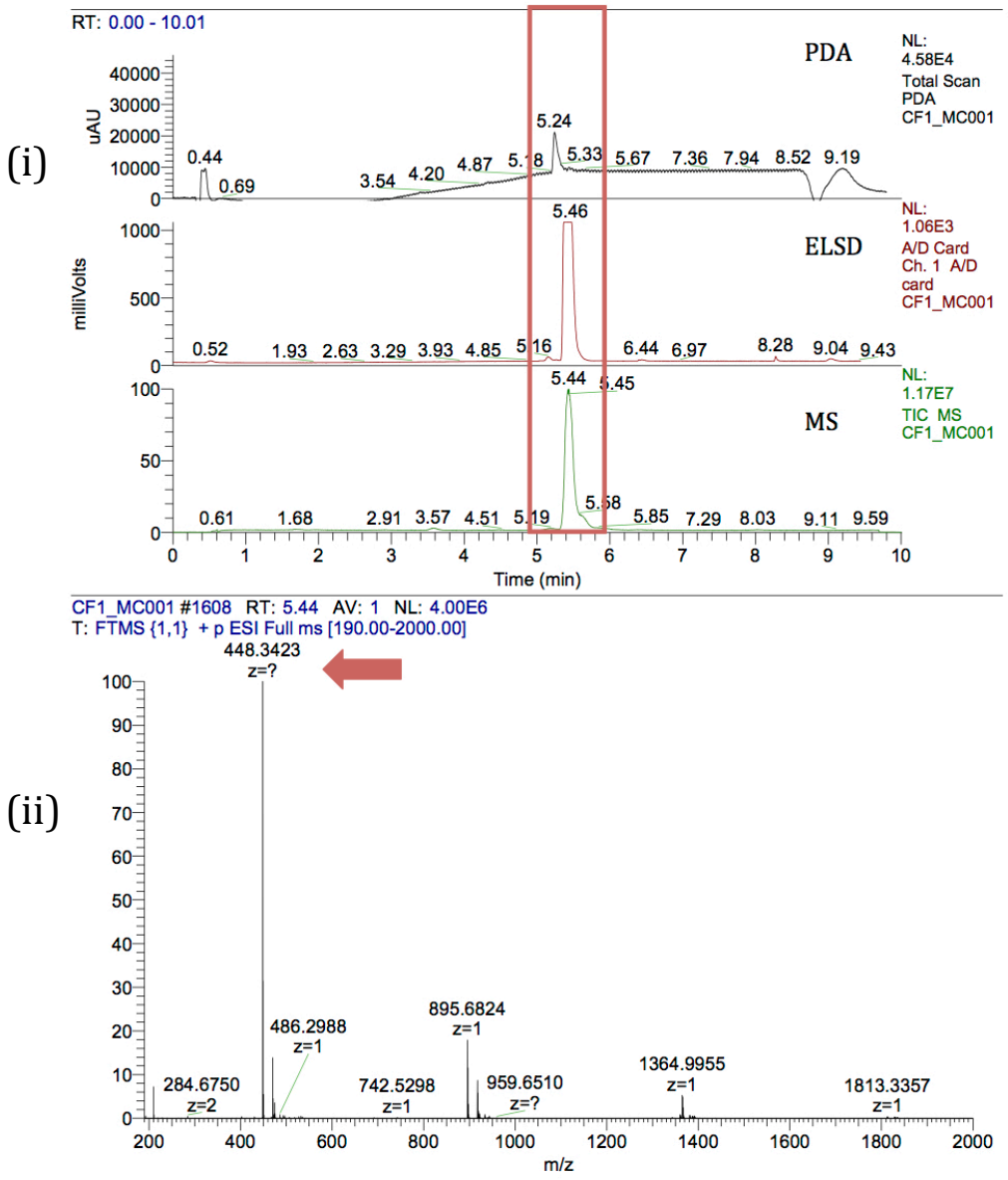


Figure 5. Chromatogram of purified metabolite 1. (i) Photodiode array (PDA) chromatograms measuring UV absorbance; Evaporative light scattering detector (ELSD) chromatograms illustrating compound abundance; Mass spectrum (MS) chromatograms indicating compound ionization. (ii) Mass spectrum at the highlighted retention time, arrow indicates the mass/charge (m/z) value of metabolite 1.

7. Structural Elucidation: Metabolite 1:

Elucidation of the chemical structure of metabolite 1 was accomplished using a combination of ^{1}H, ^{13}C, Correlation Spectroscopy (COSY), Heteronuclear Single Quantum Correlation (HSQC), Heteronuclear Multiple Bond Correlation (HMBC) and Nuclear Overhauser Effect Spectroscopy (NOESY) NMR spectroscopy. ^{1}H and ^{13}C NMR spectra were acquired on a 400 MHz Bruker AVANCE III NMR spectrometer operating at 400 and 100 MHz, respectively, and equipped with a broadband SmartProbe and AVANCE III HD NanoBay console. All chemical shifts were referenced to residual solvent peaks [1H ($CDCl_3$): 7.26 ppm; 13C ($CDCl_3$): 77.16 ppm].

8. Small-Scale Fermentations for Structural Confirmation:

Four small-scale fermentations (7 mL) of RKMC9 in BFM4m were conducted as described above. Extractions were prepared either with methanol or acetonitrile for submission to UHPLC-HRMS.

Identification of Bacterial Isolates

1. Bacterial Growth and DNA Extraction:

The bacterial strains RKMC9, RKMC83, RKMC85, and RKMC97 were grown on Difco™ Marine Agar at 30°C for 3 days and used to inoculate 7 mL of Difco™ Marine Broth. These tubes were incubated for 2 days at 30°C on a rotary shaker set at 200 rpm. In order to extract genomic DNA for 16s rRNA gene amplification and sequencing, a DNeasy UltraClean Microbial Kit was used. Following the Quick-Start Protocol, 1.8 mL of the microbial cultures RKMC9, RKMC83, RKMC85, and RKMC97, was centrifuged at 10,000 x g for 30 seconds at room temperature. The supernatant was

removed and the tubes were spun again at 10,0000 x g for 30 seconds. The supernatant was completely removed. The cell pellets were resuspended in 300 µL of PowerBead Solution, vortexed gently for 5 seconds and cells were transferred to the PowerBead Tube. Fifty µL of Solution SL was added to the PowerBead Tubes, tubes were vortexed for 10 minutes, and then centrifuged at 10,000 x g for 30 seconds at room temperature. One hundred µL of IRS Solution was added to the supernatant, vortexed for 5 seconds and incubated at 4°C for 5 minutes. Tubes were centrifuged at 10,000 x g for 1 minute at room temperature. Nine hundred µL of Solution SB was added to the supernatant, and vortexed for 5 seconds. Seven hundred µL of solution was loaded into a MB Spin Column and centrifuged at 10,000 x g for 30 seconds at room temperature. The flow-through was discarded, and the remaining supernatant was added to the MB Spin Column, and centrifuged at 10,000 x g for 1 minutes at room temperature. The MB Spin Column was placed in a new collection tube, 50 µL of Solution EB was added and the solution was centrifuged at 10,000 x g for 30 seconds at room temperature. Eluted DNA was stored at -20°C. Three µL of each DNA sample was run at 110 V for 25 minutes in a 1% agarose gel (70 mL TAE; 35 µL ethidium bromide) in 1 x TAE buffer. The gel was then visualized under UV light using the UVP BioDoc-It Gel Imaging System.

2. 16S rRNA Gene Amplification:

Polymerase Chain Reaction (PCR) was used to amplify the partial 16S rRNA genes of bacterial isolates RKMC9, RKMC83, RKMC85, and RKMC97. Amplification conditions included a 25 µl reaction mixture consisting of 8.75 µL nuclease free water, 12.5 µL EconoTaq Plus Green 2X Master Mix, 1.25 µL forward primer 27F, 5'-

AGAGTTTGATCMTGGCTCAG, (10 µM), 1.25 µL reverse primer 1525R, 5'-AAGGAGGTGATCCAGCC-3, (10 µM), 1.25 µL DNA template. The Eppendorf Thermal Cycler Gradient was programmed for 90 seconds at 95°C as initial denaturation, followed by 35 cycles of 30 seconds at 95°C for denaturation, 30 seconds at 55°C as annealing, 90 seconds at 72°C for extension, and final extension at 72°C for 5 minutes. 3 µL of these generated PCR products were separated by electrophoresis at 110 V for 25 minutes in a 1% agarose gel (70 mL TAE; 35 µL ethidium bromide) in 1 x TAE buffer. The gel was then visualized under UV light using the UVP BioDoc-It Gel Imaging System. The amplified 16S rRNA genes were sent for sequencing.

3. 16S rRNA Gene Sequencing:

Full-length 16S rRNA gene sequencing was performed using the primers 16S936R, 5'-GGGGTTATGCCTGAGCAGTTTG, 16S1527R, 5-A'AGGAGGTGATCCAGCC, 16S514F, 5'-GTGCCAGCASCCGCGG and 16S1114F, 5'-GCAACGAGCGCAACCC.

4. Phylogenetic Analysis:

Sequences (~1400 bp) were manually trimmed and aligned using the Geneious-Molecular Biology and NGS Analysis Tool. Sequences were compared to sequences within the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the Basic Local Alignment Search Tool (BLASTN). Sequences with < 98.65% match similarity were defined as putatively novel.

Development of a rapid and portable method for estimating numbers of bacteria in environmental samples.

Hygiena EnSURE Luminometer Instrument Evaluation

1. Instrument Protocol:

A 200 µL sample was and pipetted onto the SuperSnap (Catalog# SUS3000) swab tip, the swap was replaced into the swab tube and the Snap-Valve was broken in two directions. The reagent reservoir was squeezed twice to dispense reagent. The tube was gently shaken for 10 seconds to bathe the swab in the reagent. Activity was then measured by inserting the tube into the EnSURE luminometers (Catalog# ENSURE).

2. Dynamic Range:

Based on a manufacturer protocol, 10 mM ATP was diluted 10-fold in Tris-acetate buffer to 1 fM, and measurements were taken using the instrument usage protocol. This experiment was conducted in triplicate and the average RLU readings for each dilution were plotted against the corresponding ATP concentrations (mM).

3. Repeatability:

A 10 mM stock of pure ATP was diluted 10-fold in Tris-acetate buffer to a concentration of 1 fM. Using the instrument usage protocol, the relative light unit (RLU) readings generated by the luminometer were recorded for the 1 nM (n=4) and 0.1 nM (n=6) dilutions. All measurements were taken within 10 minutes to limit ATP degradation.

DAPI, Plate Count and Luminometer (RLU) Correlation

1. Bacterium Growth Conditions:

The following methods were carried out in triplicate using the *Escherichia coli* stain DH5alpha grown on Thermofisher Luria-Bertani (LB) agar at 30°C and subsequently used to inoculate 25 mL of Thermofisher LB broth. Flasks were incubated overnight at 15°C on a rotary shaker set to 200 rpm. Flasks were removed from the incubator and placed at 4°C for 30 minutes. Following this period, 10 mL was taken from each flask and diluted 10-fold (to 10^{-7}) in sterile particle-free water. Dilutions (10 mL tubes) were kept on ice.

2. DAPI Staining Protocol:

Each 10 mL sample was separated into 5 mL portions, and one of those portions was fixed with 250 µL of 37% formaldehyde. To result in a final DAPI concentration of 1 mg/L, 0.5 mL of DAPI work solution, 1 µg/mL (in sterile methanol), was added to each fixed sample. Samples were stained for 15 minutes in the dark, at room temperature, and filtered through a 0.2 µm pore-sized cyclopore track etched black polycarbonate, 25 mm diameter, membrane filter (Whatman Cat# 7063-2502). Filters were then washed with 5 mL of sterile particle-free water. Filters were placed on microscope slides, covered with a small amount of non-fluorescent immersion oil and mounted with a cover slip. Slides were visualized on an AxioPlan 2 imaging microscope at the 63x objective and photographed using an AxioCam camera in combination with the AxioVision software. The above methodology was adapted and refined to achieve adequate cell staining and photographs. Fifteen to twenty photos were taken of the dilution that had the best

combination of countable cell number and reduced background luminescence. In each photo, an area of 5000 um^2 was indicated and cell numbers were manually counted (Figure 6). The average number of cells per area were then multiplied by the total number of areas on the membrane, and divided to yield the approximate number of cells/ 200 µL.

3. Plate counts:

From dilution 10^{-4} to 10^{-7}, 100 µL of sample was used to inoculate a plate of Thermofisher LB agar using the spread plate method. This step was done in triplicate, and plates were grown for 3 days at 30°C. Following incubation, cells were manually counted. The average number of cells per mL was used to determine the approximate number of cells present within 200 µL of each dilution of the sample.

4. Luminometer readings:

Following instrument protocol, readings were taken for 200 µL of each sample until it was determined which samples fell within the linear range of the instrument. Based on this range, luminometers readings were taken on 200 µL of sample. This was performed in quadruplicate.

5. Correlations:

The cell counts/ 200 µL obtained using both fluorescence microscopy and plate counts were compared to the average RLU reading generated by the luminometer for 200 µL of the same sample. Calibration curves were then generated.

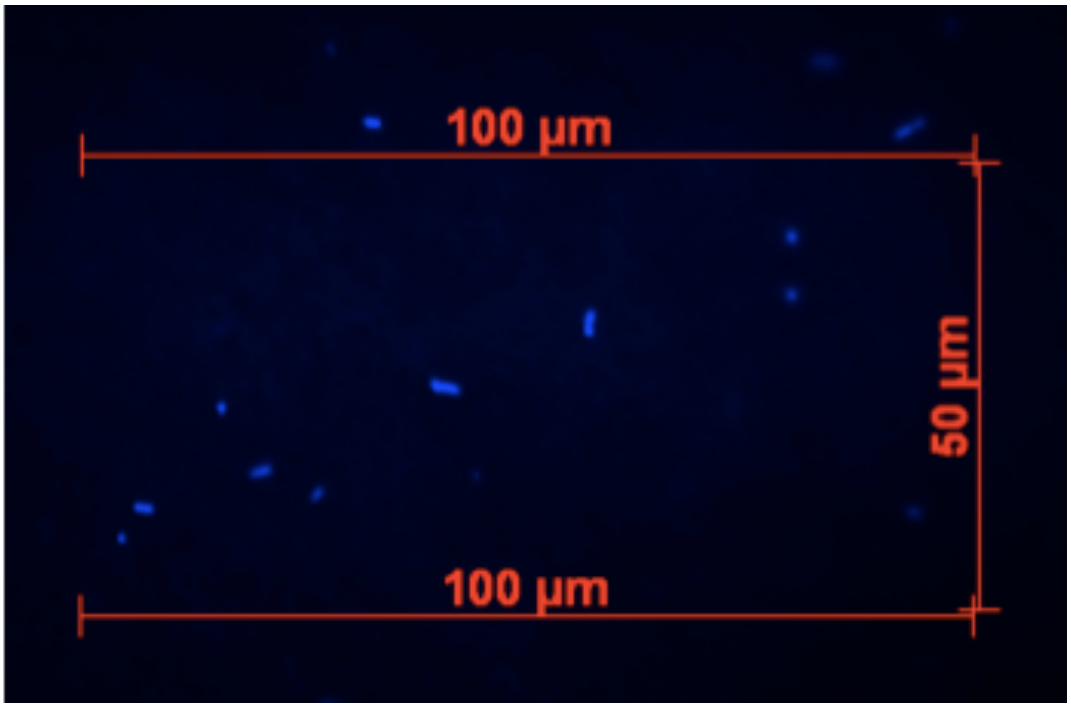


Figure 6. A representative slide of *E. coli* stained using 4',6-Diamidino-2-Phenylindole (DAPI). Cells were visualized on an AxioPlan 2 imaging microscope at the 63x objective and photographed using an AxioCam camera in combination with the AxioVision software.

RESULTS

Investigating Natural Product Biosynthesis From A Library Of Bacteria Isolated From Two Sponges Using An ichip.

Natural Product Discovery

1. Metabolomics:

The 161 fermentations generated 267 metabolites (Figure 7). Manual screening and comparison of chromatograms resulted in the identification of seven abundantly produced metabolites.

2. Metabolite Identification:

Of these 7 metabolites, four did not yield hits within 5 ppm from a bacterial species within the 2017 AntiBase Natural Product Identification database and consequently proved to be of interest as potential novel metabolites. Metabolite 1 had a mass/charge of 448.3419 (**metabolite 1**) and was produced by RKMC9 in all media (Figure 8), metabolite 2 had a mass/charge of 408.3107 (**metabolite 2**) and was produced by RKMC97 in BFM8m (Figure 9), metabolite 3 had a mass/charge of 846.2903 (**metabolite 3**) and was produced by RKMC83 and RKMC85 in BFM11m and BFM8m (Figure 10) and metabolite 4 had a mass/charge of 345.1544 (**metabolite 4**) and was produced by RKMC83 in BFM4m (Figure 11).

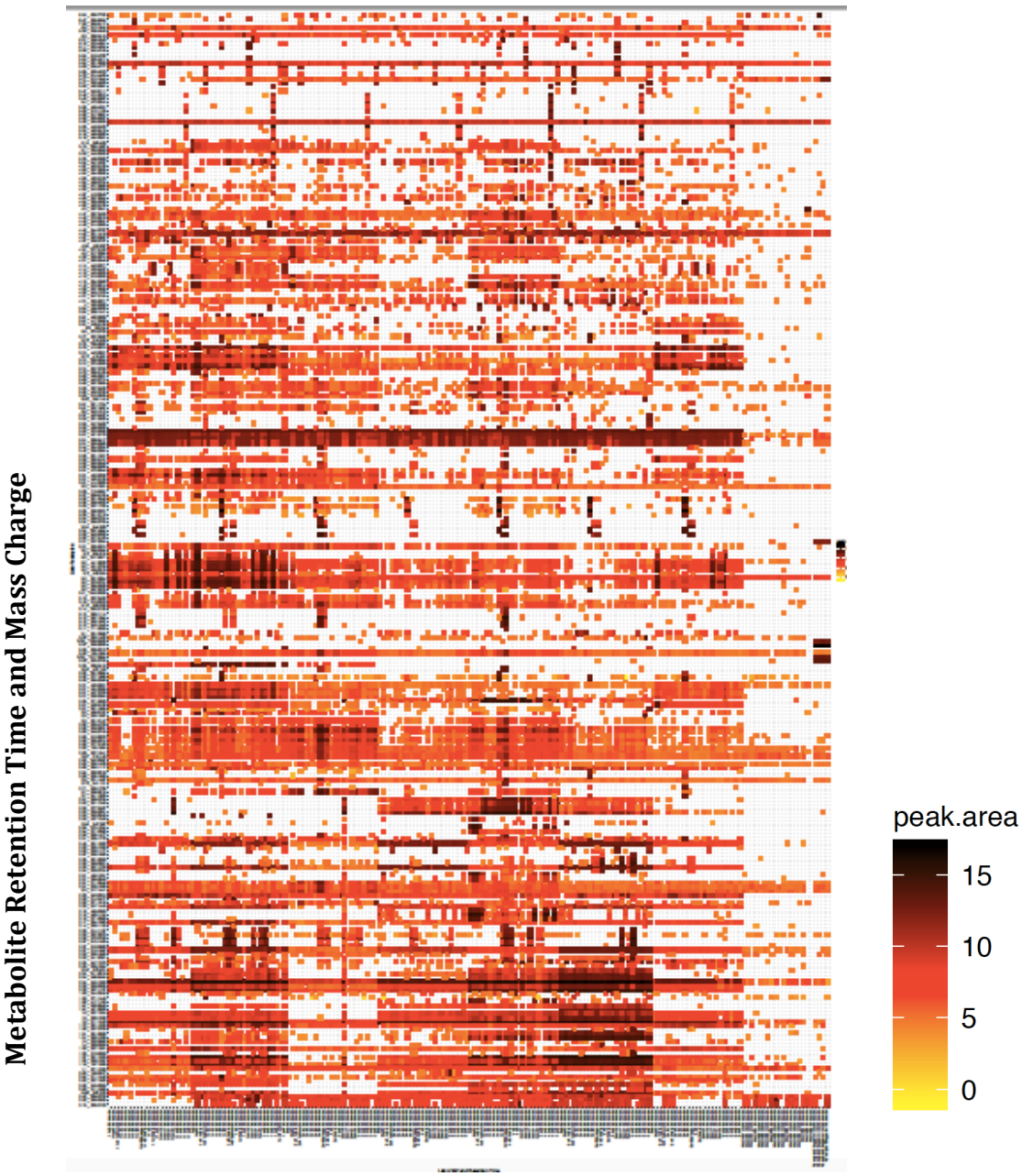


Figure 7. Graphical representation of metabolite production and abundance. The colour intensity denotes relative peak area, which corresponds to metabolite abundance. The image was generated using the Rstudio extension ggplot.

(i)

(ii)

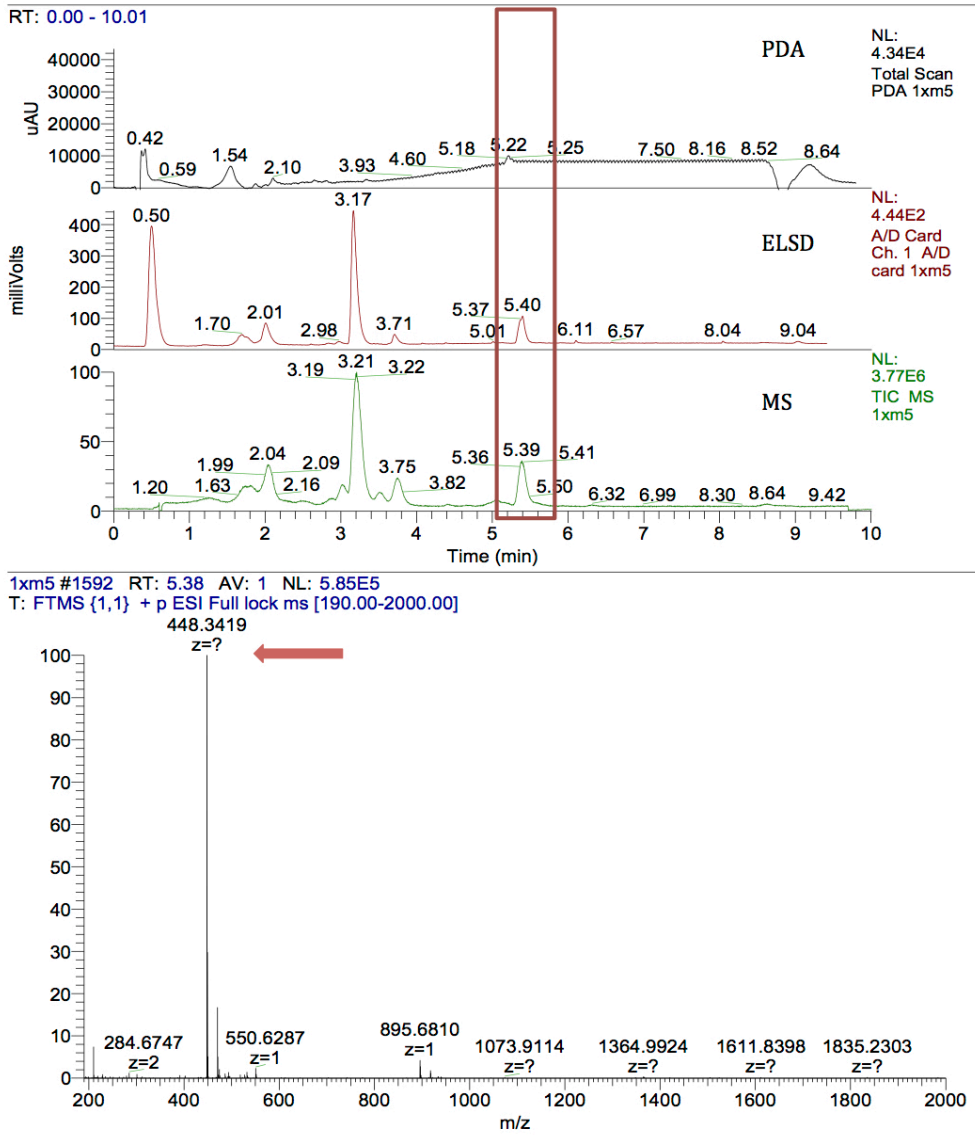


Figure 8. The UHPLC-HRMS chromatogram for metabolite 1.

The compound was produced by strain RKMC9. (i) Photodiode Array (PDA) chromatogram illustrates UV absorbance; Evaporative light scattering detector (ELSD) chromatogram illustrates compound abundance; Mass Spectrum (MS) chromatogram indicates compound ionization. (ii) The arrow indicates the mass/charge (m/z) value of the novel metabolite and the highlighted box indicates the retention time/ mass spectrum being viewed.

(i)

(ii)

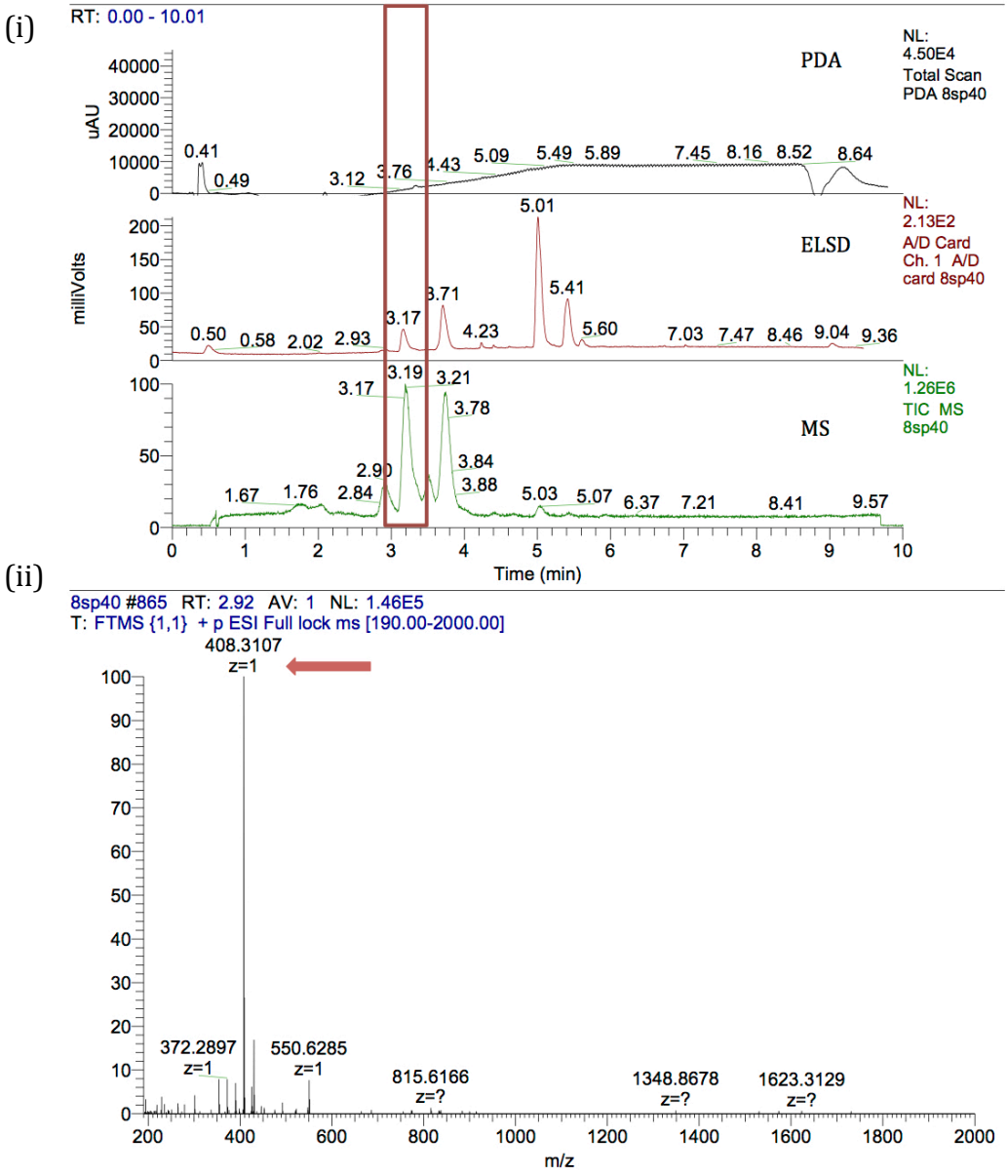


Figure 9. The UHPLC-HRMS chromatogram for metabolite 2.

The compound was produced by strain RKMC97. (i) Photodiode array (PDA) chromatogram illustrates ultra-violet (UV) absorbance; Evaporative light scattering detector (ELSD) chromatogram illustrates compound abundance; Mass spectrum (MS) chromatogram indicates compound ionization. (ii) The arrow indicates the mass/charge (m/z) value of the novel metabolite and the highlighted box indicates the retention time/ mass spectrum being viewed.

(i)

(ii)

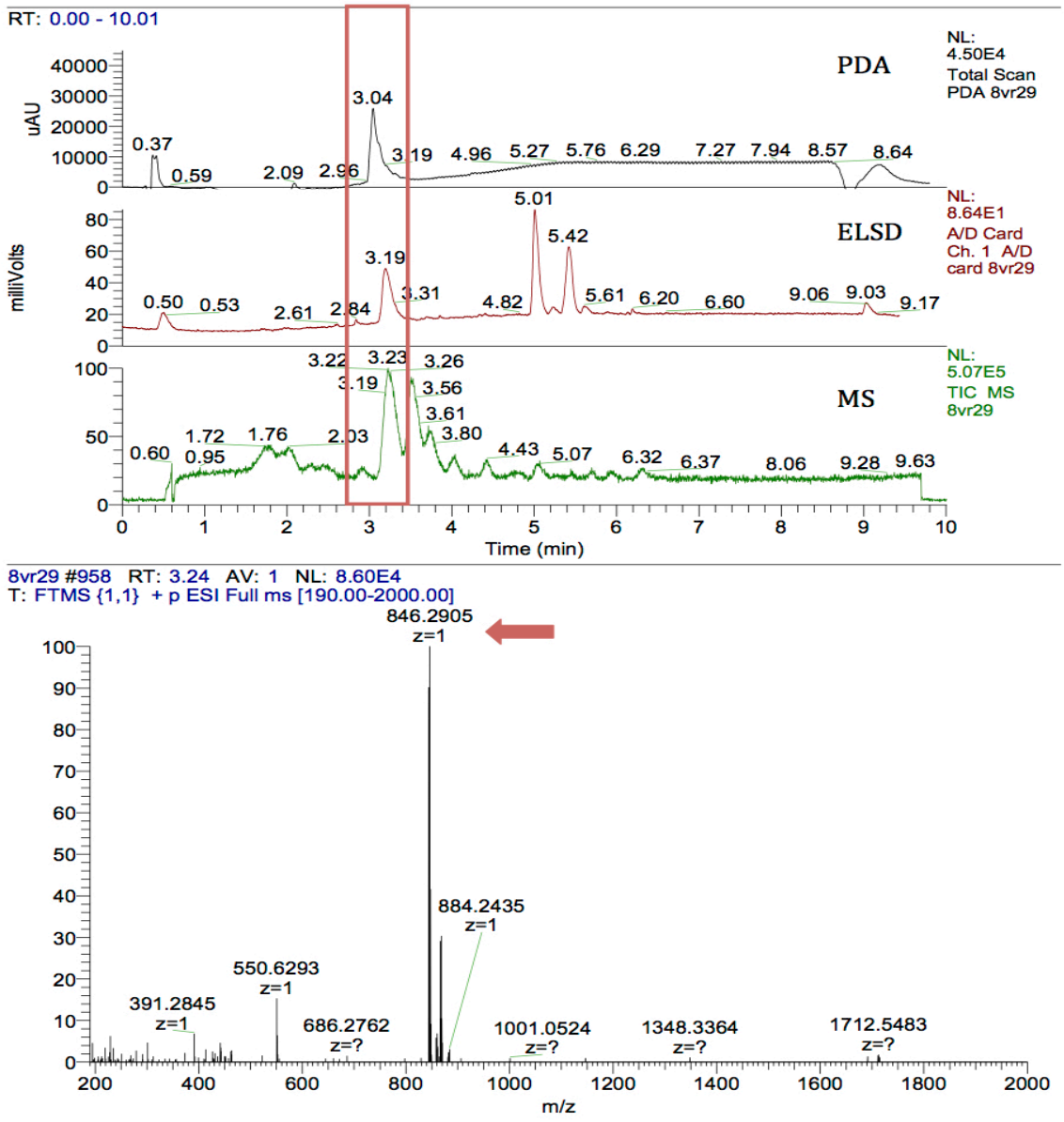


Figure 10. The UHPLC-HRMS chromatogram for metabolite 3.

The compound was produced by strain RKMC83 and RKMC85. (i) Photodiode array (PDA) chromatogram illustrates UV absorbance; Evaporative light scattering detector (ELSD) chromatogram illustrates compound abundance; Mass spectrum (MS) chromatogram indicates compound ionization. (ii) The arrow indicates the mass/charge (m/z) value of the novel metabolite and the highlighted box indicates the retention time/ mass spectrum being viewed.

(i)

(ii)

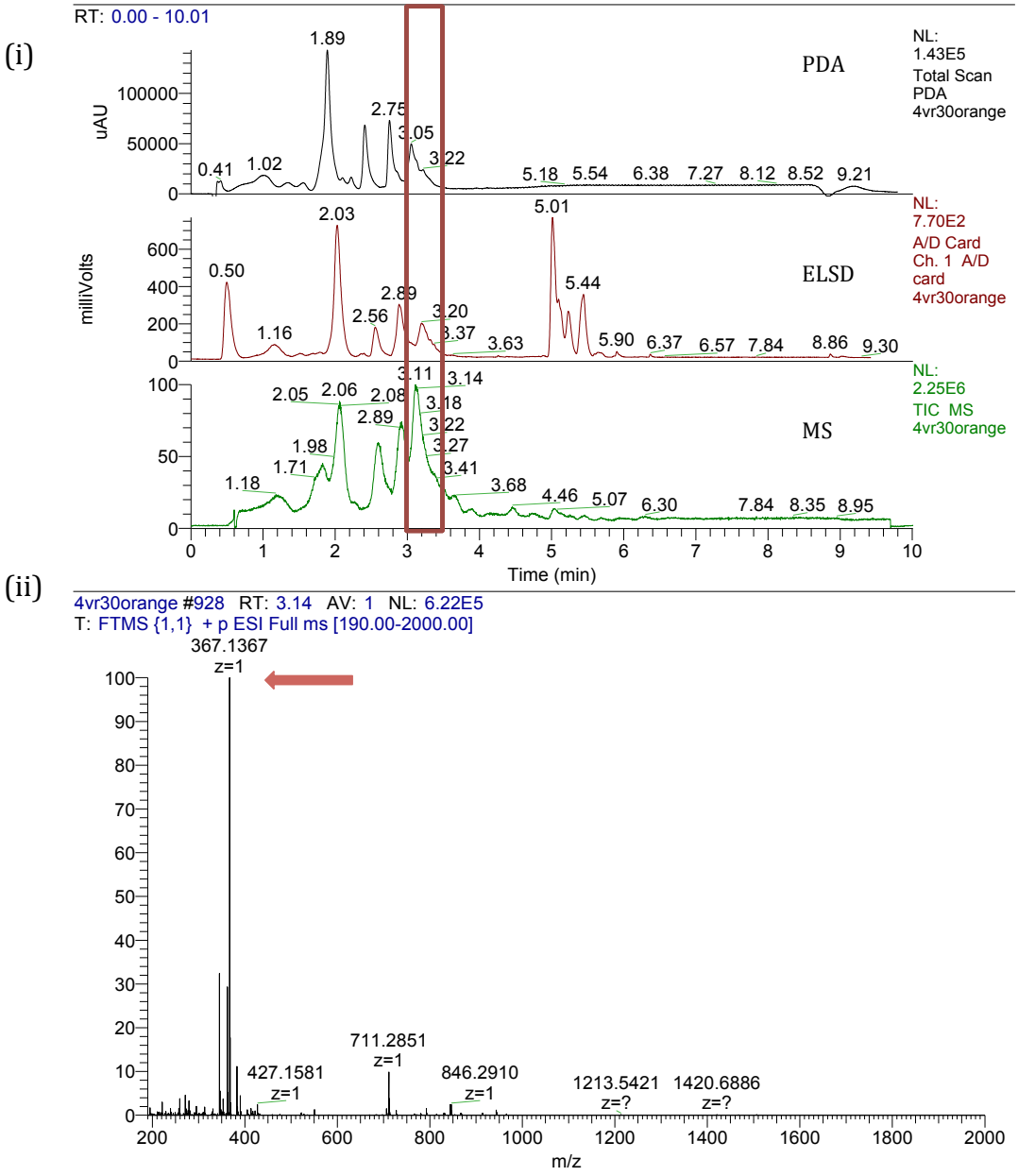


Figure 11. The UHPLC-HRMS chromatogram for metabolite 4.

The compound was produced by strain RKMC83. (i) Photodiode array (PDA) chromatogram illustrates UV absorbance; Evaporative light scattering detector (ELSD) chromatogram illustrates compound abundance; Mass spectrum (MS) chromatogram indicates compound ionization. (ii) The arrow indicates the mass/charge (m/z) value of the novel metabolite and the highlighted box indicates the retention time/mass spectrum being viewed.

Based on the results in the Antibase database, three remaining compounds, were Caprolactin A, an anti-cancer and anti-herpes simplex agent, produced by RKMC76 in all media (Figure 12), Daidzein, a topoisomerase inhibitor, a wide variety of bacteria in many media (Figure 13), and Topostin B567, a DNA topoisomerase 1 inhibitor, produced by RKMC83 in BFM4m (Figure 14). Due to its abundance, potential ease of purification, and production in all media, it was decided that **metabolite 1** would be the primary focus of this project. As the 600 mL extraction in BFM4m yielded 117 mg of crude extract; subsequent RKMC9 fermentations would be grown in BFM4m on a 600 mL scale.

Metabolite 1

1. Bioactivity:

At a concentration of 100 µg/mL, the crude extract containing metabolite 1 did not exhibit activity against the pathogens MRSA, VRE, *S.warneri, P.aeruginosa, P.vulgaris,* and *C.albicans*. The crude extract containing metabolite 1 did not appear to have cytotoxic effects against human cell lines ATCC HCT-116, ATCC MCF-7, ATCC HTB-26 and Vero, ATCC CCL-81. The crude extract of metabolite 1 inhibited the growth of *M. tuberculosis* at 64 µg/mL, followed by a lack of inhibition at 32 µg/mL (Figure 15). The purified extract of metabolite 1 inhibited the growth of *M. tuberculosis* with a half maximal inhibitory concentration (IC_{50}) of 27.71 µg/mL (Figure 16).

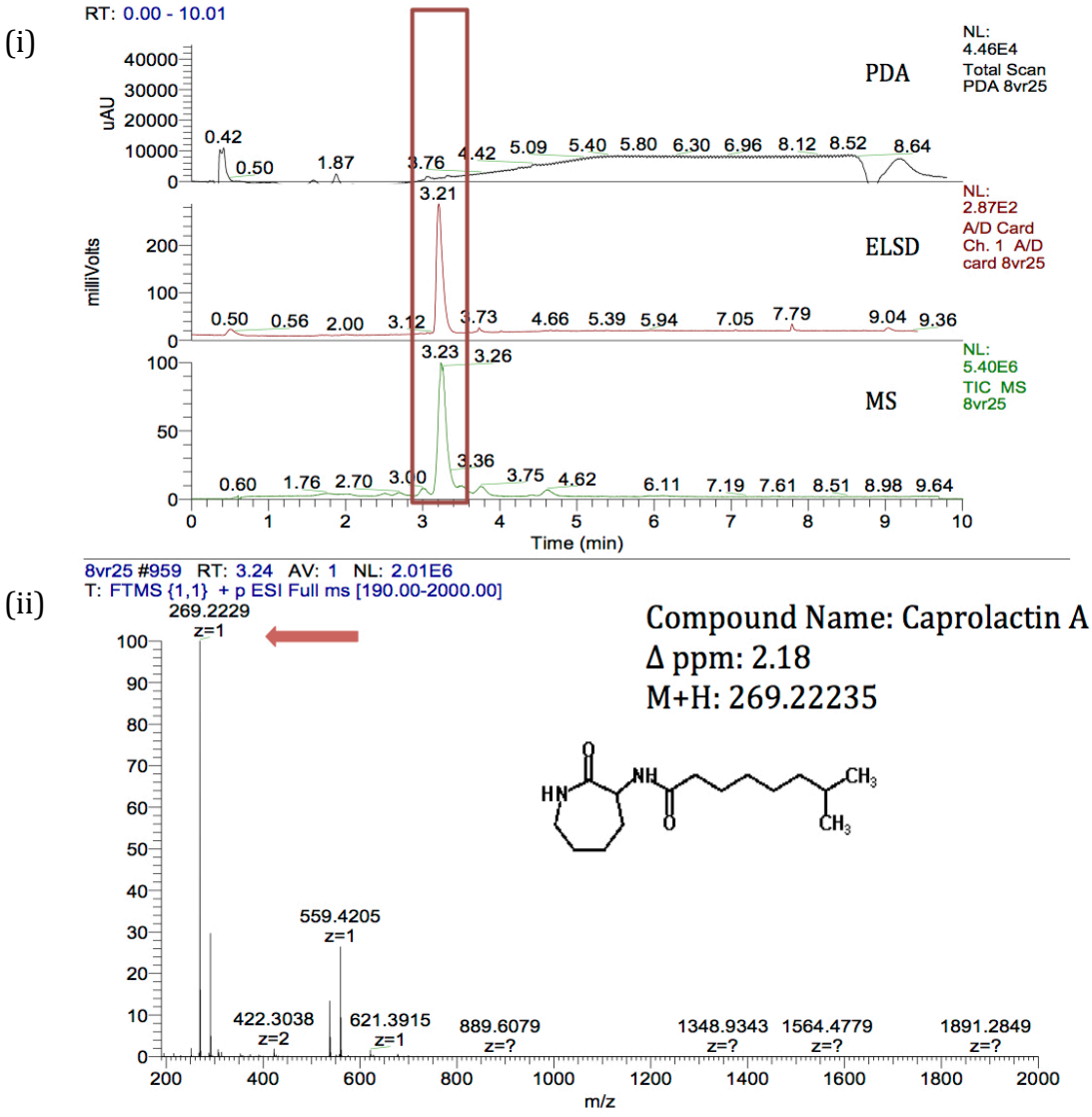


Figure 12. The UHPLC-HRMS chromatogram for Caprolactin A.

The compound was produced by strain RKMC76 in all media. (i) Photodiode array (PDA) chromatogram illustrates UV absorbance; Evaporative light scattering detector (ELSD) chromatogram illustrates compound abundance; Mass spectrum (MS) chromatogram indicates compound ionization. (ii) The arrow indicates the mass/charge (m/z) value of Caprolactin A and the highlighted box indicates the retention time/mass spectrum being viewed.

(i)

(ii)

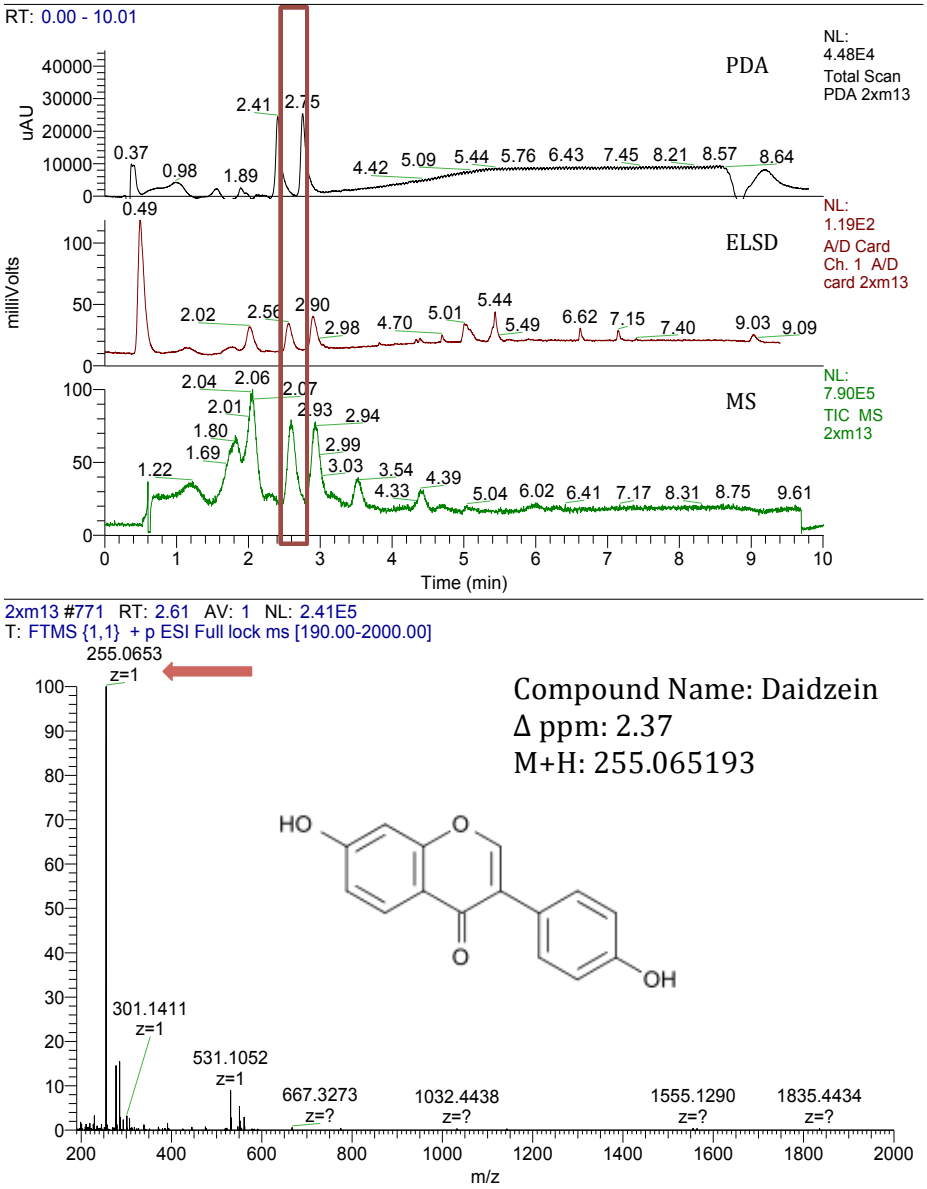
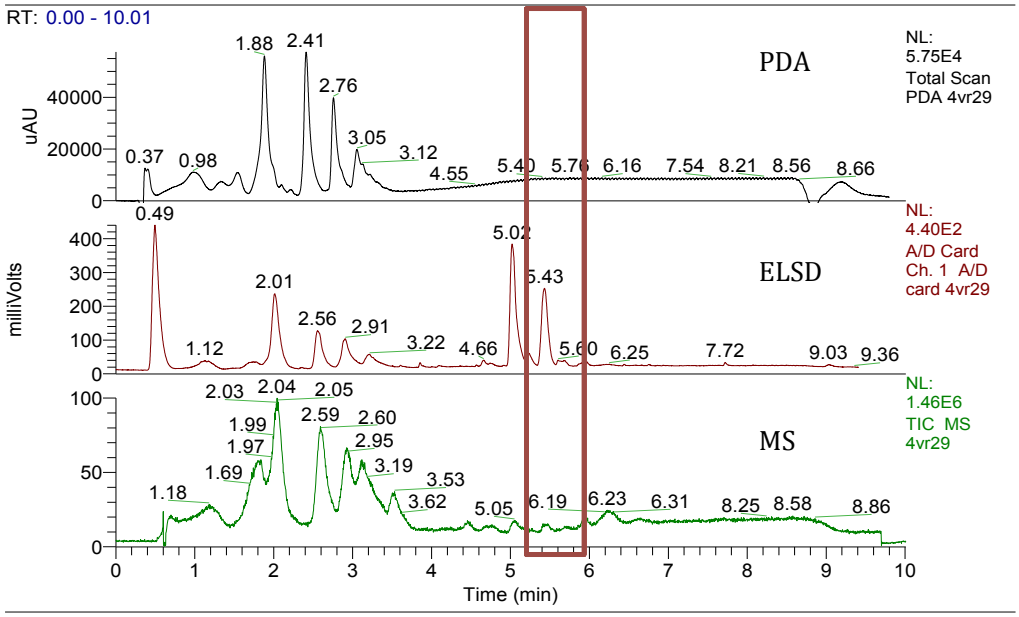


Figure 13. The UHPLC-HRMS chromatogram for Daidzein.

The compound was produced by many strains in a variety of media. (i) Photodiode array (PDA) chromatogram illustrates UV absorbance; Evaporative light scattering detector (ELSD) chromatogram illustrates compound abundance. Mass spectrum (MS) chromatogram indicates compound ionization. (ii) The arrow indicates the mass/charge (m/z) value of Daidzein and the highlighted box indicates the retention time/ mass spectrum being viewed.

(i)



(ii)

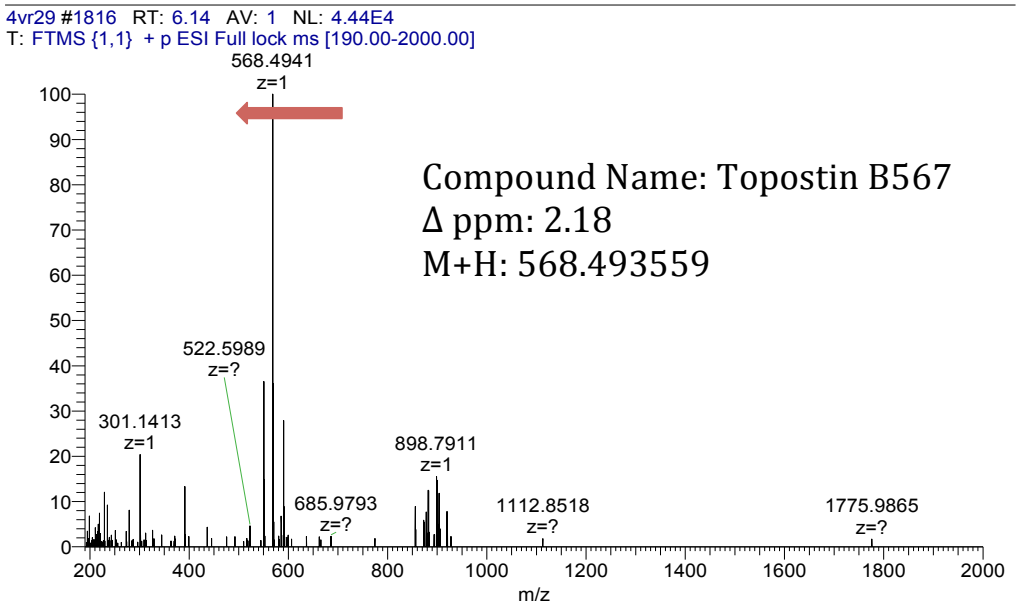


Figure 14. The UHPLC-HRMS chromatogram for Topostin B567.

The compound was produced by strain RKMC83 in BFM4m. (i) Photodiode array (PDA) chromatogram illustrates UV absorbance; Evaporative light scattering detector (ELSD) chromatogram illustrates compound abundance; Mass spectrum (MS) chromatogram indicates compound ionization. (ii) The arrow indicates the mass/charge (m/z) value of Topostin B567 and the highlighted box indicates the retention time/mass spectrum being viewed.

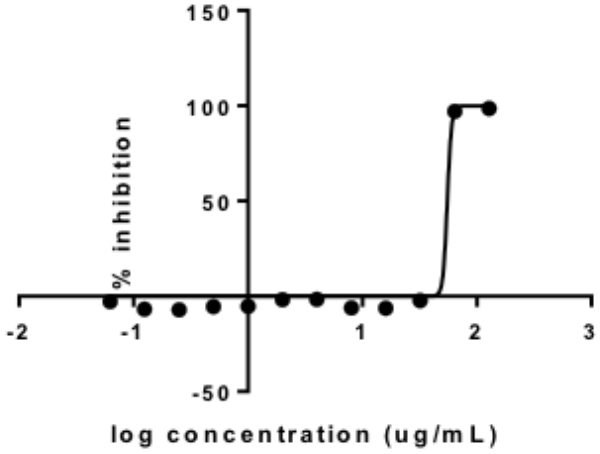


Figure 15. Dose response curve of the crude extract of metabolite 1 against *M. tuberculosis*. Courtesy of Trevor Clark (UNB)

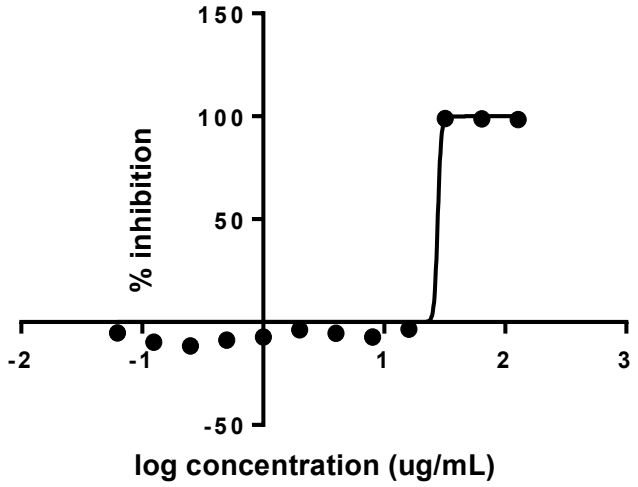


Figure 16. Dose response curve of partially purified metabolite 1 against *M. tuberculosis.* Metabolite 1 was 95% pure. Courtesy of Trevor Clark (UNB).

2. Purification:

Using reverse-phase flash chromatography (CombiFlash Rf system), a 15.5 g C18 column and a linear gradient from 10% methanol/water to 100% methanol over 20 minutes, the metabolite had a retention time of approximately 12 minutes. This purification resulted in the collection of 10.11 mg of product and from this collection 4.15 mg of sample was submitted for NMR analysis. Subsequent purification using reverse-phase flash chromatography (CombiFlash Rf system) with a 15.5 g C18 column and a linear gradient from 5% methanol/water to 100% methanol over 40 minutes, the compound had a retention time of approximately 26.5 minutes. Based on UHPLC-HRMS analysis, two analogues of metabolite 1 were separated.

3. Structural Elucidation:

The 1D and 2D NMR data suggest that metabolite 1 is a new amino acid. However, no further structural information can be provided to avoid disclosing potentially patentable data.

4. Small-Scale Fermentations for Structural Confirmation:

The extractions prepared for analysis by UHPLC-HRMS using methanol, and those prepared using acetonitrile, resulted in identical chromatograms. Additionally, the metabolite ionized in both positive and negative mode when analyzed by UHPLC-HRMS.

Identification of Bacterial Isolates

1. Phylogenetic Analysis:

The sequences for both RKMC85 (1421 bp) and RKMC83 (1432 bp) matched *Pseudoalteromonas piscicida* (NBRC 103038 NR_114190.1) with 99.93% and 99.86% similarity respectively. The sequence for RKMC99 (1439 bp) matched *Pseudoalteromonas shioyasakiensis* (SE3 NR_125458.1) with 99.89% similarity. The sequence for RKMC9 (1369 bp) matched *Alteromonas confluentis* (DSSK2-12 NR_137375) with a 98.24% similarity; this low similarity (<98.65%) indicates that RKMC9 is a putatively novel species.

Development of a rapid and portable method for estimating numbers of bacteria in environmental samples.

Hygiena EnSURE Luminometer Instrument Evaluation

1. Dynamic Range and Repeatability:

Using pure ATP, the instrument provided linear readings within the range of 10 and 1500 relative light units (Figure 17). The coefficients of variation (CV) were 20% and 23% for the 1 nM and 0.1 nM dilutions of pure ATP, respectively.

2. Correlation of Fluorescence Microscopy, Plate Counts, RLU Readings:

The luminescence readings were compared to the cell counts determined by standard plating and by fluorescence microscopy (Table 2).

(i)

(ii)

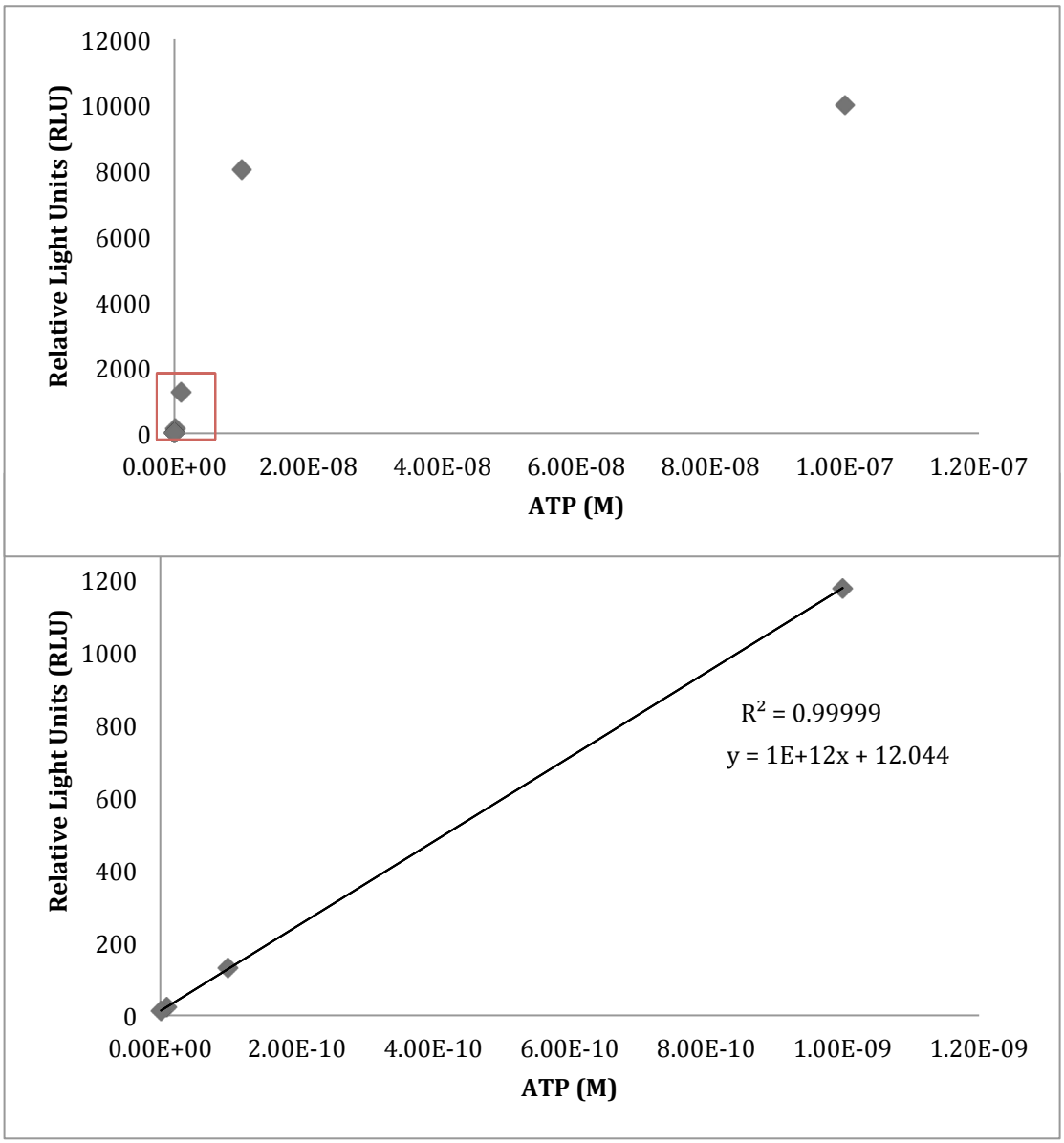


Figure 17. Hyigena EnSure Luminometer Calibration Curve for pure ATP.
(i) The average RLU readings for each ATP dilution were plotted against the corresponding ATP concentration. The highlighted area denotes the region of the graph shown in ii. (ii) The instrument provided linear readings within the approximate range of 10 and 1500 RLU.

Table 2. The luminescence relative to cell counts determined by standard plating and by fluorescence microscopy. Data points are sorted by ascending RLU.

Sample ID Trial (dilution)	Viable cell count (cfu)	DAPI cell counts	RLU
C (10^{-3})	533.3	5,391.7	20
A (10^{-4})	2,000	3,883.0	39
B (10^{-4})	2,920	19,155.709	79
C (10^{-2})	5333	53,917	158
A (10^{-3})	20,000	38,830	428
B (10^{-3})	29,200	191,557	1080
C (10^{-1})	53,333	539,170	1696

The cell counts obtained by DAPI staining were 2-10 fold greater than the amount of colony forming units (cfu) counted for the same sample. Luminescence readings were plotted against the cell counts obtained by fluorescence microscopy (Figure 18) as well as against the viable cell counts obtained by standard plating (Figure 19). Each DAPI stained cell was equivalent to approximately 0.0058 ± 0.0033 RLU and each viable cell was equivalent to approximately 0.0300 ± 0.0070 RLU.

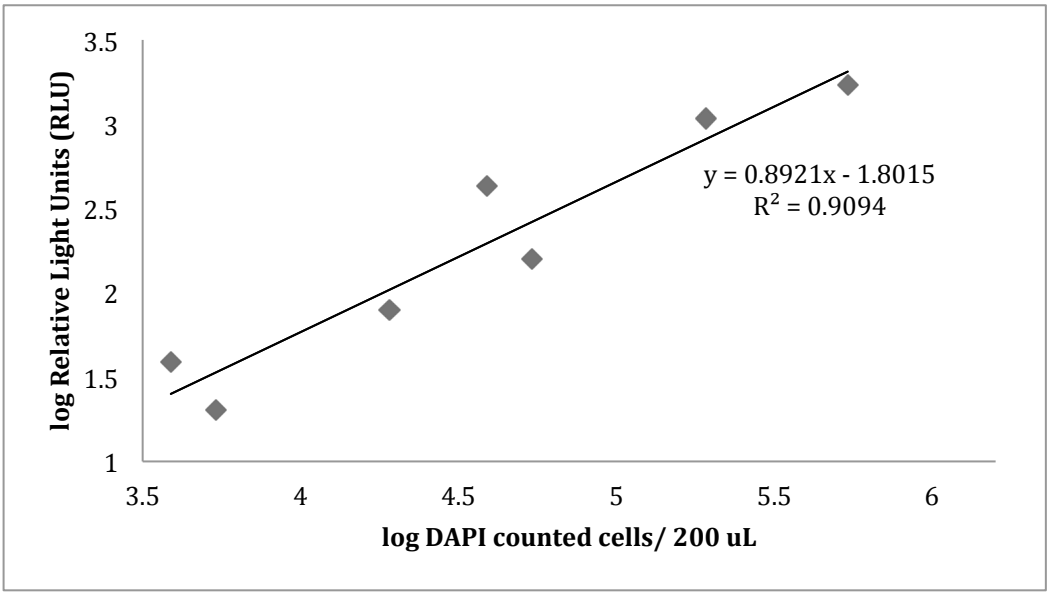


Figure 18. Calibration Curve for the Hyigena EnSURE luminometer. The luminescence readings plotted against the cell counts obtained by fluorescence microscopy.

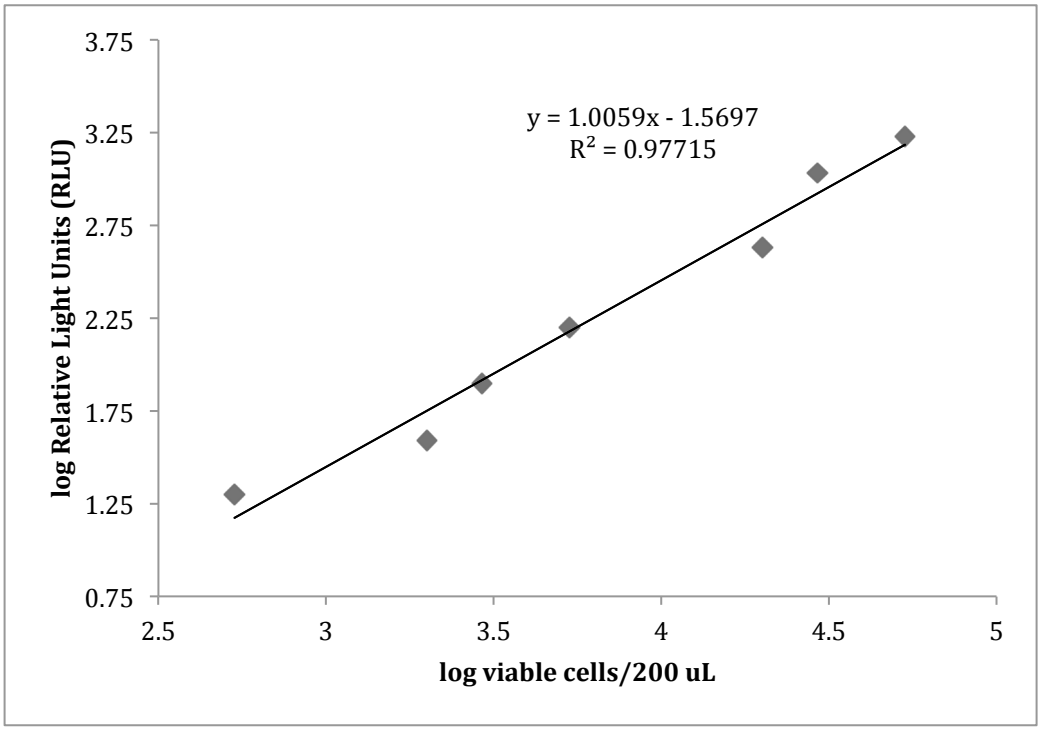


Figure 19. Calibration curve for the Hygiena EnSURE luminometer. The luminescence readings plotting against the viable cell counts obtained by standard plating.

DISCUSSION

Investigating Natural Product Biosynthesis From A Library Of Bacteria Isolated From Two Sponges Using An ichip.

Natural Product Discovery

1. Secondary Metabolite Production:

Bacteria were isolated from two marine sponges using standard plating, as well as through the use of a novel isolation method, the isolation chip (ichip). A representative group of these bacteria was fermented in different marine media with the goal of revealing novel natural product biosynthesis. In the past, secondary metabolite production has been determined by nutrient availability; therefore, the range of media compositions chosen for this project included different carbon and nitrogen sources to expand the range of metabolite production. This fermentation study resulted in the production of 267 metabolites. A combination of automated and manual chemical screening was used to determine which compounds were produced in abundance. Seven metabolites were obtained through this screening process; four of these did not yield search hits within the AntiBase Database, and were thus designated as putatively novel. The AntiBase 2017 database is a Natural Compound Identifier database that contains more than 40,000 natural compounds and their masses for M+, M+/-H, M+Na ions that have been sourced from primary and secondary literature. Three compounds did yield results within this database and were included in the results for interest. Of the bacteria selected for

fermentation, those that produced novel metabolites belonged to the phylum Proteobacteria; these bacteria were gram negative and belonged either to the genus *Alteromonas* or the genus *Pseudoalteromonas*. These genera have been known to produce secondary metabolites; however, historically, the largest producers of clinically and pharmaceutically relevant secondary metabolites have been the genus *Streptomyces*, of the phylum Actinobacteria.

2. A Novel Metabolite Produced by a Putatively Novel Bacterial Strain:

In a previous partial 16S rRNA gene sequencing and analysis, one of the bacterial strains, RKMC9, was identified as a putatively novel species. Metabolite 1 was solely produced by RKMC9 in all seven media. Additionally, metabolite 1 did not yield hits within the Antibase Database, and appeared easy to purity. Based on these results, this metabolite was chosen as the subsequent focus of the project. The three other metabolites could be the focus of future research.

3. Bioactivity of Metabolite 1:

As other compounds from the Kerr lab were being tested in various assays, and as the time required for assay results is lengthy, it was decided that preliminary activity data could be generated for a crude extract containing metabolite 1. The crude extract did not exhibit cytotoxic effects against selected human cell lines, or activity against the pathogens, MRSA, VRE*, S.warneri*, *P.aeruginosa*, *P.vulgaris,* and *C.albicans*. However, the crude extract showed 100% inhibition against *M. tuberculosis*. Re-testing, with a more highly purified compound, resulted in a half maximal inhibitory concentration

(IC50) of 27.71 µg/mL. These results are promising as they indicate that this extract has selective activity against a critical human pathogen.

4. Tuberculosis and Antitubercular Drugs:

Tuberculosis (TB) is a contagious disease caused by *Mycobacterium tuberculosis*. Annually, this pathogen causes nearly 2 million deaths and over 9 million infections (Stop TB Partnership, 2011). For several decades TB was successfully treated with antibiotics; however, current chemotherapeutic measures are unsuccessfully preventing the spread of the disease due to the emergence of drug resistance (Delogu et al, 2013). Resistant strains are emerging against the major first-line anti-TB drugs, including isoniazid and rifampicin, as well as against second-line drugs. To overcome the limitations of the present drugs and to cease the development of antibiotic resistance by mycobacteria, the discovery of new compounds with antitubercular activity is essential. Data for the half inhibitory concentration of metabolite 1 was calculated to be 27.71 µg/mL, this level of activity is not as potent as the first line drugs, which typically show activity at the ng/mL levels. However, second-line drugs show activity in the low ng/mL range. With activity in this range, metabolite 1 shows promise as a lead compound: a compound which has pharmacological activity, but whose structure could be synthetically altered to improve activity.

5. Structural Elucidation:

Through the use of 1D and 2D NMR data, metabolite 1 was determined to be a new amino acid. Part of the structure contained a methoxy group; small-scale fermentations and extractions confirmed that this group was not artefact from the

isolation procedure. As previously stated, the full structure cannot be disclosed at this time to avoid issues with patent filing.

Identification of Bacterial Isolates

1. Phylogenetic Analysis:

Full sequencing of the 16s rRNA gene of strain RKMC9 resulted in a 1369 bp sequence that matched that of *Alteromonas confluentis* with a 98.24% similarity. This low similarity suggests that RKMC9 is a novel species in the genus *Alteromonas*. As it is solely a new species, and not a new genus or family, this new bacterium will not substantially contribute to improving the great plate count anomaly. Nevertheless, these results are encouraging as they indicate that the Kerr lab isolation chip was able to aid in the isolation of a novel bacterial strain from a sponge species, *X. muta*, which has historically been mined for microbes.

Development Of A Rapid And Portable Method For Estimating Numbers Of Bacteria In Environmental Samples

Hygiena EnSURE Luminometer Instrument Evaluation:

Using pure ATP, the instrument provided linear readings within the range of 10 and 1500 relative light units. This indicates that to use the device properly, samples with RLU readings outside of this threshold must be concentrated or diluted. The level of variance between readings may be due to unequal dispersal of lysing reagent from the SuperSnap or from uneven distribution of bacterial cells in a liquid sample. It is therefore important that multiple readings are taken for the same sample while using the device.

Bacterial Cells Quantification and Correlations:

Laboratory methods of bacterial quantification were explored and tested in a preliminary step to correlate the RLU readings generated by the EnSURE Luminometer with accurate measures of bacterial sample density. There was a linear correlation between both forms of cell counts and the readings obtained from the Hygiena EnSURE Luminometer; however, the standard plate count curve had greater linearity. This trend might be due to the facts that only viable cells grow on laboratory plates, that dead cells can be stained and viewed by fluorescence microscopy, and that dead cells do not release ATP, and thus would not contribute to RLU readings. The calculations of approximate RLU per DAPI stained cell had a large standard deviation. It should be noted that it was difficult to count cells for the first replicate of this study (A) due to large amounts of background fluorescence and a high amount of cells on the slide. Removing this replicate increases both the linearity of the calibration curve and decreases the size of the standard deviation for the fluorescent cell counts; therefore, it would be reasonable to assume that the amount of bacteria in this sample were largely underestimated by manual cell counting. Overall, it appears that these calibration curves may be used to provide an estimate of bacterial density. The numbers generated by the luminometer are not exact, but can be used to limit the number of dilutions attempted while in the field.

CONCLUSION AND FUTURE WORK

The objectives of this study were to investigate the metabolites produced by the bacterial library collected from two marine sponges and to develop a bacterial quantification method to be used in the field in conjunction with the isolation chip, and future isolation devices.

Metabolite Investigation

The fermentation of 22 bacteria in 7 different media resulted in the production of 4 putatively novel compounds. Of these compounds, one was produced by a novel species. Subjection of the compound, metabolite 1, to bioassays resulted in selective activity against the rapidly reemerging human pathogen, *Mycobacterium tuberculosis*. The fully purified compound will be tested against *M. tuberculosis* as well as against the other pathogens and various human cancer cell-lines to confirm its selective activity. Marfey's method will be used to determine the stereochemistry, at the single stereocenter. The genome of the bacterial strain RKMC9 will be sequenced to identify the gene cluster responsible for the synthesis of metabolite 1 and further phylogenetic tests will be conducted in order to characterize the species.

Bacterial Quantification

Given that the purpose for this device was not to provide the user with the exact number of cells in the sample, but rather to give the user a range from which dilutions could be made, preliminary results from the evaluation of the Hygiena EnSURE luminometer system are promising. Future work will include increasing the number of replicates and testing the luminometer's effectiveness with sediment samples. However, given the fact that environmental samples contain ATP from multiple sources, it will also be critical to develop a method to remove the bacteria from the original sample.

LITERATURE CITED

Abdel-Lateff A. 2006. Natural products from marine organisms and their associated microbes. Chembiochem 7(2):229-38.

Alain K and Querellou J. 2009. Cultivating the uncultured: Limits, advances and future challenges. Extremophiles 13(4):583-94.

Alonso D, Khalil Z, Satkunanthan N, Livett, B. 2003. Drugs from the sea: Conotoxins as drug leads for neuropathic pain and other neurological conditions. Mini Rev. Med. Chem. 3:785–787

Amann J. 1911. Die direkte Zahlung der Wasserbakterien mittels des Ultramikroskops. Centralbl. f. Bakeriol. 29:381-384.

Amann RI, Ludwig W, Schleifer KH. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol Rev 59(1):143-69.

Armstrong E, Yan L, Boyd KG, Wright PC, Burgess JG. 2001. The symbiotic role of marine microbes on living surfaces. Hydrobiologia 461(1):37-40.

Baltz R. 2005. Antibiotic discovery from actinomycetes: Will a renaissance follow the decline and fall?186 p.

Bergmann W and Feeney RJ. 1951. Contributions to the study of marine products. xxxii. the nucleosides of sponges. i.1. J Org Chem 16(6):981-7.

Bollmann A, Lewis K, Epstein SS. 2007. Incubation of environmental samples in a diffusion chamber increases the diversity of recovered isolates. Appl Environ Microbiol 73(20):6386-90.

Collins, L. A.; Franzblau, S. G. Antimicrob. Agents Chemother. 1997, 41,1004.

Crombrugge J, Waes G. 1991. ATP method. In: Heeschen W, ed. Methods for assessing the bacteriological quality of raw milk from the farm. Brussels: International Dairy Federation: 53–60

Chin Y, Balunas MJ, Chai HB, Kinghorn AD. 2006. Drug discovery from natural sources. The AAPS Journal 8(2):E253.

Davis KER, Joseph SJ, Janssen PH. 2004. Effects of growth medium, inoculum size, and incubation time on culturability and isolation of soil bacteria. Appl Environ Microbiol 71(2):826-34.

Delogu, G., Sali, M., Fadda, G. 2013. The Biology of Mycobacterium Tuberculosis Infection. Mediterr J Hemato Infect Dis 5(1)

Egeberg PK. Paull, C.K., Matsumoto, R., Wallace, P.J., and Dillon, W.P. 2000. Adenosine-5-Triphosphate as a proxy for bacterial number in deep-sea sediment and correlation with geochemical parameters. Proceedings of the Ocean Drilling Program, Scientific Results 164

Ferrari BC, Binnerup SJ, Gillings M. 2005. Microcolony cultivation on a soil substrate membrane system selects for previously uncultured soil bacteria. Appl Environ Microbiol 71(12):8714-20.

Fischbach MA and Walsh CT. 2009. Antibiotics for emerging pathogens. Science 325(5944):1089-93.

Forner D, Berrue F, Correa H, Duncan, K, Kerr, RG. 2013. Chemical dereplication of marine actinomycetes by liquid chromatography–high resolution mass spectrometry profiling and statistical analysis. Analytica chimica acta. 805 (70):9

Koopmans M, Martens D, Wijffels HR. 2009. Towards commercial production of sponge medicines. Marine Drugs 7(4).

Ling LL, Schneider T, Peoples AJ, Spoering AL, Engels I, Conlon BP, Mueller A, Schberle TF, Hughes DE, Epstein S, and others. 2015. A new antibiotic kills pathogens without detectable resistance. Nature 517(7535).

Manivasagan P, Venkatesan J, Sivakumar K, Kim S. 2014. Pharmaceutically active secondary metabolites of marine actinobacteria. Microbiol Res 169(4):262-78.

National Committee for Clinical Laboratory Standards. 2003. Methods for dilution antimicrobial susceptibility test for bacteria that grow aerobically, 6th ed. Approved standard M7-A6. National Committee for Clinical Laboratory Standards, Wayne, Pa.

Newman DJ and Cragg GM. 2016. Natural products as sources of new drugs from 1981 to 2014. J Nat Prod 79(3):629-61.

Newman DJ, Cragg GM, Snader KM. 2000. The influence of natural products upon drug discovery. Nat Prod Rep 17(3):215-34.

Nichols D, Calhoon N, Trakhtenberg E, Pharm L, Mehta A, Belanger A, Kanigan T, Lewis K and Epstein S. 2010. Use of Ichip for High-Throughput In Situ Cultivation of "Uncultivable" Microbial Species. Appl. Environ. Microbiol 76(8):2445-2450.

Osburne MS, Grossman TH, August PR, MacNeil IA. 2000. Tapping into microbial diversity for natural products drug discovery some microbiologists are probing the rich diversity of their backyards instead of going far afield to find useful natural products. Asm News- American Society for Microbiology 66:411-7.

Selvin J, Shanmugha Priya S, Seghal Kiran G, Thangavelu T, Sapna Bai N. 2009. Sponge-associated marine bacteria as indicators of heavy metal pollution. Microbiol Res 164(3):352-63.

Staley JT and Konopka A. 1985. Measurement of in situ activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. Annu Rev Microbiol 39(1):321.

Stevenson BS, Eichorst SA, Wertz JT, Schmidt TM, Breznak JA. 2004. New strategies for cultivation and detection of previously uncultured microbes. Appl Environ Microbiol 70(8):4748-55.

Stewart EJ. 2012. Growing unculturable bacteria. J Bacteriol (15-16):4151.

Stop TB Partnership. 2011. The Global Plan to Stop TB 2011–2015: Transforming the Fight towards Elimination of Tuberculosis; World Health Organization: Geneva, Switzerland.

Watve MG, Tickoo R, Jog MM, Bhole BD. 2001. How many antibiotics are produced by the genus streptomyces? Arch Microbiol 176(5):386-90.

APPENDIX

A-1. Bacterial Fermentation Media

Bacterial Fermentation Medium 1 Marine

MEDIUM CODE: BFM1m	g/L
Dextrin	20
Soluble Starch	20
Beef extract	10
Peptone	5
$(NH_4)_2SO_4$	2
$CaCO_3$	2
Instant Ocean	18
Nanopure H_2O	1 Q.S./L

Bacterial Fermentation Medium 2 Marine

MEDIUM CODE: BFM2m	g/L
Soluble starch	5
Pharmamedia	5
Instant Ocean	18
Nanopure H_2O	1 Q.S./L

Bacterial Fermentation Medium 3 Marine

MEDIUM CODE: BFM3m	g/L
$MgSO_4$*7H2O	0.5
KCl	0.5
K_2HPO_4	3
NaCl	5
Agar	0.4
Glycerol	12
Soy Peptone	5
Instant Ocean	18
Nanopure H_2O	1 Q.S./L

Bacterial Fermentation Medium 4 Marine

MEDIUM CODE: BFM4m	g/L
Nutrisoy	12
NH_4Cl	1
Dextrose	12
Agar	0.4
Calcium Carbonate	1
NZ-amine A	3
Instant Ocean	18
pH	6.8
Nanopure H_2O	1 Q.S./L

Bacterial Fermentation Medium 5 Marine

MEDIUM CODE: BFM5m	g/L
Pancreatic Digest of Casein	17
Enzymatic Digest of Soybean Meal	3
Sodium chloride	5
Dipotassium Phosphate	2.5
Dextrose	2.5
Instant Ocean	18
pH	7.3+/-0.2
Nanopure H_2O	1 Q.S./L

Bacterial Fermentation Medium 8 Marine

MEDIUM CODE: BFM8m	g/L
Peptone	5
Yeast Extract	1
Ferric Citrate	0.1
Instant ocean	18
pH	7.6 +/- 0.2
Nanopure H_2O	1 Q.S./L

Bacterial Fermentation Medium 11 Marine

MEDIUM CODE: BFM11m	g/L
Starch (potato)	10
Yeast Extract	4
Peptone	2
KBr Stock (20 g/L)	5 mL
$FeSO_4$-$7H_2O$ (8 g/L; pH 7)	5 mL
Instant ocean	18
pH	7.0 +/- 0.2
Nanopure H_2O	1 Q.S./L