Isolation and Analysis of Antibiotic Compounds from Soil Microbes

A Major Qualifying Project Report:

Submitted to the Faculty

Of the

WORCESTER POLYTECHNIC INSTITUTE

In partial fulfillment of the requirements for the

Degree of Bachelor of Science

In

Biology and Biotechnology

and

Chemistry and Biochemistry

by

Matthew Googins (BBT), Colin Harrington (BBT), and Parsant Jotikasthira (CBC)

Date: 4/25/2017

Project Advisor

Prof. Michael A. Buckholt (BBT)

Table of Contents

Acknowledgements

We would like to acknowledge the following individuals for their assistance with this project, firstly our project advisor Professor Michael A. Buckholt for his constant support and guidance throughout the duration of the project. We would also like to thank Professor JoAnn Whitefleet-Smith for her help in using the lyophilizer in Goddard and Andrew Butler for providing us with information regarding mass spectroscopy and NMR protocols.

Abstract

The growing number of antibiotic resistant strains of bacteria is a serious issue that must be addressed. New antibiotics need to be found to prevent a global epidemic. Soil bacteria isolates from the Microbes to Molecules (BB2905) course were identified and their products were purified and analyzed. We developed an identification pipeline for antibiotic discovery to proceed from the BB2905 course. The process for creating the pipeline resulted in the optimization of 16s ribosomal subunit PCR protocols. This allowed for the identification of samples 15-6 and 14-29 to be of the *Streptomyces* and *Brevibacillus* genus, respectively. The pipeline was then utilized to extract and analyze of antibiotic extracts from samples 15-6 and 14-29. The antibacterial products of 15-6 and 14-29 were isolated and purified into fractions using HPLC. The final characterization of said samples was not accomplished due to the characterization methods, nuclear magnetic resonance (NMR) and mass spectroscopy, requiring larger sample masses than what were feasibly obtained. Future experimentation should focus on further optimization of growth techniques for the isolated species and chemical analysis methods to further characterize the antibiotic compounds.

1. Background

1.1 The Problem

The growing number of antibiotic resistant strains of bacteria is a serious issue that must be addressed. Diseases that were once easily treatable are now becoming harder to treat making them more dangerous. According to the Centers for Disease Control and Prevention (CDC) at least two million people become infected with antibiotic resistant strains of bacteria in the United States every year (CDC, 2016). This is because the current arsenal of effective antibiotics used for treating bacterial infections is shrinking due to bacterial resistance. Biologists need to find new antibiotics to combat this threat and prevent a global epidemic. In response to the looming crisis, crowdsourcing efforts such as the Small World Initiative are attempting to help combat the threat of antibiotic resistant bacteria.

The Small World Initiative is a project started at Yale University in 2012 that aims to discover new, potentially useful, antibiotics from soil microbes by crowdsourcing the discovery process. The Initiative is currently made up of 150 institutions across the world, including Worcester Polytechnic Institute (WPI) (Small World Initiative, 2015). The institutions involved in the Small World Initiative contribute to the effort by having students collect soil samples, grow microbes from the soil sample, and screen these microbes for antibiotic production. The data obtained from this, such as the location from which the sample was taken and the probable species or genus of the "antibiotic" producing bacteria, is uploaded into the Small World Initiative's database on their website (Small World Initiative, 2015).

WPI is currently a part of this initiative with the course Microbes to Molecules (BB2905). This course has students collect their own soil sample and attempt to isolate antibiotic producing bacteria. However, further analysis of these bacteria and their potential antibiotic products is still required. This analysis includes detailed characterization of the bacteria, finding the best conditions to grow the bacteria, and characterizing the antibiotic compounds produced by the bacteria. The goal of this project is to complete this work and develop more optimum procedures for future Microbes and Molecules courses and future projects working with soil bacteria and antibiotic isolation.

1.2 Antibiotics

Antibiotics are compounds that are able to kill or stop the growth of bacteria. Alexander Fleming discovered the first antibiotic, Penicillin, in 1928 (ACS and RSC, 1999). Antibiotics continued to be discovered frequently until the early 1980s, when economic pressure within the pharmaceutical community shifted the focus of research towards on other drugs (Spellberg and Gilbert, 2014). This shift in the pharmaceutical industry resulted in a diminished pipeline of new antibiotics into the clinical setting.

Currently, researchers are uncertain of the exact function of antibiotics in nature, but there are two competing theories. The first is that antibiotics are used as a defensive mechanism by organisms against other microbes within the same ecosystem. The other, is that antibiotics may actually be bacterial signaling molecules (Clardy *et al*, 2009;Yim *et al*, 2007). Soil bacteria represent the largest source of already discovered antibiotic compounds (Handelsman *et al*, 1998). Despite this, soil microbes remain largely uncharacterized. It is estimated that there are 10^3 to 10^7 in any given soil sample (Fierer *et al*, 2007). However, it is only possible to cultivate a fraction of these bacteria, using standard culturing techniques (Delong and Pace, 2001). The continued exploration of the largely unexplored soil environment could yield a multitude of novel medicinal compounds.

A recent publication illustrating the continued potential of novel antibiotic discovery from soil was the discovery of teixobactin by Ling *et.al* at Northeastern University in 2015. This process involved using a device called an iChip to isolate and grow soil microbes in native conditions (Ling *et al.*, 2015). The device involves a plastic matrix filled with solid media with semipermeable membranes on either side. The device is then inserted into a sample of the native soil that the bacteria came from (Ling *et al.*, 2015). Using the iChip, the group at Northeastern University was able to isolate an antibiotic producing organism called *Eleftheria terrae* which produced the novel antibiotic teixobactin. This story demonstrates the continued importance of the relatively unexplored soil ecosystem for finding novel medicinal compounds. For more information on Teixobactin see section 1.3.9 below.

1.3 Antibiotic Classes

Antibiotics can be broken down into many structural classes. Each class has its own unique chemical properties that distinguishes it from other classes. We have included

information on the most common classes below.

1.3.1 Beta Lactams

Beta lactams are the oldest discovered class of antibiotics, the most famous of which is Penicillin, shown in Figure 1. Beta Lactams are broad-spectrum antibiotics effective against mostly gram negative bacteria and some gram positive bacteria (Tomasz, 1976). They act by inhibiting cell wall biosynthesis and are mainly bactericidal (Tomasz, 1976). Beta lactam antibiotics can be further categorized into penicillins and cephalosporins. The main similarity between members of this class of antibiotics is the beta lactam ring, seen circled in Figure 1 below.
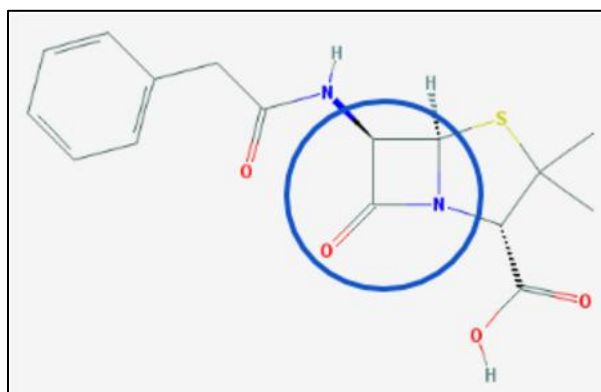
**Figure 1: Penicillin G, Pubchem CID: 5904 (National Center for Biotechnology Information, N.D.)**

Beta lactams, excluding the potential for the development of antibiotic resistant bacteria, have few known side effects. The only major side effect reported was gastrointestinal distress, yeast infection, and diarrhea. These side effects have also been reported when the Beta Lactam drugs were combined with beta lactamase inhibitor drugs (Holten and Onusko, 2000).

1.3.2 Aminoglycosides

Aminoglycosides were the first class of antibiotics to be discovered from soil bacteria. The first to be discovered and the most famous example of this class is Streptomycin shown in Figure 2, an antibiotic isolated from the soil actinobacteria *Streptomyces griseus.*

Aminoglycosides are antibiotics that act on the 16s ribosomal subunit and inhibit normal protein synthesis (Hermann, 2007). While aminoglycosides are technically bacteriostatic, cells exposed to aminoglycosides will eventually be killed due to a buildup of truncated proteins caused by the aminoglycoside binding to the ribosome which makes aminoglycosides appear bactericidal (Hermann, 2007). They inhibit mostly gram negative bacteria, but also inhibit some gram positive bacteria. Aminoglycosides are highly polar molecules (Mingeot-Leclercq *et al.*, 1999).
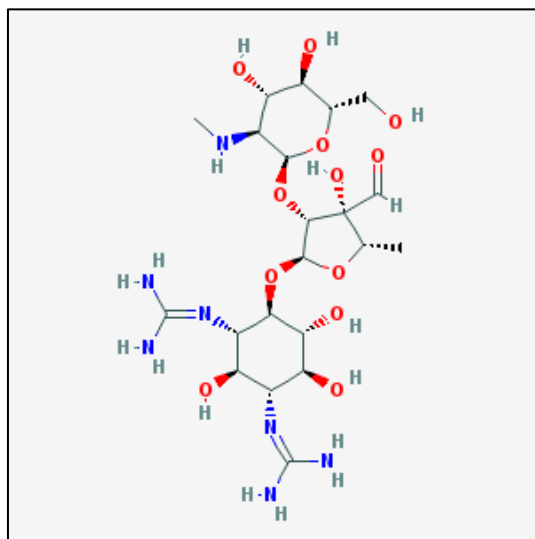

Figure 2: Streptomycin, Pubchem CID: 19649 (National Center for Biotechnology Information, N.D.)

Aminoglycosides have also been observed to have numerous detrimental side effects when used in a part of a high-dose anti-bacterial treatment. Symptoms such as kidney damage, chronic kidney disease, hearing loss, balance impairment, and lung tissue damage have been observed. These symptoms have been linked to the development of cystic fibrosis in the patience. This antibiotic has also been shown to cause different types of cellular toxicity caused by uptake of large doses of aminoglycosides, leading to cell death (Prayle *et al.*, 2010).

1.3.3 Glycopeptides

Glycopeptides are a class of antibiotics that act on the cell wall by inhibiting peptidoglycan biosynthesis. Glycopeptides are generally bactericidal and effective almost exclusively against gram positive bacteria as gram negative bacteria's outer membrane does not allow molecules as large as the glycopeptides to pass through (Malabarba *et al.*, 1997). A

notable example of a glycopeptide antibiotic is Vancomycin, shown in Figure 3. The basic structure of glycopeptides is a cyclic non-ribosomal peptide with sugars attached on the periphery of the cycle (National Center for Biotechnology Information, N.D.) (See Figure 3).
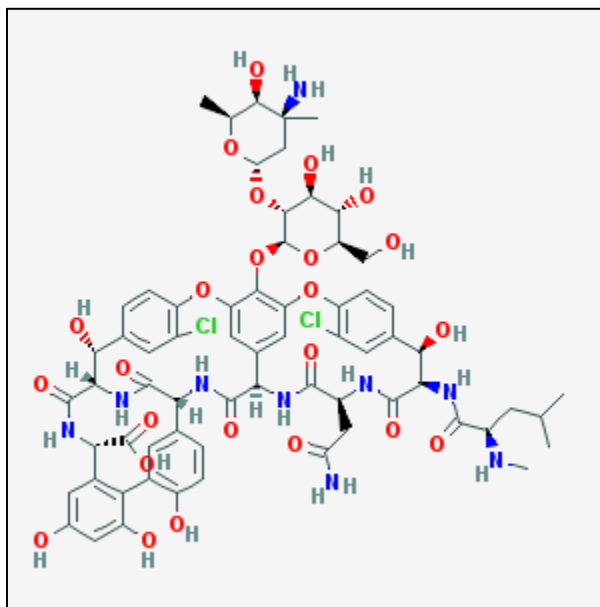

Figure 3: Vancomycin Pubchem CID: 14969 (National Center for Biotechnology Information, N.D.)

Glycopeptide antibiotics have side effects similar to aminoglycosides. Intravenous administration of glycopeptides have been observed to cause histamine based effects such as hypertensions and in a rare case, cardiac arrest. Other symptoms include renal damage, skin rashes, and inner ear damage caused by ototoxicity. The cause of these effects and tissue toxicities have been linked to impurities found when the antibiotic is isolated. Modern methods of isolating glycopeptides have increased the purities of samples and reduced the severity of many of the side effects (Finch and Eliopoulus, 2005).

1.3.4 Tetracyclines

Tetracyclines are another class of antibiotic that commonly used in modern medicine. Tetracyclines are formed from a linear fusion of a tetracyclic nucleus, shown in Figure 4, with different groups attached (Chopra and Roberts, 2001). This class of antibiotics has been shown to function as a bacteriostatic compound that is effective against Gram positive, Gram negative, and preventative against certain parasites. In bacteria, tetracyclines function by

targeting and inhibiting protein synthesis. The antibiotic does this by binding to the 30S subunit of the ribosome and preventing the tRNAs from binding. The antibiotic binding to this subunit prevents amino acid associated tRNAs from binding to the ribosome (Chopra and Roberts, 2001).
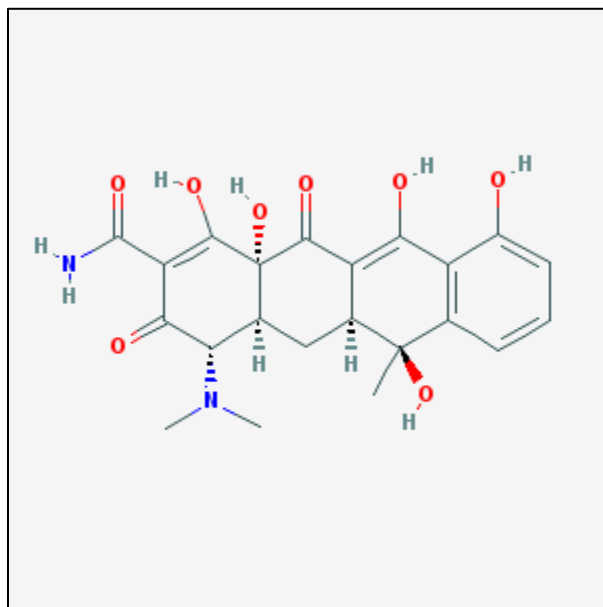

Figure 4: Tetracycline, Pubchem CID: 54675776 (National Center for Biotechnology Information, N.D.)

Many of the properties regarding tetracycline class antibiotics have become well established. Tetracyclines have been recorded having pH's ranging from 3-7 and generally have a pKa value of 3.3 at 25°C. This class of antibiotics is polar due to the different side groups branching off the tetracyclic nucleus. This polarity favors the polar environment in cells that it functions in (Tetracycline, 2016). Another trait of tetracyclines is the class' ability to fluoresce under UV light when absorbed into bone.

Long term usage of tetracycline based drugs has also been shown to cause a variety of mild and severe side effects. Short term usage of tetracycline has been shown to cause skin sensitivity to sunlight, diarrhea, and upset stomach. However, tetracycline can also cause skin rash, difficulty breathing, vomiting, and muscle weakness. These effects have been observed when taking higher doses of tetracyclines for prolonged periods of time (Citron *et al.*, 1968).

1.3.5 Macrolides

Macrolides are a large class of antibiotics produced by bacteria and fungi alike. Macrolides are generally made from a lactonic ring of 12 to 22 carbon atoms with different side groups, shown in Figure 5. Generally, macrolides have been shown to be effective against Gram positive strains of bacteria, but have also been shown to function against Gram negative bacteria (Hof, 1994). Macrolides function as bacteriostatic antibiotics, though bactericidal activity has been observed in in vitro experiments (Pankey and Sabath, 2004). These antibiotics function by targeting the 50S unit of the ribosome. By targeting this part of the ribosome, macrolides inhibit protein synthesis.
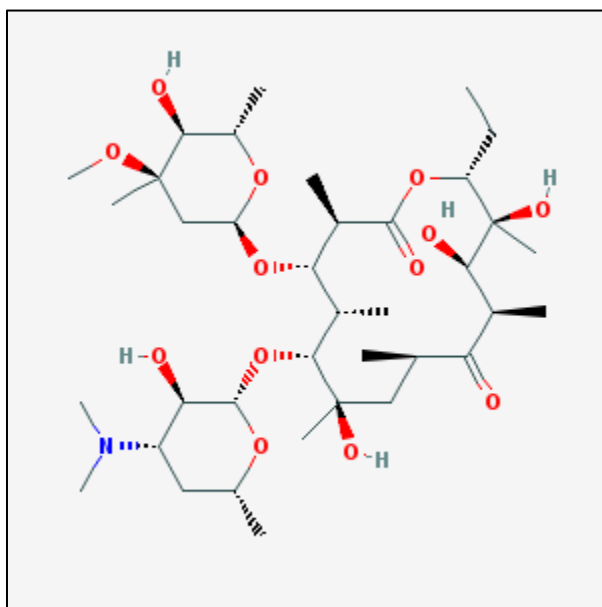

Figure 5: Erythromycin, Pubchem CID: 12560 (National Center for Biotechnology Information, N.D.)

The properties of macrolide class antibiotics have been determined due to their general structure. Macrolides are polar, due to the numerous polar side groups on the main carbon ring structure. These antibiotics have their highest activity at pHs of 7.8 to 8 (Macrolides, 2016)

Similarly to other classes of antibiotics, macrolides have been observed to cause adverse effects on patients. The milder observed side effects are abdominal pain, diarrhea, and nausea. However, macrolides have been shown to cause, on rare occasions, pancreatitis, acute hepatitis, and reversible ototoxicity (Cunha, 2001).

1.3.6 Lipopeptides

Lipopeptides are a class of antibiotics they are made of a cyclic peptide and a long hydrocarbon tail. Lipopeptides act by making the plasma membrane permeable to ions, which causes cell lysis (Yu *et al.*, 2015;Steenbergen *et al.*, 2005). They are generally bactericidal (Yu *et al.*, 2015;Steenbergen *et al.*, 2005). Daptomycin is almost exclusively effective against gram positive bacteria, while the polymyxins are mostly effective against gram negative bacteria (Yu *et al.*, 2015;Steenbergen *et al.*, 2005). Daptomycin and the polymyxins were listed in the same class as they are structurally similar in that they are made from both lipid and peptide components and mechanistically similar, in that they both depolarize the membrane and cause cell lysis. Despite their similarities however, they are effective against very different bacteria in that polymyxins are effective against a narrow spectrum of gram negative bacteria and daptomycin is effective against most gram positive bacteria (Yu *et al.*, 2015;Steenbergen *et al.*, 2005).
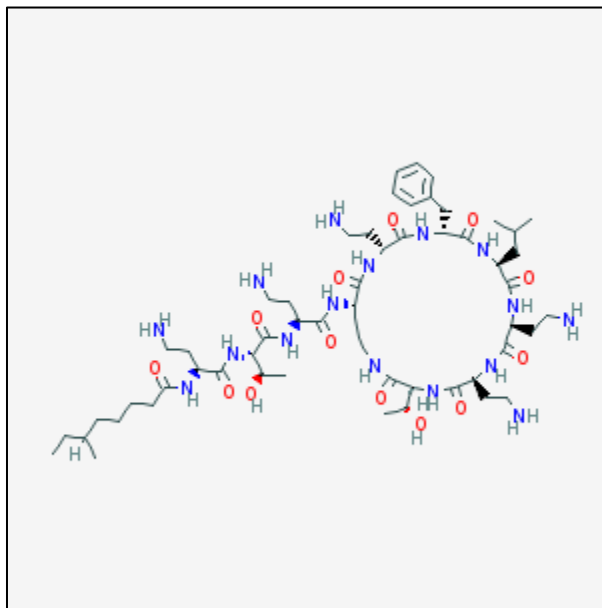

Figure 6: Polymyxin B, Pubchem CID: 49800004 (National Center for Biotechnology Information, N.D.)

The majority of lipopeptide based drugs have been shown to cause few mild unwanted effects on patients. Those that have been observed to cause adverse effects have only occurred in 2% of patients in pre-clinical trials. The known side effects of lipopeptide drugs are nausea,

constipation, headache, rash, and skin reactions at injection site (Steenbergen *et al.*, 2005).

Daptomycin is another example of a lipopeptide antibiotic. Daptomycin is a natural product of the soil bacterium *Streptomyces roseosporus*. This drug is used for treatments of complicated skin infections by gram-positive bacteria. High dosage of this drug can cause effects on skeletal muscle, including myalgia and myositis (Eisenstein *et al.*, 2010) . The bacteria was discovered by scientists at Eli Lilly from a soil sample from Mount Ararat in Turkey during the late 1980s, and later developed by Cubist Pharmaceuticals (Eisenstein *et al.*, 2010). The structure of daptomycin is shown in Figure 7.
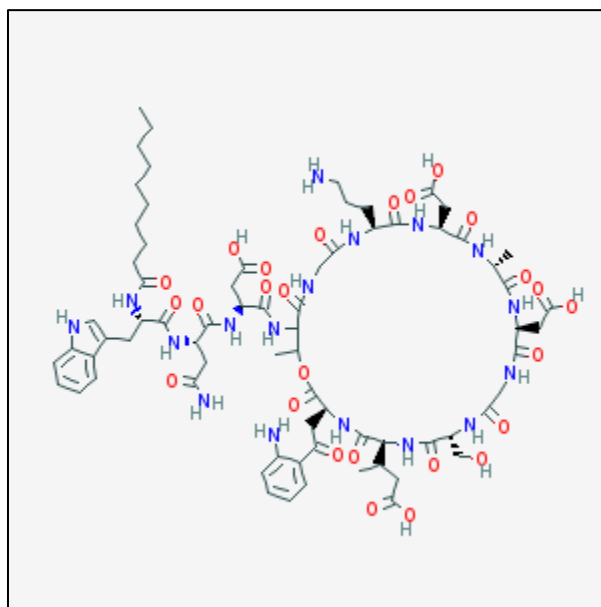

Figure 7: Daptomycin, Pubchem CID: 16129629 (National Center for Biotechnology Information, N.D.)

1.3.7 Chloramphenicols

Chloramphenicols are one of the more simplistic antibiotics in regards to its structure the 2,2-dichloro-N-[(1R,2R)-1,3-dihydroxy-1-(4-nitrophenyl)propan-2-yl]acetamide shown in Figure 8, differs from most antibiotics due to the two chloride atoms (National Center for Biotechnology Information, N.D.). The only other antibiotic to have chloride atoms is vancomycin. Chloramphenicol has been shown to target both Gram positive and Gram negative bacteria. Generally, chloramphenicol acts as a bacteriostatic antibiotic. However, it has shown bactericidal activity against certain strains of bacteria similarly to macrolides,

chloramphenicol works by binding to the 50S subunit of the ribosome, inhibiting protein synthesis (Pankey and Sabath, 2004).
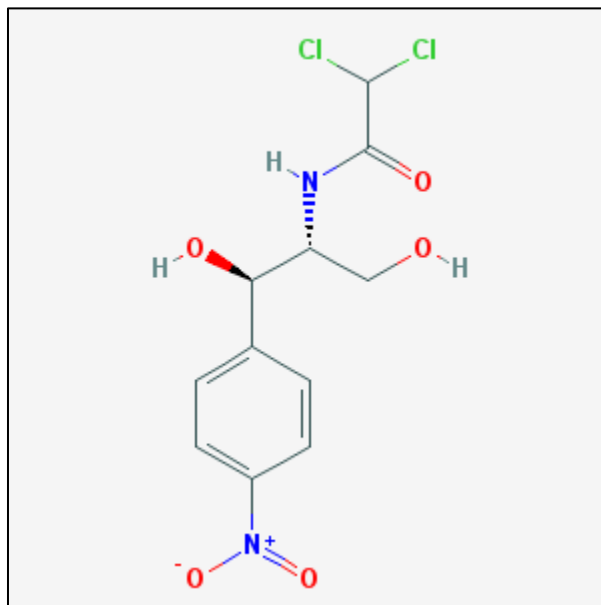
Figure 8: Chloramphenicol, Pubchem CID: 5959 (National Center for Biotechnology Information, N.D.)

The commonly shared and simplistic structure of chloramphenicol made identifying its chemical properties easier. Like other antibiotics, chloramphenicol has a polar charge allowing it to function properly within the cell's inner environment. Additionally, chloramphenicol has shown to have a neutral pH utilizing litmus tests (National Center for Biotechnology Information, N.D.).

Chloramphenicol based therapy for bacterial infection has been known to cause similar side effects to other antibiotic treatments. These adverse effects include nausea, vomiting, headache, and diarrhea. However, chloramphenicol drugs have also been known to cause rash, muscle weakness, swelling, stomach cramps, and bloody stool (U.S. National Library of Medicine: Chloramphenicol, 2016).

1.3.8 Ansamycins

Ansamycins are class of antibiotics that are characterized by a handle (*ansa* in latin) like structure that loops around a central ring structure (Wehrli, 1977). Most ansamycins (Rifamycins, streptovaricins, and tolypomycins) inhibit prokaryotic DNA-dependent RNA synthesis (Wehri, 1977). They are effective against mycobacteria, a large percentage of gram

positive bacteria, and some gram negative bacteria (Floss and Yu, 2005). They act on bacteria in a bacteriostatic way. Rifamycin is a naturally occurring example of this class (See Figure 9).
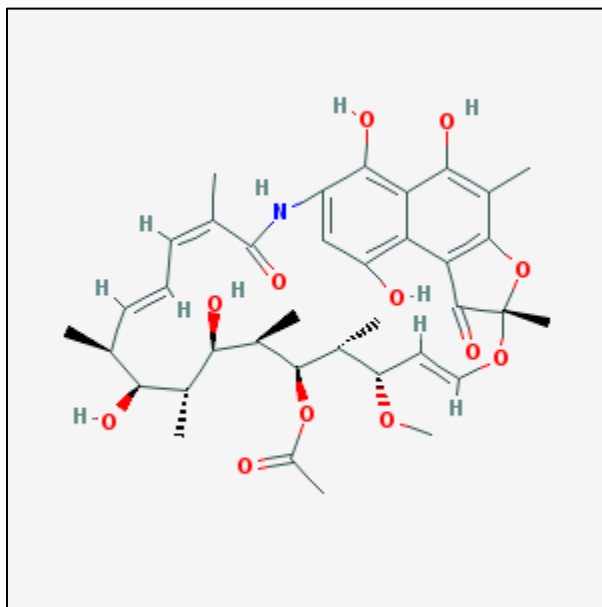

Figure 9: Rifamycin SV, Pubchem CID: 6324616 (National Center for Biotechnology Information, N.D.)

Ansamycins based drugs have been observed to have few known negative side effects. These adverse effects include rash, blisters, loss of coordination, and other common side effects shared among most antibiotics such as nausea and diahrrea (U.S. National Library of Medicine: Rifampin, 2016).

1.3.9 Teixobactin

Teixobactin acts by inhibiting cell wall synthesis by binding to a precursor of peptidoglycan (Lipid II) and a precursor of teichoic acid, both of which are necessary to construct the cell wall (Ling *et al.*, 2015). Teixobactin does not belong to an already established class of antibiotics and instead is the first member of a new class of antibiotics that bind to the Lipid II peptidoglycan precursor (Ling *et al.*, 2015). A structure of teixobactin is shown in Figure 10 below.
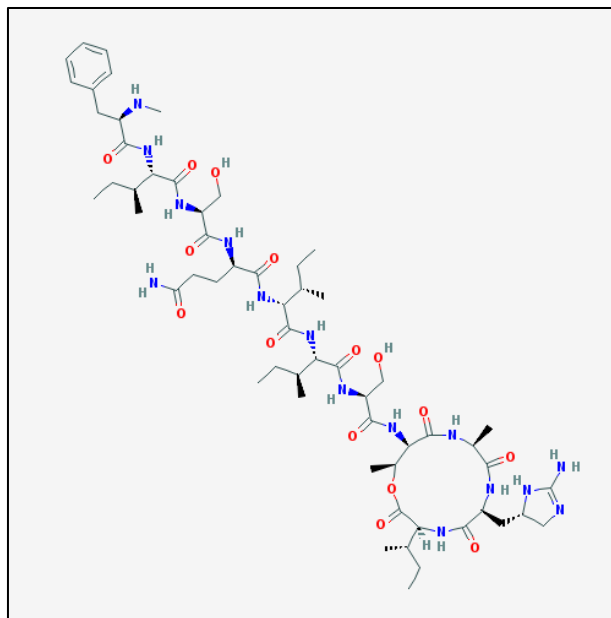
Figure 10: Teixobactin, Pubchem CID: 86341926 (National Center for Biotechnology Information, N.D.)

1.4 Project Outline

The focus of this project was on two main topics: Soil bacteria and the antibiotics they produce. For the soil bacteria, optimum methods for the culturing, maintenance, and analysis of isolated samples were investigated. These protocols were established concurrently with the antibiotic based research. This research focused on determining which isolated soil bacteria produce antimicrobial molecules as secondary metabolites. The conditions for them to produce their secondary metabolites (potential antibiotics) from these selected samples were established. Then, techniques for properly isolating these metabolites were determined. Once isolated the characterization of these metabolites was attempted using different analytical techniques where possible to determine if it belongs to a known class of antibiotics. All techniques and protocols can then be used by the Microbes and Molecules course to further this type of research. The project is outlined visually in *Figure 11*.
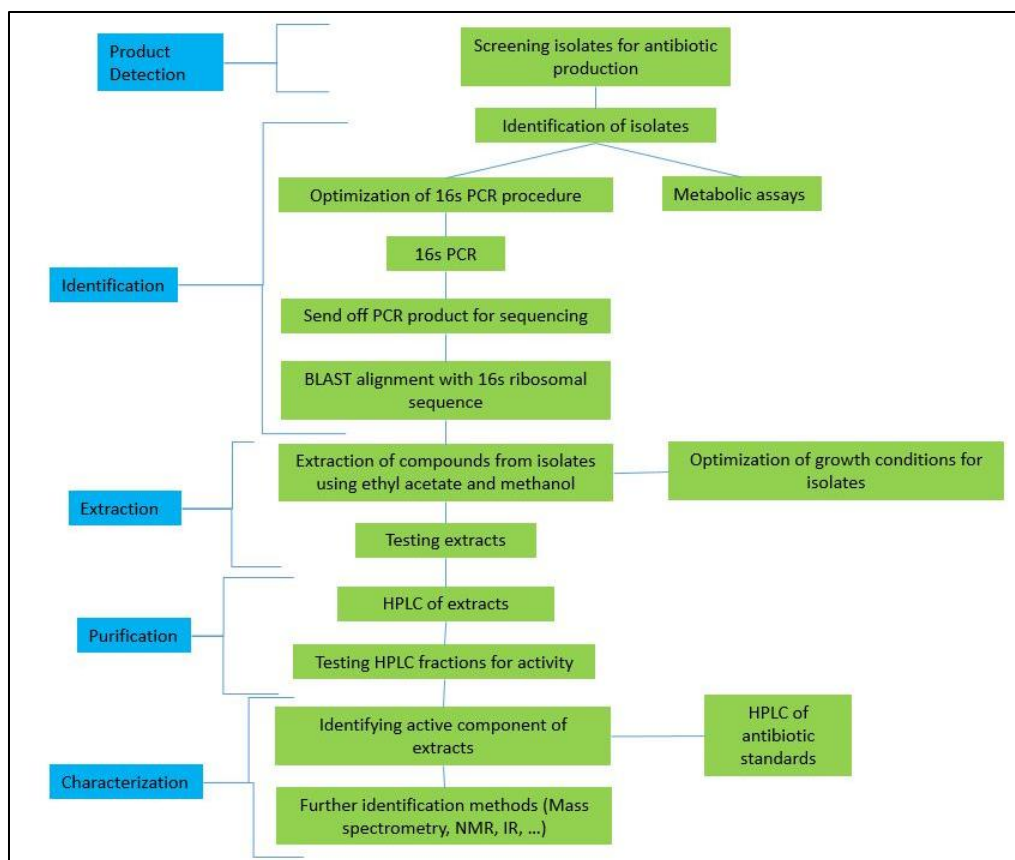
Figure 11: Project Overview Flowchart

2. Methods

2.1 Screening Microbes to Molecules Freezer Isolates for Antibiotic Activity

All isolates from previously run Microbes to Molecules courses (2014, 2015, and 2016) were streaked from freezer stocks onto Luria-Bertini (LB) agar and allowed to incubate at room temperature for 72 hours. For a complete list of isolates see Appendix I.

The isolates were screened for antibiotic production using a top agar method which was adapted from the top agar method present in the Small World Initiative research protocols document (Hernandez *et al.*, 2015). The isolates were transferred from the freezer stocks to the LB plates using a sterilized inoculation loop. The isolates were plated clockwise in numbered order (See Appendix I for full list of isolates plated and their corresponding ID numbers) in a circular pattern around the plate. Each year of isolates were plated on separate plates in order to minimize the risk of confusion. A 3 mL liquid culture was made for *E. coli* (ATCC 11795) and *B. Subtilis* and allowed to incubate at 37 degrees Celsius for 24 hours. The

top agar itself was made of 50% agar LB. 1 mL of the liquid culture of each species were added to separate 4 ml aliquots of the top agar. Each aliquot was poured onto a single plate of small world samples. Antibiotic activity of the isolates was indicated by zones of inhibition of the species tested against. *Figure 12* represents an example of a zone of inhibition for the top agar method. .
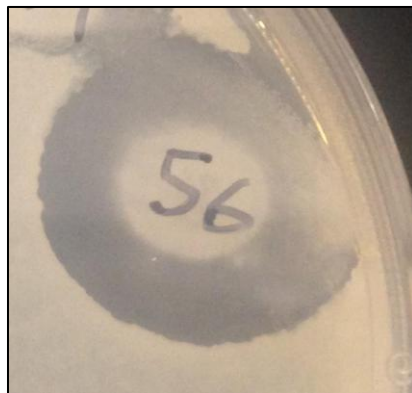
Figure 12: Example of a Zone of Inhibition

2.2 Determining the Identity of the Isolates

      Before continuing work with the isolates in liquid culture, the identity of the isolates was determined. The identities of the bacterial isolates were determined through both PCR, metabolic assays, and Gram staining.

2.2.1 Metabolic Assays

    In order to help characterize the bacterial isolates, a variety of metabolic assays were performed. These assays include the indole assay (Appendix II), the glucose fermentation assay (Power and Johnson, 2009), the lactose fermentation assay (Power and Johnson, 2009), the MacConkey agar assay (Hernandez *et al.*, 2015), and the amylase assay (Buckholt, 2014).

      In both the glucose and lactose fermentation assays, a yellow colored result means ferments with acid, while bubbles indicated gas production due to fermentation of the carbohydrate (Power and Johnson, 2009).

2.2.2 PCR

    PCR was utilized to better identify the provided soil bacteria samples. Two different sets of primers were used for the 16s ribosomal subunit amplification (27F and 1492R, 8F and

1391R, ordered from Integrated DNA technologies). The mix used for all PCR samples was 10 uL Deionized water, 1.3 uL of 10 uM of the first primer, 1.3 uL of 10 uM of the second primer, and 12.5 uL of 12.5 uM of New England Biolabs 2x Q5 Master Mix. This procedure was later redone using OneTaq 2x PCR Mix. The PCR protocols differed only in annealing temperature due the different primers. The general protocol was 95 degrees C for 2 minutes, then 30 cycles of 95 for 30 seconds, 49 degrees (54 degrees for the 8F and 1391R primer combination) for 45 seconds, 72 degrees for two minutes, followed by 72 degrees for 10 minutes, and finally held at 10 degrees.

An additional set of primers and protocols were used in an attempt to identify several *Bacillus* genus microbe samples. These primers (UP-1 and UP-2r) and sequencing primers (UP-1S and UP-2Sr) were designed to target bacterial gyrase genes. Gyrase genes were known to be used by bacteria to power its flagella. The PCR utilized 12.5 uL of Q5 2x Master Mix, 1.3 uL of 1 uM UP-1 primer, 1.3 uL of 1uM UP-2r primer, and 10 uL Deionized water. The PCR protocol used was 30 cycles of 94 degrees C for 1 minute, 60 degrees C for 1 minute, and 72 degrees C for 2 minutes. The PCR products were then held at 10 degrees C until they were removed from the PCR machine (Yamamoto and Harayama, 1995).

Primer Sequences:
16s ribosomal subunit
27F:5'-AGAGTTTGATCMTGGCTCAG-3'
1492r:5′-GGTTACCTTGTTACGACTT-3'
8F:5'-AGAGTTTGATCCTGGCTCAG-3'
1391r:5'-GACGGGCGGTGTGTRCA-3'
gyrB
UP-1:5'-GAAGTCATCATGACCGTTCTGCA(TC)GC(TCAG)GG(TCAG)AA(AG)TT(TC)GA-3'
UP-1S:5'-GAAGTCATCATGACCGTTCTGCA-3'
UP-2r:5'-AGCAGGGTACGGATGTGCGAGCC(AG)TC(TCAG)AC(AG)TC(TCAG)GC(AG)TC(TCAG)GTCAT-3'
UP-2Sr:5'-AGCAGGGTACGGATGTGCGAGCC-3'

2.2.2.2 Gradient PCR

A gradient PCR was performed to optimize the PCR protocols for future experiments. OneTaq Master Mix was used instead of Q5, but the rest of the mix was kept the same. PCR for the outside primers (1442R and 27F) was performed using a gradient of annealing temperatures from 44 to 54 degrees Celsius, placing samples at 44 degrees (5 degrees cooler), 48 degrees (normal annealing temperature), and at 54 degrees (5 degrees warmer). Standard PCR cycle times and temperatures were kept the same. The gradient PCR for the inside primers (8F and 1391R) using annealing temperatures from 44 to 54 degrees Celsius, placing samples at 44 (10 degrees cooler), 48 (5 degrees cooler), and 54 degrees Celsius (normal annealing temperature).

2.2.3 Gram Staining

Gram staining was used as a supplementary identification method to distinguish between Gram positive and Gram negative organisms. The specific protocol used was from the Small World Initiative manual (Hernandez *et al.*, 2015).

2.2.4 Gel Electrophoresis

Gel electrophoresis of the PCR products was run at 90 volts to check for the correctly sized DNA fragment. The gel was created using 60 mL of 10% agarose gel in 1x TAE buffer and the DNA was stained using SYBR green. When using the 2x Q5 Master Mix, 0.5 uL of 10x SYBR was mixed with 5ul of the sample and 0.5 uL of loading dye. The protocol for using OneTaq was just to add 1uL of SYBR to the sample, as OneTaq contained its own loading dye. When it was determined that the correct product was made, the samples were sent off to Eton Biosciences for sequencing. The sequencing was run twice for each sample using the same primers used for PCR. When the sequences were returned from Eton Biosciences, they were inputted into the NCBI nucleotide BLAST search (Altschul *et al.*, 1990) with the 16s bacterial ribosomal option selected. The likely genus and species of the isolates was determined using the max score and the identification percent. This method however does not identify the isolates with complete certainty, and is only accurate to the genus level. The genus was determined due to all visible BLAST results sharing the same genus.

2.3 Extraction of Secondary Metabolites

Organic extractions using both ethyl acetate and methanol were performed on solid media cultures (grown on LB agar) of the isolates that had been frozen by placing them in the -80°C freezer for 24 hours (Hernandez *et al.*, 2015). Liquid nitrogen was also used to freeze samples by placing their conical tube in the liquid until frozen. Before freezing, the solid media cultures were removed from the petri dishes and placed in a 50 mL conical centrifuge tube. After the freezing period 12 mL of ethyl acetate and 8 mL of water or 20 mL of methanol were added depending on which solvent was used. The tubes were then allowed to gently rock at 25°C for 24 hours. After 24 hours the tubes were centrifuged at 2056.32 RCF for 15 minutes. The solvent phase was then pipetted off the top of the tube and deposited into a glass scintillation vial. It should be noted that the methanol extractions did not have a phase separation and therefore all liquid was pipetted off into a 50 mL tube and then centrifuged again. The liquid was then pipetted into a glass scintillation vial. The vials were then allowed to dry down completely by passing air over them in the fume hood and then they were suspended in 1 mL of the original solvent. The extracts were tested by applying 10 uL of extract on 6 mm diameter paper disks, made by using a paper hole puncher on Whatman 1mm filter paper, letting them dry, and overlaying these disks on plates of test species such as *E. coli* (ATCC 11795) and *B. subtilis* and allowing incubation for 24 hours. Zones of inhibition indicated a positive result for an active antibiotic extract.

Organic extractions were also performed on liquid cultures of isolates 15-6 and 14-29. 30 mL of liquid culture was grown at 25° C for 5 days for both isolates. Ethyl acetate was used to extract the 15-6 isolate and methanol was used to extract the 14-29 isolate. Fifty percent of the volume of the liquid culture of solvent was added to the top of the liquid cultures in 50 mL conical tubes. The tubes were then allowed to shake at 25° C for 24 hours. The rest of the liquid culture extraction method matches the solid media extraction procedure described in Hernandez *et al.*, 2015. These extracts were tested the same way as the solid media extracts. For the methanol extracts further filtering was required after centrifugation. A 0.2 micron test tube filter was utilizes to remove any remaining solid particles from the extracts.

2.3.2 Alternate Media Extractions

The 15-6 sample was grown and extracted from a variety of media types to determine if other media types could produce antibacterial product more efficiently. The agar media tested were LB, Potato Dextrose Agar (PDA), Trypticase Soy Agar (TSA), Reasoner's 2a Agar (R2A), and Todd Hewitt Agar (THA) media. Samples from the 15-6 freezer stock were plated on each plate and left to grow at room temperature. After three days the plates were examined and growth documented. The plates were then left to grow for an additional two days (totaling five days of growth at room temperature), before they were extracted. All plates were extracted using the ethyl acetate extraction technique described in Section 2.3. All resulting extracts were analyzed with a zone of inhibition test and then were further analyzed and purified using techniques described in Section 2.4 to determine concentrations relative to each other.

2.4 Chemical Analysis of Extracts

High pressure liquid chromatography (HPLC) was used as both an analytical technique and as a separation technique for both the 15-6 and 14-29 extracts using a 100 uL injection volume of the samples. The HPLC machine used was an Agilent 1100 series. The column used was the Zorbax RX-C18 (4.6 x 25 mm column, 5 μL, 300 Å) and the solvents used were 0.1% Formic acid in H_2O (Solvent A) and 0.1% Formic acid in Acetonitrile. Table 1 shows the elution profile used for this HPLC. The particular HPLC method used was taken from Wobbe (2015).

Table 1: HPLC Elution profile used (Wobbe, 2015)

Time (minutes)	% Solvent A	% Solvent B
0	92	8
4.25	92	8
29.25	20	80
37.55	20	80
37.56	10	90
45.85	10	90
45.86	92	8
54.15	92	8

Fractions were taken manually at five minute intervals for the HPLC runs. Starting at time zero the elution was collected from the column into a 15 mL conical tube, every five minutes the 15 mL tube was replaced being sure to keep and label all tubes in order of collection. The extracts of the 15-6 and the 14-29 isolates were run as well as the solvents present in the extracts (ethyl acetate and methanol respectively).

Initial attempts to dry the fractions down was done by passing nitrogen over the top of the liquid. This method however was too inefficient and lyophilization was chosen as an alternative.

Lyophilization was used to dry down both sets of the HPLC fractions. The lyophilizer used was a Savant Novalyphe-NL500. Each 5 mL fraction was transferred into either a large glass test tube or a 50 mL conical tube. The fractions were then frozen into shells using liquid nitrogen and placed into glass lyophilization canisters. A vacuum was pulled and the fractions were allowed to lyophilize for 24 hours or until no frozen solvent was visible. The test tubes/50 mL conical tubes were then thoroughly rinsed with the solvent used in the original extraction (ethyl acetate or methanol) and this solution was transferred to 15 mL conical tubes and dried down by passing air over them. The fractions were then resuspended in 100 µL of the respective solvent. 100 µL of solvent was used as this is the volume of the original extract that was injected into the HPLC column.

In order to test the fractions, 10 uL of each fraction was spotted onto small filter paper disks in the same procedure as described above for the extract testing, and allowed to dry. Control fractions of just the extraction solvent (ethyl acetate or methanol) collected in the same way as the extract fractions were also spotted onto the filter paper disks in the same manner. The disks were then placed on an inoculated *E. coli* (ATCC 11795) or *B. subtilis* LB plate, all fractions were tested against both species. The plates were then allowed to incubate at 37°C for 24 hours. Zones of inhibition indicated fractions that contain the antibiotic compound.

2.4.2 HPLC of Antibiotic Standards

Antibiotic standards were run through the same HPLC method as above in order to determine similarities between the extractions and known antibiotics. The antibiotic standards

run included a penicillin/streptomycin mix (10,000 ug/uL), carbenicillin (50 mg/mL), ampicillin (10 mg/mL), tetracycline (15 mg/mL), gentamicin (10 mg/mL), cycloheximide (25 ug/uL), and amphotericin B (10 mg/mL).

3. Results

The purpose of these experiments was the optimization of different laboratory methods used in the Small World soil bacteria project as well as the identification of isolated antibacterial compounds from these soil bacteria. These methods were designed to identify bacterial strains isolated from soil samples that showed antibacterial activity, extract bacterial products from these samples, and characterize these products to determine their identity.

3.1 Initial Antibiotic Production Screening

Soil bacteria samples from the 2014, 2015, and 2016 Microbes to Molecules (BB 2905) laboratory course were taken from their freezer stocks and plated on LB agar to determine which were still viable.
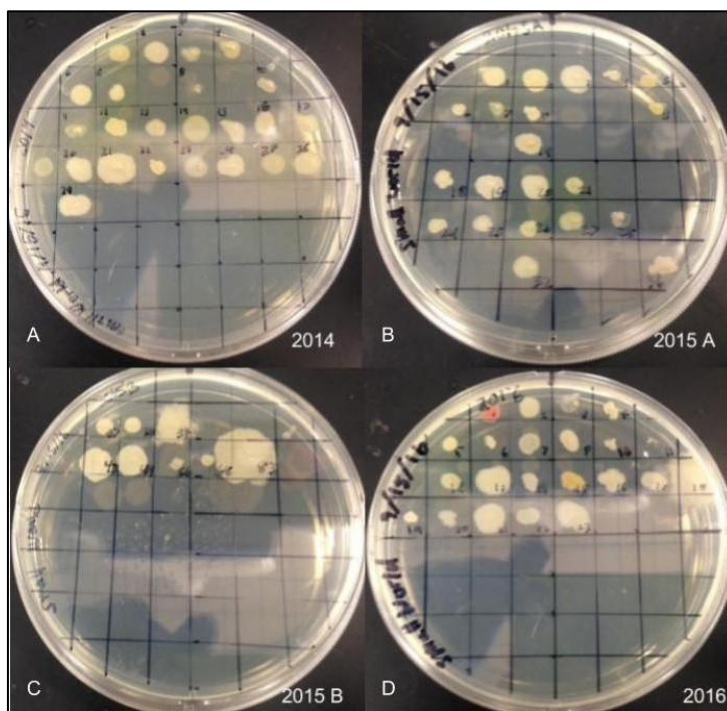
Figure 13: Microbes to Molecules isolates plated by year of class (Isolates in photo were grown for 48 hours)

After 48 hours, colonies of various sizes and colors were observed from each sample shown in *Figure 13*. It was also observed that certain strains grew larger colonies than others, but this information was not relevant as they had not been tested for antibacterial activity. These results showed that the cryo-storage method used by the class worked and that all of the samples were viable for further testing. Any potential antibacterial activity of the samples was tested using a top agar assay. A colony of each sample was grown on two separate LB agar plates. The two plates allowed for each soil bacteria sample to be tested for antibacterial activity against gram positive (*B.subtilis*) and gram negative (*E. coli*) bacteria. After the top agar mixed with its respective bacteria culture was added to the plates, they were left to grow as described above.
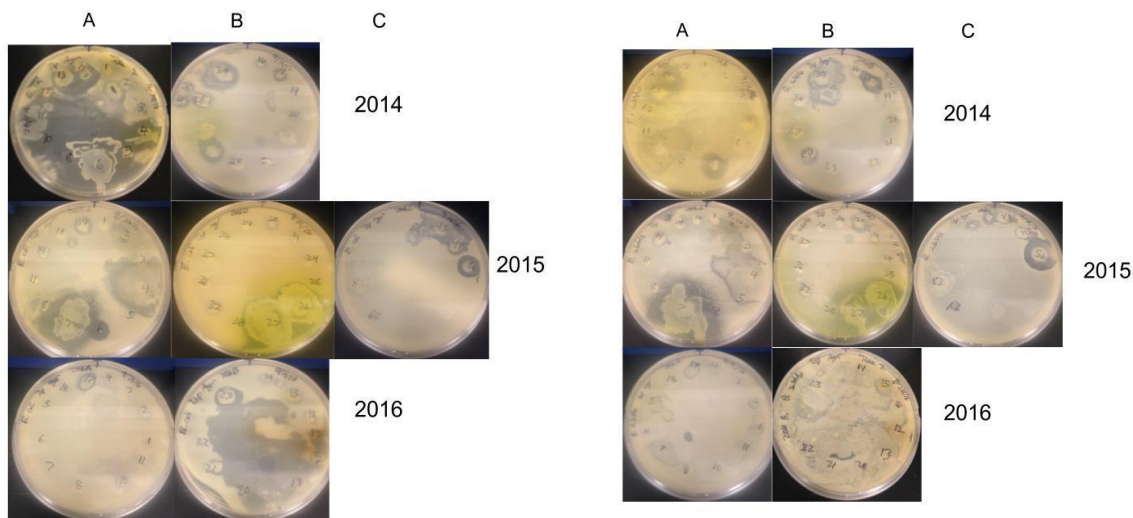
Figure 14: E.coli (Left) and B. subtilis (Right) top agar screening plates. Note that 14-29 is mislabeled in this figure as 14-39.

The plates, after incubation, showed varying levels of antibacterial activity in *Figure 14*. Antibacterial activity was observed on both the *E. coli* and *B. subtilis* plates. In some instances, the zone of inhibition was much bigger than others, such as the zone created by one of the colonies in the first row of column A of the *E. coli* plate. Some samples were also observed to only inhibit the growth of one type of bacteria, such as the 15-19 sample that only created a zone of inhibition on the *E.coli* plate. Soil bacteria samples that showed to have antibacterial activity were selected for further analysis. Specifically, samples that showed strong inhibited of both gram positive and gram negative bacteria were selected for further

screening. The results of the antibiotic production screening for all isolates tested are summarized in Table 2 below.

Table 2: Antibiotic Production Screening Summary

2014			2015			2016		
ID #	Against E. coli	Against B. subtilis	ID #	Against E. coli	Against B. subtilis	ID #	Against E. coli	Against B. subtilis
14-1	-	-	15-1	-	-	16-1	Weak zone	Strong zone
14-2	-	-	15-2	Strong zone	Weak zone	16-2	Weak zone	Strong zone
14-3	-	-	15-3	-	-	16-3	-	-
14-4	-	Weak zone	15-4	Strong zone	Strong zone	16-4	-	-
14-5	-	-	15-5	-	-	16-5	-	-
14-6	-	Strong zone	15-6	Strong zone	Strong zone	16-6	-	-
14-8	-	-	15-7	Strong zone	Strong zone	16-7	-	-
14-10	-	Strong zone	15-8	-	-	16-8	-	-
14-11	-	-	15-11	-	-	16-10	Weak zone	-
14-12	-	-	15-14	Weak zone	Strong zone	16-11	Weak zone	-
14-13	-	Strong zone	15-18	-	-	16-12	Strong zone	-
14-14	-	Strong zone	15-19	Strong zone	-	16-13	-	-
14-15	-	-	15-20	Strong zone	Strong zone	16-14	Weak zone	-
14-16	Strong zone	-	15-21	Strong zone	Strong zone	16-15	Weak zone	-
14-17	Strong zone	Strong zone	15-24	-	-	16-16	Weak zone	-
14-19	-	-	15-25	-	-	16-17	Strong zone	Weak zone
14-20	-	-	15-26	Strong zone	Strong zone	16-18	-	-
14-21	-	Weak zone	15-27	Strong zone	Strong zone	16-19	-	-
14-22	-	-	15-28	-	-	16-20	-	-
14-23	-	-	15-32	-	Strong zone	16-21	Strong zone	-
14-24	Strong zone	Strong zone	15-36	-	-	16-22	-	-
14-29	Strong zone	Strong zone	15-37	-	-	16-23	Strong zone	-
14-36	Weak zone	Weak zone	15-38	-	-			
			15-39	-	-			
			15-43	Strong zone	-			
			15-44	Strong zone	-			
			15-56	Strong zone	-			
			15-68	-	-			
			15-83	Weak zone	-			

3.2 Organic Extraction of Isolate Cultures

Selected isolates were then grown on their own LB agar plates, transferred into 50 mL conical tubes, and then frozen using either liquid nitrogen or the -80 ^{o}C freezer. These growth plates were then used to extract any bacterial products using the ethyl acetate extraction method. The extraction samples were then applied to paper circles and placed on LB agar plates that were previously inoculated with liquid media of *E.coli* and *B.subtilis*.
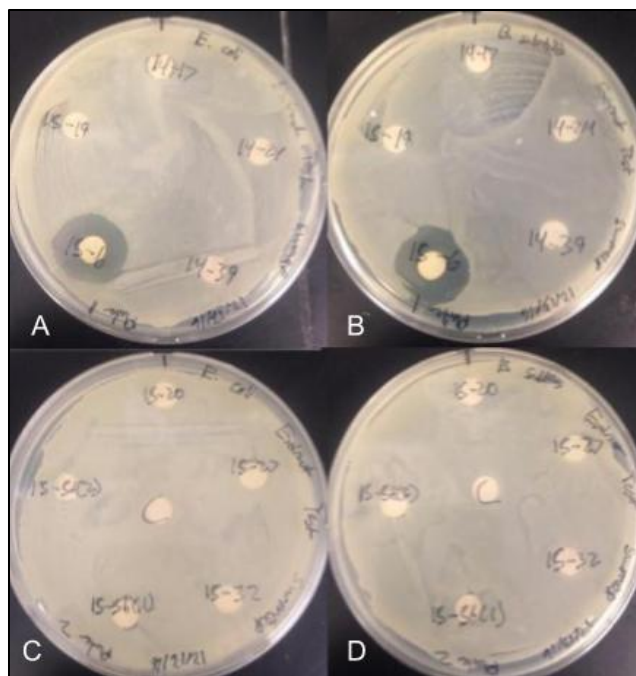
Figure 15: Bacterial extracts extracted with ethyl acetate from solid media tested on *E. coli* and *B. subtilis*

Out of all of the samples collected from the ethyl acetate extraction, only the 2015-6 (dubbed 15-6) samples showed to still have a product that had antibacterial characteristics, shown in *Figure 15*'s frames A (against *E. coli*) and B (against *B. subtilis*). The remaining samples and the control (which was a filter disk with only ethyl acetate added) showed no zones of inhibition.

The bacterial isolates whose extracts did not produce zones on either test species were used again to see if any antibacterial products would be extracted out of them using methanol. It was noted that during this extraction, many of the samples did not separate well and many contained large amounts of solid particulate. This solid particulate was filtered from samples before other analytical techniques were performed.
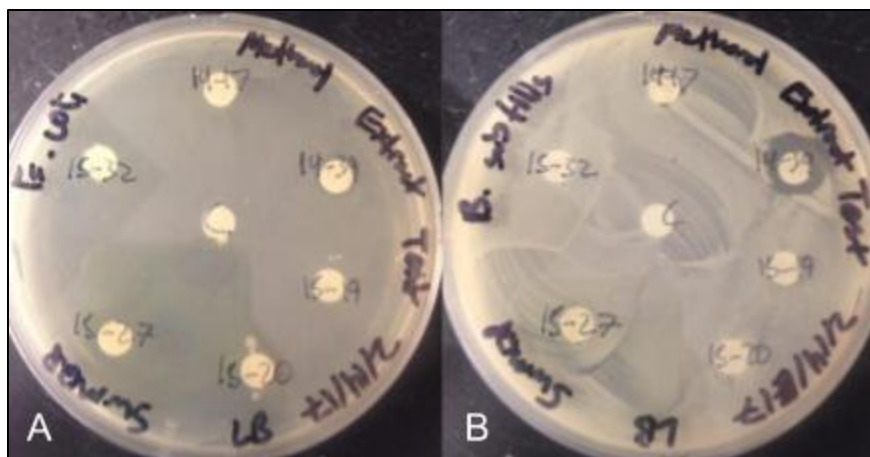
Figure 16: Bacterial extracts extracted in methanol from solid media tested against *E. coli* and *B. subtilis*

The samples were tested using the same technique used for the ethyl acetate extractions. Observed in *Figure 16*, only sample 2014-29 (dubbed 14-29) was observed to produce zones of inhibition on the plates. The remaining samples and the control (made by placing a paper circle on the agar that only had methanol added to it) failed to produce any zones of inhibition.

3.3 General PCR Results

Colony PCR was utilized to help identify the soil bacteria. Primers for the 16s subunit of the ribosome were selected to produce DNA samples to be sequenced. Using the normal PCR protocol for the 16s subunit showed poor results for most samples. Using a gradient of annealing temperatures, it was determined that the PCR produced more quality product at 44 C when using the 27F and 1492R primers.

The gyrase gene targeted PCR was then conducted on samples identified as being in the *Bacillus* genus. The attempted PCR did not produce sufficient amounts of DNA. Due to limited time and repeated insufficient results from the gyrase PCR, attempts to sequence the *Bacillus* samples were not pursued further.

3.4 Isolate 15-6

The 15-6 isolate produced an antibiotic compound that could be extracted using the ethyl acetate extraction technique. 15-6 grew slowly on LB agar at room temperature, taking at least 72 hours before colonies are visible. It was able however able to readily produce the

antibiotic compound on LB agar. 15-6 also grew slowly in LB liquid media taking at least 72 hours before the media becomes cloudy or clumps of colonies are apparent. On LB agar the colonies are very small, circular, and have a hard and waxy texture, making them difficult to pick up when inoculating other media. After 96 hours a yellow-brown precipitate could be seen excreting from the colonies on the plate.
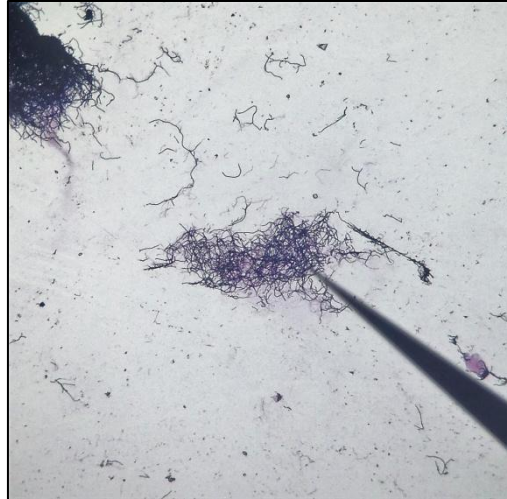
Figure 17: Gram stain of isolate 15-6 at 400x magnification

When 15-6 was gram stained it appeared gram positive and filamentous (seen in *Figure 17*).

3.4.1 15-6 Metabolic Assays

The results of four metabolic assays for the 15-6 isolate were recorded in *Figure 18*. These assays in clockwise order are glucose fermentation, lactose fermentation, amylase assay, and the MacConkey agar assay.
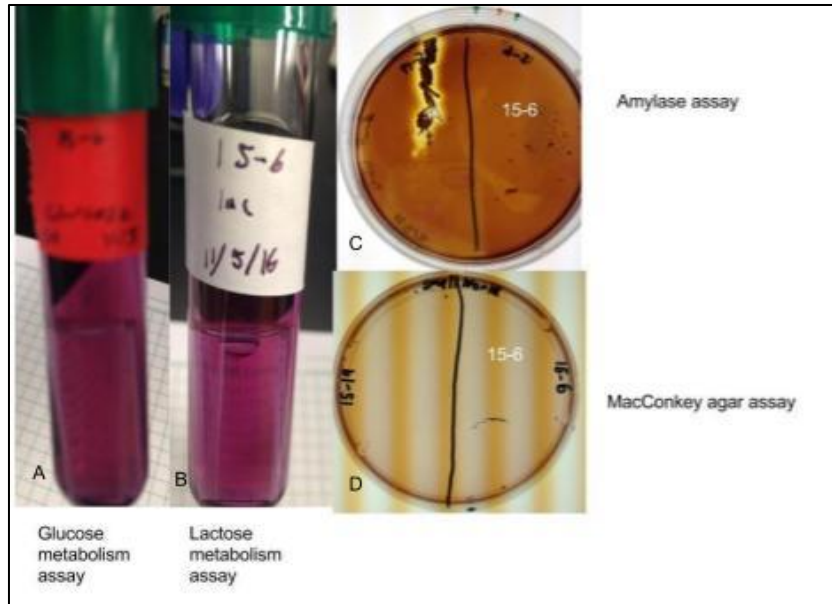
Figure 18: 15-6 isolate metabolic assay results

The physical results shown in Figure 18 are summarized in Table 3 below.

Table 3: 15-6 metabolic assay summary of results

Assay	Color of media	Presence of gas	Assay	Growth
Glucose fermentation assay	Purple	No	Amlyase assay	No growth
Lactose fermentation test	Purple	Yes	MacConkey agar assay	No growth

The 15-6 isolate appears to be unable to ferment glucose as no acid or gas was formed in the glucose metabolism assay. In the lactose metabolism assay gas was present, but the media did not turn yellow indicating that no acid was fermented from the carbohydrate.

15-6 did not grow on the starch plate; therefore the presence of amylase could not be determined. The 15-6 isolate also did not grow on MacConkey agar. This result is expected as 15-6 appears to be gram positive and MacConkey agar is designed to select against gram positive bacteria.

3.4.2 15-6 PCR results

According to the 16s ribosomal nucleotide BLAST search alignment (Altschul *et al.*, 1990) 15-6 is likely a *Streptomyces* species. The exact species cannot be determined with the sequences that were obtained. The likely species include *Streptomyces costaricanus*, *Streptomyces murinus*, *Streptomyces phaeogriseichromatogenes*, and *Streptomyces misionensis. Figure 19* summarizes the BLAST 16s ribosomal subunit sequence alignment results for isolate 15-6. The 16s ribosomal nucleotide alignments for the top three matched species are shown in *Figures 20-22*.

Description	Max score	Total score	Query cover	E value	Ident	Accession
Streptomyces costaricanus strain NBRC 100773 16S ribosomal RNA gene, partial sequence	1304	1304	89%	0.0	91%	NR_041414.1
Streptomyces murinus strain NBRC 14802 16S ribosomal RNA gene, partial sequence	1304	1304	89%	0.0	91%	NR_112445.1
Streptomyces murinus strain NBRC 12799 16S ribosomal RNA gene, partial sequence	1304	1304	89%	0.0	91%	NR_041072.1
Streptomyces costaricanus strain NRRL B-16897 16S ribosomal RNA gene, partial sequence	1304	1304	89%	0.0	91%	NR_115455.1
Streptomyces phaeogriseichromatogenes strain NRRL 2834 16S ribosomal RNA gene, partial sequence	1288	1288	88%	0.0	91%	NR_042095.1
Streptomyces lanatus strain NBRC 12787 16S ribosomal RNA gene, partial sequence	1243	1243	87%	0.0	91%	NR_041220.1
Streptomyces rameus strain NBRC 3782 16S ribosomal RNA gene, partial sequence	1242	1242	87%	0.0	91%	NR_112497.1
Streptomyces lanatus strain ISP 5090 16S ribosomal RNA gene, partial sequence	1230	1230	86%	0.0	91%	NR_114826.1
Streptomyces misionensis strain JCM 4497 16S ribosomal RNA gene, partial sequence	1227	1227	89%	0.0	90%	NR_044138.1
Streptomyces misionensis strain NBRC 13063 16S ribosomal RNA gene, partial sequence	1227	1227	89%	0.0	90%	NR_112355.1
Streptomyces coacervatus strain IFM 11055 16S ribosomal RNA gene, partial sequence	1219	1219	87%	0.0	90%	NR_112916.1
Streptomyces phaeoluteichromatogenes strain NRRL B-5799 16S ribosomal RNA gene, partial sequence	1210	1210	89%	0.0	90%	NR_042096.1
Streptomyces gilvifuscus strain T113 16S ribosomal RNA, partial sequence	1208	1208	87%	0.0	90%	NR_137389.1
Streptomyces antibioticus strain NBRC 12838 16S ribosomal RNA gene, partial sequence	1208	1208	87%	0.0	90%	NR_112299.1
Streptomyces antibioticus strain CSSP528 16S ribosomal RNA gene, partial sequence	1208	1208	87%	0.0	90%	NR_043348.1
Streptomyces levis strain NRRL B-16370 16S ribosomal RNA gene, partial sequence	1205	1205	87%	0.0	90%	NR_115778.1
Streptomyces levis strain NBRC 15423 16S ribosomal RNA gene, partial sequence	1205	1205	87%	0.0	90%	NR_041184.1
Streptomyces filipinensis strain NBRC 12860 16S ribosomal RNA gene, partial sequence	1205	1205	89%	0.0	89%	NR_041083.1

Figure 19: 15-6 16s ribosomal subunit sequence alignment results (sequenced with 27F primer)

Figure 20: 15-6 and *Streptomyces costaricanus* (strain NBRC 100773) 16s ribosomal subunit partial sequence alignment

Figure 21: 15-6 and *Streptomyces murinus* (strain NBRC 12799) 16s ribosomal subunit partial sequence alignment

Figure 22: 15-6 and *Streptomyces phaeogriseichromatogenes* (strain NRRL 2834) 16s ribosomal subunit partial sequence alignment

The BLAST results, shown in *Figure 19*, indicated that the sequences generated from the 16s PCR was equally probable to belong to numerous bacteria strains. This was demonstrated in *Figures 20-22* when the DNA sequences of the top three BLAST matches were aligned with the DNA generated from PCR. Each DNA sequence from BLAST results showed equally high levels of similarity with the 15-6 16s Ribosomal subunit DNA.

3.4.3 15-6 Growth and antibiotic production on various solid media

Over the course of the project, the growth of 15-6 was noted to be very slow, requiring three days to grow to concentrations with defined colonies. Different agar media types were then used to determine if 15-6 grew more efficiently on other media besides LB. The agar media tested were LB, Potato Dextrose Agar (PDA), Trypticase Soy Agar (TSA), Reasoner's 2a Agar (R2A), and Todd Hewitt Agar (THA) media. The plates were left to grow for five days and the resulting bacterial growth was documented.
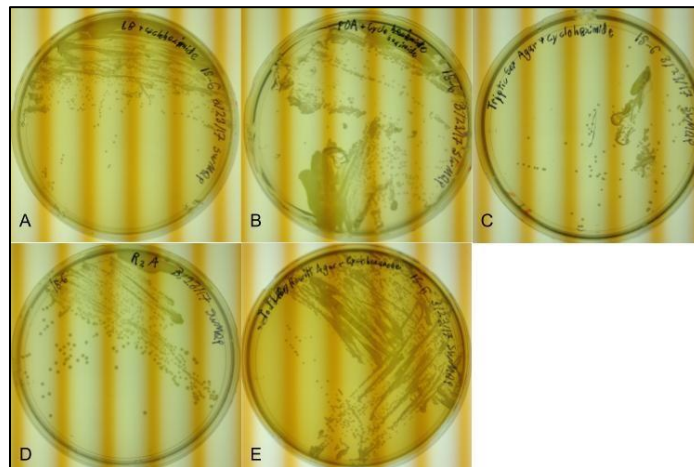
Figure 23: 15-6 growth on LB (A), PDA (B), TSA (C), R2A (D), and THA (E)

The plates, *Figure 23*, showed that the 15-6 sample grew at different rates for each media. The PDA and THA media showed the largest concentration of colonies. TSA media showed the lowest, with R2A and LB media showing about the same concentrations of colonies. Every plate had its bacterial product extracted using ethyl acetate extraction.

The extracts were then used on a zone of inhibition tests against *E.coli* and *B.subtilis*. The goal of this test was to determine if any of the extracted samples produced a more concentrated sample. The theory being, more concentrated samples would show larger zones of inhibition on the plates.
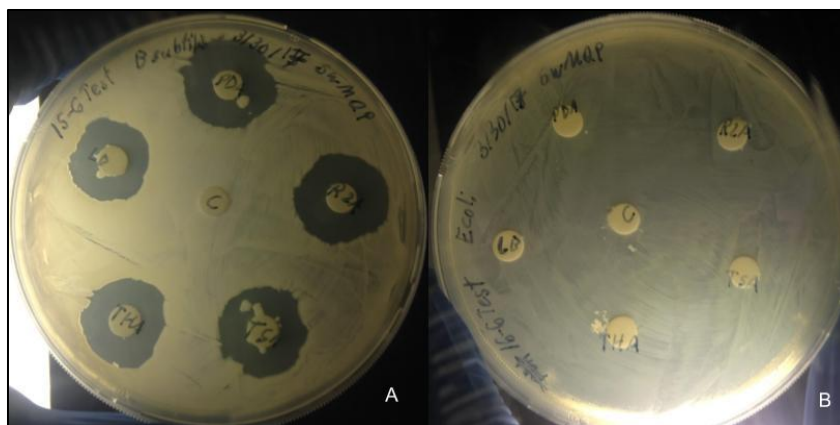
Figure 24: Testing of 15-6 extracts extracted from LB, PDA, TSA, R2A, and THA media against *B. subtilis* (A) and *E. coli* (B)

After letting the *E.coli* and *B.subtilis* grow on the treated plates for 24 hours, the plates shown in *Figure 24* were obtained. Each of the extracts showed to produce clear zones of inhibition on the *B.subtilis* plate and very faint zones of inhibition on the *E.coli* plate. Along with the control showing no zones of inhibition, as it served as a negative control, the R2A and PDA extracts were observed to have slightly larger zones of inhibition. The THA, LB, and TSA extracts were observed to have smaller zones of inhibition.

3.4.3.2 Various Solid Media 15-6 Extract HPLC Results

Further analysis of the extracts along with the purification of the extracts was conducted using an HPLC column. Previously grown 15-6 extract, grown on LB media, was used to determine elution times. Elution times and protocols from this experiment, shown in section **3.6,** were used to design the HPLC graphs shown in *Figures 25-29*. Each extract was run through the HPLC for 30 minutes and the fraction from the 25-30 minute time period was collected.
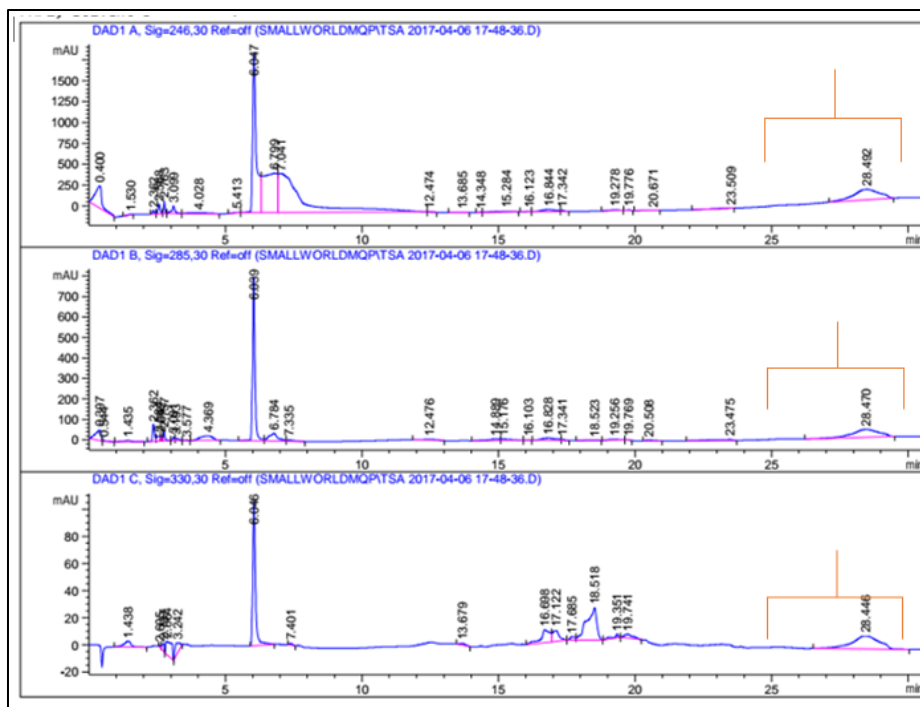
Figure 25: HPLC chromatogram of 15-6 grown on TSA extracted in ethyl acetate
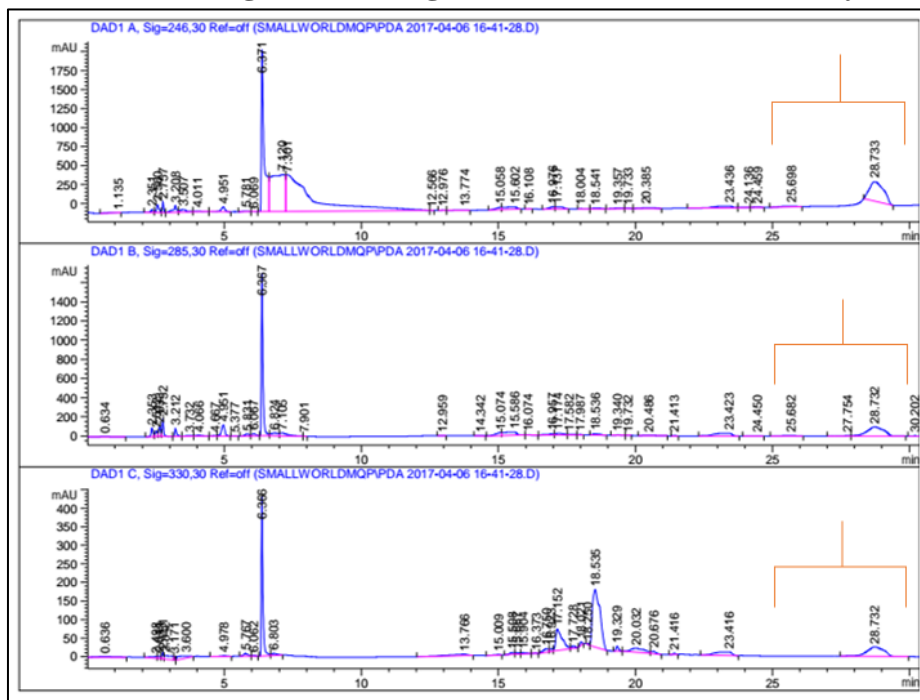

Figure 26: HPLC chromatogram of 15-6 grown on THA extracted in ethyl acetate
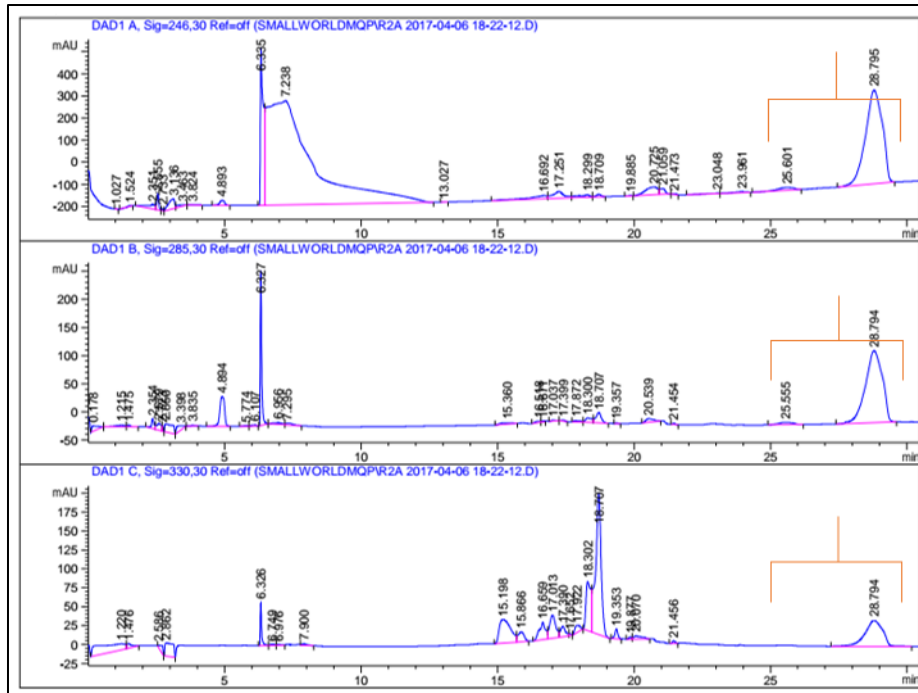
Figure 27: HPLC chromatogram of 15-6 grown on R2A extracted in ethyl acetate
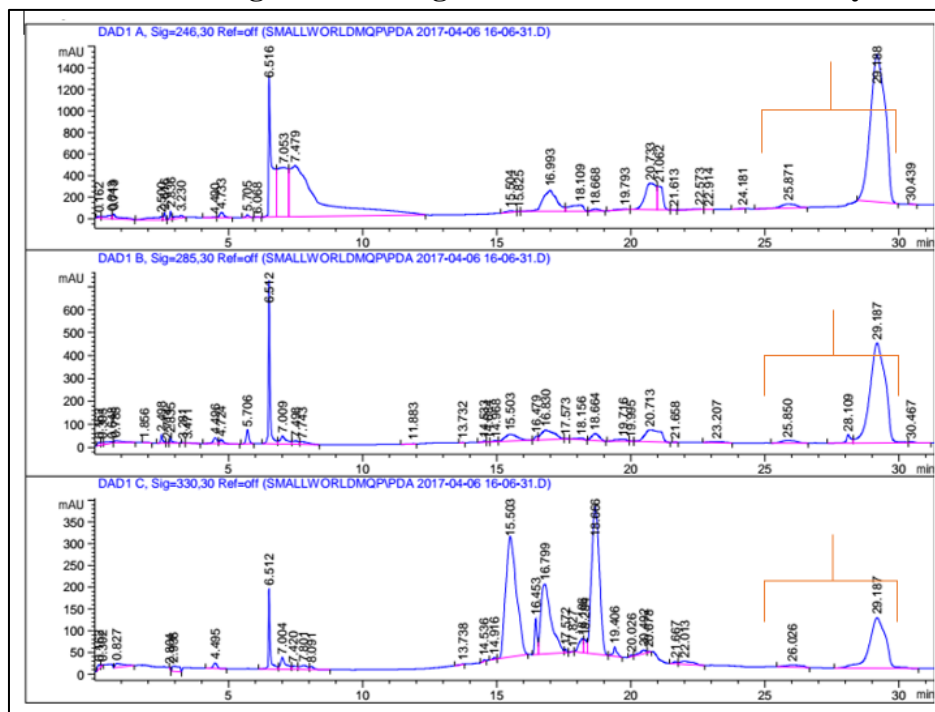
Figure 28: HPLC chromatogram of 15-6 grown on PDA extracted in ethyl acetate
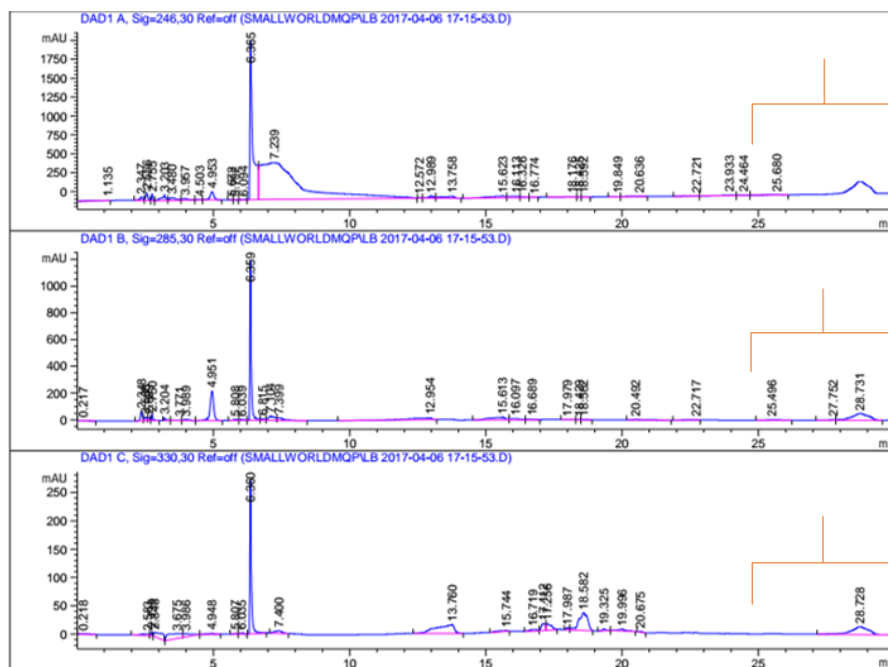
Figure 29: HPLC chromatogram of 15-6 grown on LB extracted in ethyl acetate

The generated HPLC chromatograms from the 15-6 samples grown on various media showed unique results for each media type. Previous testing indicated that the 15-6 antibacterial compound eluted at the 25-30 minute fraction, indicated on each graph (Data recorded in Section 3.6). TSA, THA and LB media, shown in *Figures 25, 26,* and *29* showed the smallest peaks at the 25-30 minute time interval. The 15-6 extract grown from R2A media showed the second highest peaks of solution at the designated time interval, shown in *Figure 27*. The PDA media sample was observed to have the highest peak of at the designated time interval, shown in *Figure 28*. Despite these results, LB media was used for the rest of the laboratory experiments. This was due to limited supply of alternate media types as well as time.

3.5 Isolate 14-29

The 14-29 isolate grows relatively fast at room temperature on LB agar with colonies appearing within 24 hours. 72 hours is however necessary for a full lawn of growth to appear. Colonies appear soft, irregularly shaped, and cream colored.
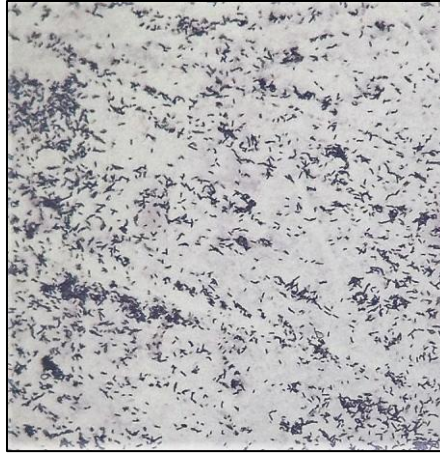
Figure 30: Gram stain of isolate 14-29 at 400x magnification

When Gram stained the bacteria appear to be gram positive rods (*Figure 30*).

3.5.1 14-29 Metabolic assays

The results of four metabolic assays for the 14-29 isolate were recorded in *Figure 31*. These assays in clockwise order are glucose fermentation, lactose fermentation, amylase assay, and the MacConkey agar assay.
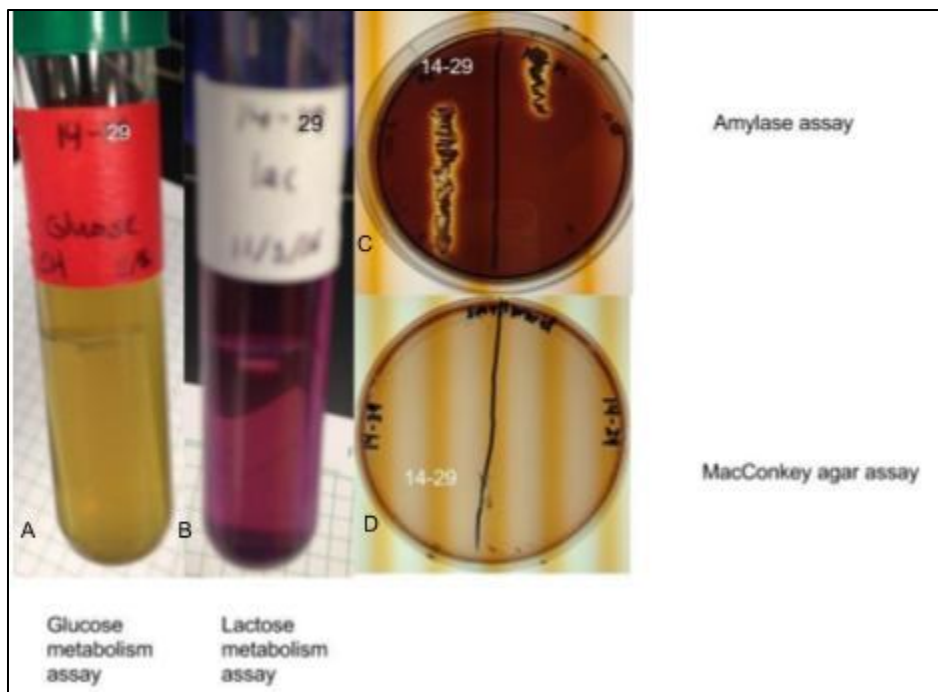
Figure 31: 14-29 isolate metabolic assay results

The physical results shown in Figure 31 are summarized in Table 4 below.

Table 4: 14-29 metabolic assay summary of results

Assay	Color of media	Presence of gas	Assay	Growth	Result
Glucose fermentation assay	Yellow	Yes	Amlyase assay	Growth	Amylase positive
Lactose fermentation test	Purple	Yes	MacConkey agar assay	No growth	N/A

The 14-29 isolate appears to be able to ferment glucose as the media turned yellow, indicating the presence of an acid, and gas was formed. In the lactose fermentation test, gas was formed but the media did not turn yellow, indicating no acid was present.

14-29 appears to be amylase positive (*Figure 31*). On the MacConkey agar plate in Figure 19 14-29 is mislabeled as 14-39. 14-29 did not grow on MacConkey agar. This result is expected as 14-29 appears to be gram positive and MacConkey agar is designed to select against gram positive bacteria.

3.5.2 14-29 PCR results

According to the 16s ribosomal nucleotide BLAST alignment (Altschul *et al.*, 1990), 14-29 is likely a *Brevibacillus* species. The exact species cannot be determined with the sequences that were obtained. The most likely species include *Brevibacillus laterosporus, Brevibacillus inocatus,* or *Brevibacillus fomnosus.* A summary of the top sequence alignment results can be seen in *Figure 32*. The nucleotide alignments for the top five matches are shown in *Figures 33-37.*

Description	Max score	Total score	Query cover	E value	Ident	Accession
Brevibacillus laterosporus strain DSM 25 16S ribosomal RNA gene, partial sequence	1055	1055	97%	0,0	96%	NR_112212.1
Brevibacillus laterosporus strain IAM 12465 16S ribosomal RNA gene, partial sequence	1055	1055	97%	0,0	96%	NR_037005.1
Brevibacillus laterosporus strain NBRC 15654 16S ribosomal RNA gene, partial sequence	1051	1051	97%	0,0	95%	NR_112727.1
Brevibacillus invocatus strain LMG 18962 16S ribosomal RNA gene, partial sequence	1000	1000	97%	0,0	94%	NR_041836.1
Brevibacillus invocatus strain NCIMB 13772 16S ribosomal RNA gene, partial sequence	1000	1000	97%	0,0	94%	NR_112210.1
Brevibacillus formosus strain NBRC 15716 16S ribosomal RNA gene, partial sequence	994	994	97%	0,0	94%	NR_113801.1
Brevibacillus brevis strain NBRC 15304 16S ribosomal RNA gene, partial sequence	994	994	97%	0,0	94%	NR_041524.1
Brevibacillus agri strain DSM 6348 16S ribosomal RNA gene, partial sequence	994	994	97%	0,0	94%	NR_040983.1
Brevibacillus formosus strain DSM 9885 16S ribosomal RNA gene, partial sequence	994	994	97%	0.0	94%	NR_040979.1
Brevibacillus brevis strain DSM 30 16S ribosomal RNA gene, partial sequence	994	994	97%	0,0	94%	NR_112204.1
Brevibacillus agri strain NBRC 15538 16S ribosomal RNA gene, partial sequence	990	990	97%	0,0	94%	NR_113767.1
Brevibacillus limnophilus strain DSM 6472 16S ribosomal RNA gene, partial sequence	989	989	97%	0,0	94%	NR_024822.1
Brevibacillus parabrevis strain IFO 12334 16S ribosomal RNA gene, partial sequence	989	989	97%	0,0	94%	NR_040981.1
Brevibacillus parabrevis strain NBRC 12334 16S ribosomal RNA gene, partial sequence	985	985	97%	0.0	94%	NR_113589.1
Brevibacillus nitrificans strain DA2 16S ribosomal RNA gene, partial sequence	983	983	97%	0.0	94%	NR_112926.1
Brevibacillus panacihumi strain DCY35 16S ribosomal RNA gene, partial sequence	983	983	97%	0.0	94%	NR_044485.1
Brevibacillus centrosporus strain DSM 8445 16S ribosomal RNA gene, partial sequence	983	983	97%	0.0	94%	NR_112211.1
Brevibacillus centrosporus strain NBRC 15540 16S ribosomal RNA gene, partial sequence	977	977	97%	0,0	94%	NR_113768.1
Brevibacillus choshinensis strain NBRC 15518 16S ribosomal RNA gene, partial sequence	977	977	97%	0,0	94%	NR_113763.1

Figure 32: 14-29 16s ribosomal subunit sequence alignment results (sequenced with 1492R primer)

Brevibacillus laterosporus strain DSM 25 16S ribosomal RNA gene, partial sequence
Sequence ID: NR_112212.1 Length: 1486 Number of Matches: 1
Range 1: 778 to 1435

Score	Expect	Identities	Gaps	Strand	Frame
1055 bits(571)	0.0()	634/663(96%)	10/663(1%)	Plus/Minus	

Figure 33:14-29 and *Brevibacillus laterosporus* **(strain DSM 25) 16s ribosomal subunit partial sequence alignment**

Figure 34:14-29 and *Brevibacillus laterosporus* (strain IAM 12465) 16s ribosomal subunit partial sequence alignment

Brevibacillus laterosporus strain NBRC 15654 16S ribosomal RNA gene, partial sequence
Sequence ID: **NR_112727.1** Length: 1461 Number of Matches: 1
Range 1: 780 to 1437

Score	Expect	Identities	Gaps	Strand	Frame
1051 bits(569)	0.0()	633/663(95%)	10/663(1%)	Plus/Minus	

Features:

```
Query  15    CTTC-GCGGCTGG-TCCTTACGGGTACCTCACCGACTTCGGGTGTTGCAAACTCCCGTGG   72
             |||| ||||||| |||||||| |||||||||||||||||||||||||||||||||| |||
Sbjct  1437  CTTCGGCGGCTGGCTCCTTNCGGTTACCTCACCGACTTCGGGTGTTGCAAACTCCCGTGG   1378

Query  73    TGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGA   132
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1377  TGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGA   1318

Query  133   TTACTAGCGATTCCGACTTCATGTAGGCGAGTTGCAGCCTACAATCCGAACTGAGATTGG   192
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1317  TTACTAGCGATTCCGACTTCATGTAGGCGAGTTGCAGCCTACAATCCGAACTGAGATTGG   1258

Query  193   TTTTAAGAGATTAGCATCTTCTCGCGAAGTAGCATCCCGTTGTACCAACCATTGTAGCAC   252
             ||||||||||||| |||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1257  TTTTAAGAGATTAGCATCTTCTCGCGAAGTAGCATCCCGTTGTACCAACCATTGTAGCAC   1198

Query  253   GTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCGCCTTCCTCCGTC   312
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1197  GTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCGCCTTCCTCCGTC   1138

Query  313   TTGTCGACGGCAGTCTCTCTAGAGTGCCCAACTGAATGCTGGCAACTAAAGATAAGGGTT   372
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1137  TTGTCGACGGCAGTCTCTCTAGAGTGCCCAACTGAATGCTGGCAACTAAAGATAAGGGTT   1078

Query  373   GCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACC   432
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1077  GCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACC   1018

Query  433   ACCTGTCACCACTGCCCCGAAGG-AAGCTCTATCTCTAGAGCGGTCAGTGGGATGTCAAG   491
             ||||||||||||||||||||||| |||||||||||||||||||||||||||||||||||||
Sbjct  1017  ACCTGTCACCACTGCCCCGAAGGGAAGCTCTATCTCTAGAGCGGTCAGTGGGATGTCAAG   958

Query  492   ACCTGGTAGGGTTCTTCGCGGTGCTTCGAATTAAAACCACATGCTCCACCGCTTGTGCG   551
             ||||||||||| |||||||||||||||||||| ||| |||||||||||||||| |||||
Sbjct  957   ACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAA-CCACATGCTCCACCGCTT-GTGCG   900

Query  552   GGGCCCCGTCAATTTCCTTTAAGTT-CACTTCTTGCCAGCGTACTCCCGAGCCGGAGTGC   610
             ||||||||||||| |||| ||||| | ||| ||||||||||||||||||||||||||||||
Sbjct  899   GGCCCCCGTCAATT-CCTTTGAGTTTCAC-TCTTGCGAGCGTACTCCCCAGGCGGAGTGC   842

Query  611   TTTATTGCGT-AGCCTCAGGACTAAGGGTATTGAAATCGCTAAAACATAGCGTTCATTGT   669
             ||| ||||| |||| |||||||| |||||||||||||| |||||| ||||| ||| |||
Sbjct  841   TT-ATTGCGTTAGCTGCGGCACTAAGGGTATTGAAACCCCTAACACCTAGCACTCATCGT   783

Query  670   TTA   672
             |||
Sbjct  782   TTA   780
```

Figure 35:14-29 and *Brevibacillus laterosporus* (strain NBRC 15654) 16s ribosomal subunit partial sequence alignment

Figure 36:14-29 and *Brevibacillus invocatus* (strain LMG 18962) 16s ribosomal subunit partial sequence alignment

```
Brevibacillus invocatus strain NCIMB 13772 16S ribosomal RNA gene, partial sequence
Sequence ID: NR_112210.1 Length: 1488 Number of Matches: 1
Range 1: 780 to 1437
```

Score	Expect	Identities	Gaps	Strand	Frame
1000 bits(541)	0.0()	624/663(94%)	10/663(1%)	Plus/Minus	

Features:

```
Query  15    CTTC-GCGGCTGG-TCCTTACGGGTACCTCACCGACTTCGGGTGTTGCAAACTCCCGTGG   72
Sbjct  1437  CTTCGGCGGCTGGCTCCTTGCGGTTACCTCACCGACTTCGGGTGTTGCAAACTCCCGTGG   1378
Query  73    TGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGA   132
Sbjct  1377  TGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGA   1318
Query  133   TTACTAGCGATTCCGACTTCATGTAGGCGAGTTGCAGCCTACAATCCGAACTGAGATTGG   192
Sbjct  1317  TTACTAGCGATTCCGACTTCATGTAGGCGAGTTGCAGCCTACAATCCGAACTGAGATTGG   1258
Query  193   TTTTAAGAGATTAGCATCTTCTCGCGAAGTAGCATCCCGTTGTACCAACCATTGTAGCAC   252
Sbjct  1257  TTTTAAGAGATTGGCATCCTCTCGCGAGGTAGCATCCCGTTGTACCAACCATTGTAGCAC   1198
Query  253   GTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCGCCTTCCTCCGTC   312
Sbjct  1197  GTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCGCCTTCCTCCGTC   1138
Query  313   TTGTCGACGGCAGTCTCTCTAGAGTGCCCAACTGAATGCTGGCAACTAAAGATAAGGGTT   372
Sbjct  1137  TTGTCGACGGCAGTCTCTCTAGAGTGCCCAACTGAATGCTGGCAACTAAAGATAAGGGTT   1078
Query  373   GCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACC   432
Sbjct  1077  GCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACC   1018
Query  433   ACCTGTCACCACTGCCCCGAAGG-AAGCTCTATCTCTAGAGCGGTCAGTGGGATGTCAAG   491
Sbjct  1017  ACCTGTCACCGCTGCCCCGAAGGGAAGCTCTGTCTCCAGAGCGGTCAGTGGGATGTCAAG   958
Query  492   ACCTGGTAGGGTTCTTCGCGGTGCTTCGAATTAAAACCACATGCTCCACCGCTTTGTGCG   551
Sbjct  957   ACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAA-CCACATGCTCCACCGCTT-GTGCG   900
Query  552   GGGCCCCGTCAATTTCCTTTAAGTT-CACTTCTTGCCAGCGTACTCCCGAGCCGGAGTGC   610
Sbjct  899   GGGCCCCGTCAATT-CCTTTGAGTTTCAG-TCTTGCGACCGTACTCCCAGGCGGAGTGC   842
Query  611   TTTATTGCGT-AGCCTCAGGACTAAGGGTATTGAAATCGCTAAAACATAGCGTTCATTGT   669
Sbjct  841   TT-ATTGCGTTAGCTGCGGCACTAAGGGTATTGAAACCCCTAACACCTAGCACTCATCGT   783
Query  670   TTA   672
Sbjct  782   TTA   780
```

Figure 37: 14-29 and *Brevibacillus invocatus* (strain NCIMB 13772) 16s ribosomal subunit partial sequence alignment

The BLAST results, shown in *Figure 32*, indicated that the sequences generated from the 16s PCR was equally probable to belong to numerous bacterial species or strains. This was demonstrated in *Figures 33-37* when the DNA sequences of the top three BLAST matches were aligned with the DNA generated from PCR. Each DNA sequence from BLAST results showed relatively equal levels of similarity with the 14-29 16s Ribosomal subunit DNA.

3.6 LB Media Extracts HPLC Results of Samples 15-6, 14-29, and Solvents

The extraction samples were then run through an HPLC column to help purify the antibacterial product out of the extracts as well as gather chemical data on the extractions. Additionally, HPLC was used for ethyl acetate and methanol by themselves to create standard chromatograms to compare with the chromatograms of the extracts. The ethyl acetate standard is *Figure 38* and the methanol standard is *Figure 41*.

After samples 15-6 and 14-29 were filtered through the HPLC column, the solution that ran through the column was collected in five minute fractions. These fractions were dried down and resuspended in 100 µL of the original solvent used for the extraction of that particular isolate (ethyl acetate for 15-6 and methanol for 14-29). Fractions were collected from the ethyl acetate and methanol standards and were tested alongside the extract fractions as a control. Each fraction was then applied to paper tested using the same inhibition zone assay as before. This assay is shown in *Figure 40* for 15-6 and *Figure 43* for 14-29.
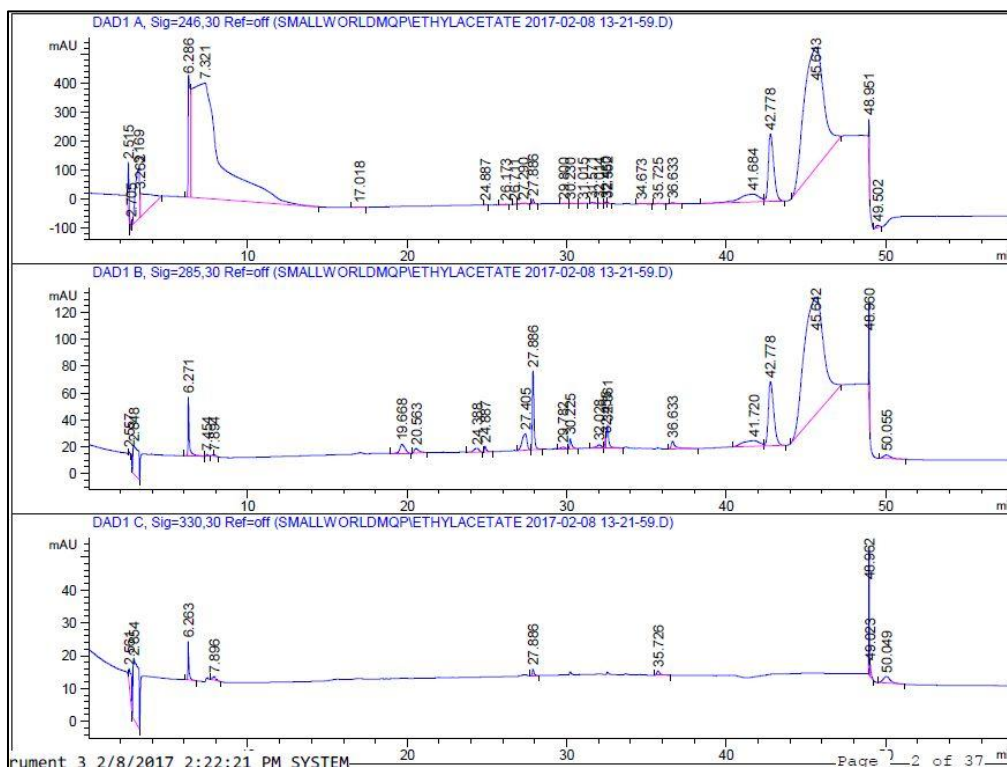
Figure 38: HPLC chromatogram of ethyl acetate

The ethyl acetate chromatogram (*Figure 38*) is characterized by a peak at approximately seven minutes and two peaks at 42 minutes and 43-50 minutes respectively. These peaks are also present in the 15-6 chromatogram seen in *Figure 39*.
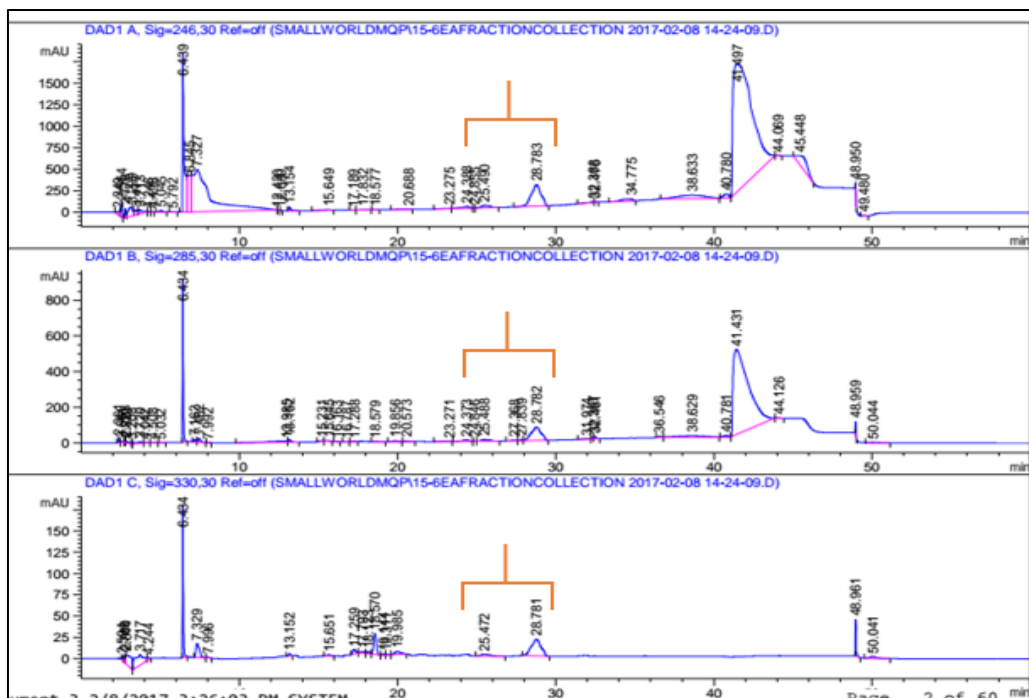
Figure 39: HPLC chromatogram of the 15-6 extract in ethyl acetate

The HPLC result of the 15-6 sample showed three distinct peaks (seen in *Figure 39*).
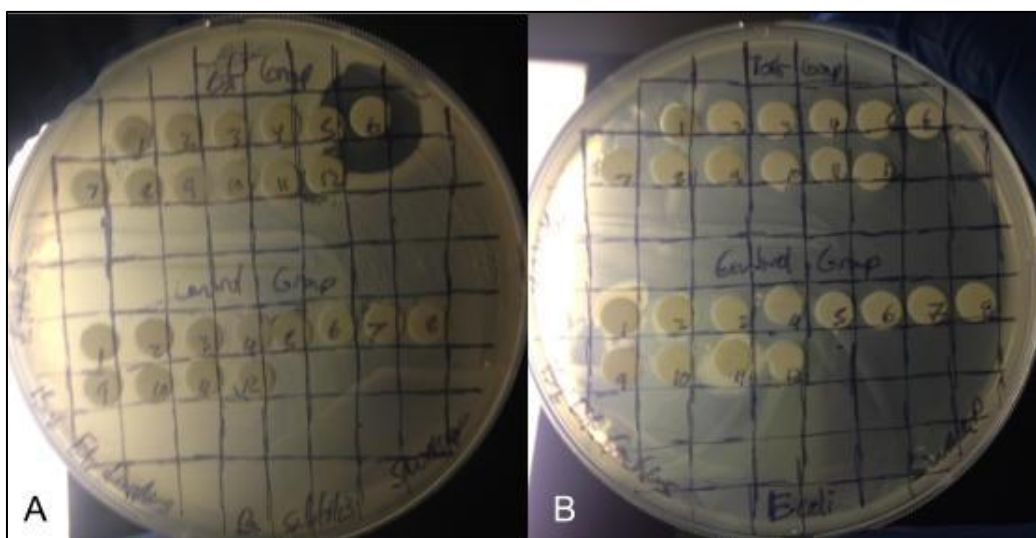
Figure 40: 15-6 extract fraction testing against *B. subtilis* (Panel A) and *E. coli* (Panel B)

The fractions collected from the 15-6 sample, shown in *Figure 40* appear to have zones of inhibition at fraction 6 for the gram positive *B. subtilis* plate (A). There was also a small zone of inhibition from fraction 12 on the *B. subtilis* plate. There was a faint zone present on the *E. coli* plate (B) for fraction 6 as well. This zone from fraction six corresponds to a peak on the 15-6 chromatogram outlined with an orange bracket.
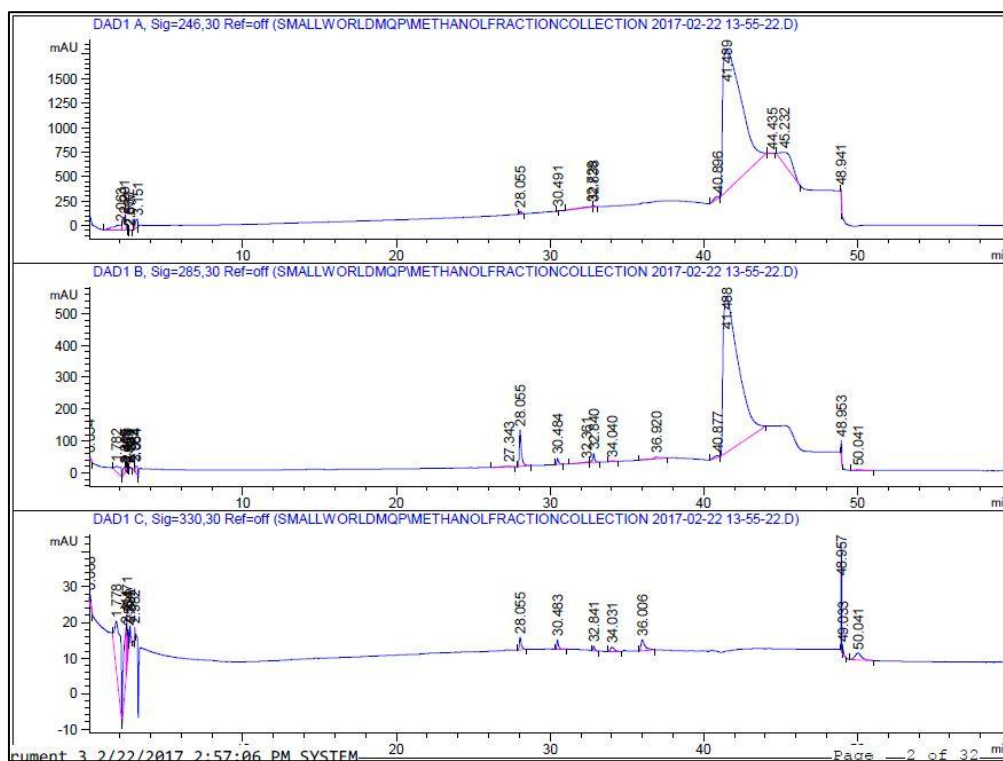
Figure 41: HPLC chromatogram of methanol

The HPLC chromatogram (*Figure 41*) of methanol defining feature is one large peak from approximately 41 minutes to 49 minutes. This feature is shared in the 14-29 chromatogram (*Figure 42*).
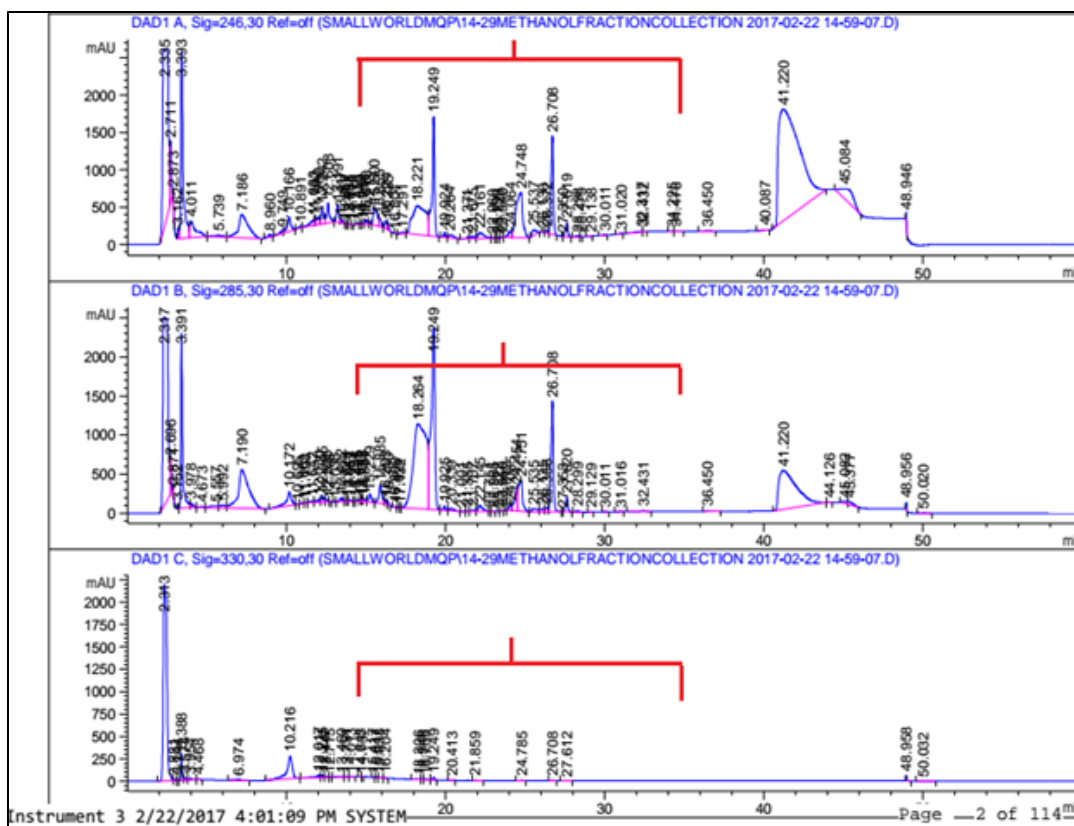
Figure 42: HPLC chromatogram of the 14-29 extract in methanol

The chromatogram generated when the 14-29 extract was run through HPLC resulted in numerous peaks. Most notably were three distinct peaks in the 15-20, 20-25, and 25-30 minute time intervals. It was noticed however, that these three peaks vanished when exposed to 330 nm wavelengths of light. Despite this, all fractions were tested using zone of inhibition tests to determine with fractions contained compounds with antibacterial activity.
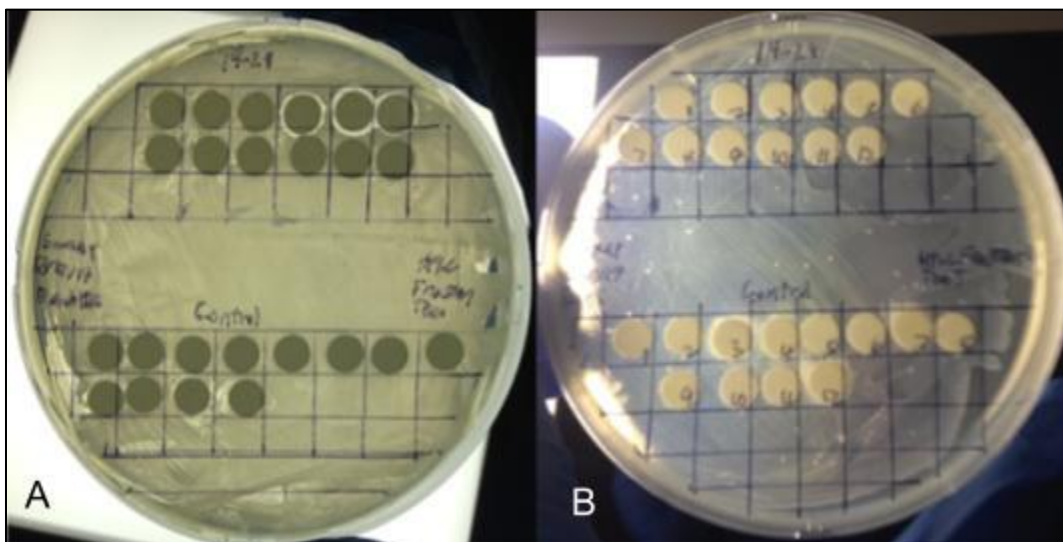
Figure 43: 14-29 extract fraction testing against *B. subtilis* (Panel A) and *E. coli* (Panel B)

The zone of inhibition tests run for the fractions collected from the HPLC purification of the 14-29 sample showed several zones of inhibition. Shown in *Figure 43*, fractions 4, 5, and 6 (collected from time intervals 15-20 minutes, 20-25 minutes, and 25-30 minutes respectively), created small zones of inhibition on the *B.subtilis* plate, *Figure 43A*, and minimal inhibition on the *E.coli* plate, *Figure 43B*. It was noted that in these fractions, peaks of activity were noted in the chromatogram at the same time intervals, indicated in *Figure 42*.

3.6.2 Antibiotic Standards HPLC Results

Antibiotic standards were run through the HPLC column using the same protocols used for the extract samples (15-6 and 14-29) and their solvents. The antibiotic stock standards were used in order to find similarities in the chromatograms between the standards and the selected samples.
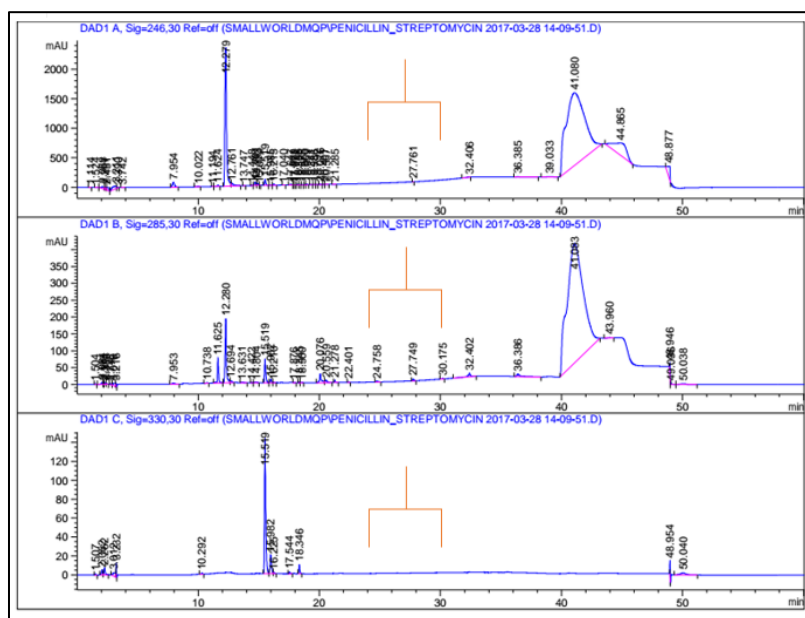
Figure 44: HPLC chromatogram of penicillin and streptomycin

The HPLC chromatogram (*Figure 44*) of penicillin and streptomycin is characterized by a sharp narrow peak at approximately 12 minutes on the 246 nm and 285 nm wavelength chromatograms, a sharp narrow peak at approximately 16 minutes on the 330 nm wavelength chromatogram, and a large broad peak from approximately 40 minutes to 50 minutes.
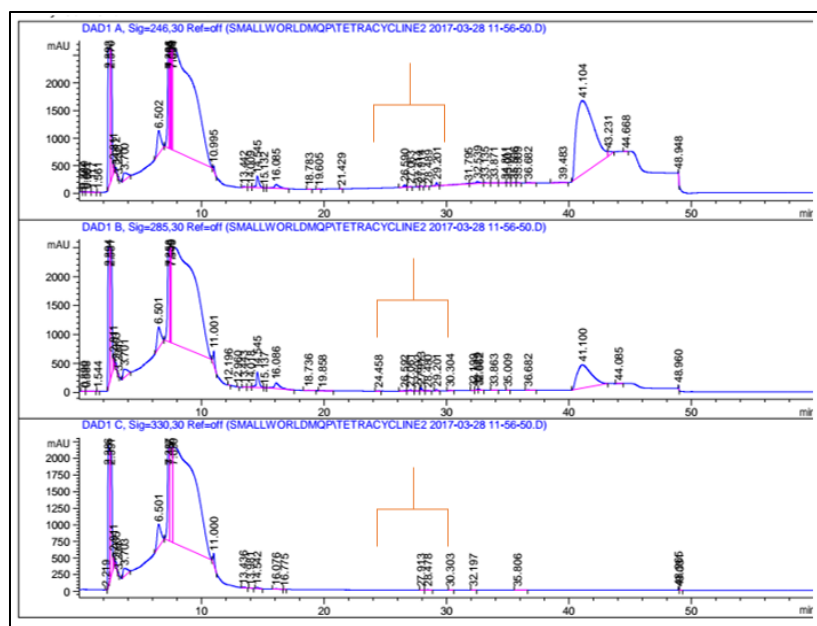
Figure 45: HPLC chromatogram of tetracycline

The HPLC chromatogram (*Figure 45*) of tetracycline is characterized by a mass of four peaks from approximately 2-11 minutes and a peak from 40 minutes to 50 minutes. This fifth peak however did not appear on the third wavelength (330 nm).
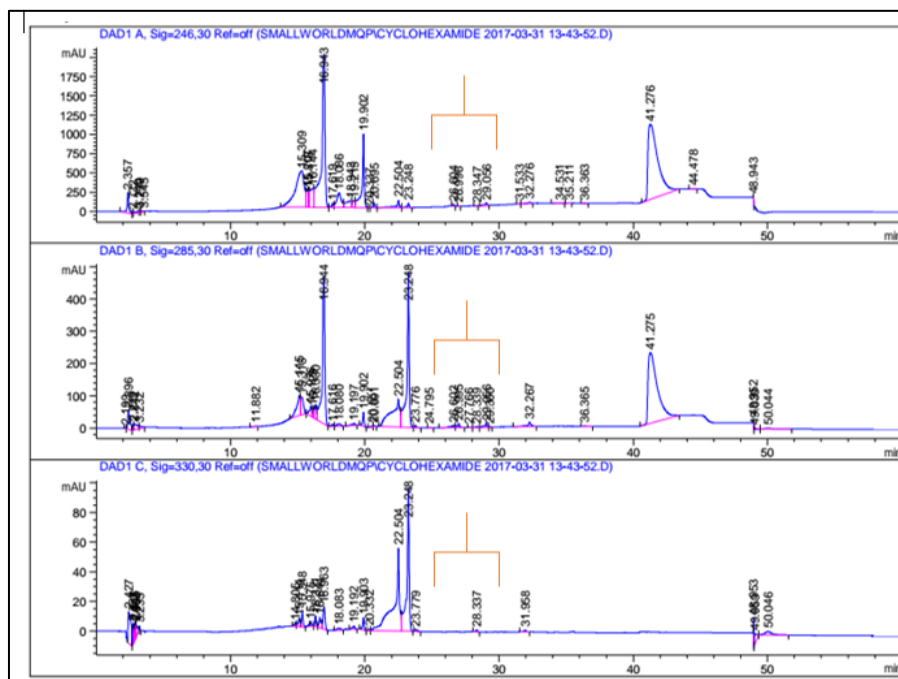
Figure 46: HPLC chromatogram of cycloheximide

The HPLC chromatogram (*Figure 46*) of cycloheximide is characterized by a cluster of peaks ranging from approximately 15-24 minutes. A large peak is also present from approximately

40-49 minutes on the 246 nm and 285 nm wavelength chromatograms.
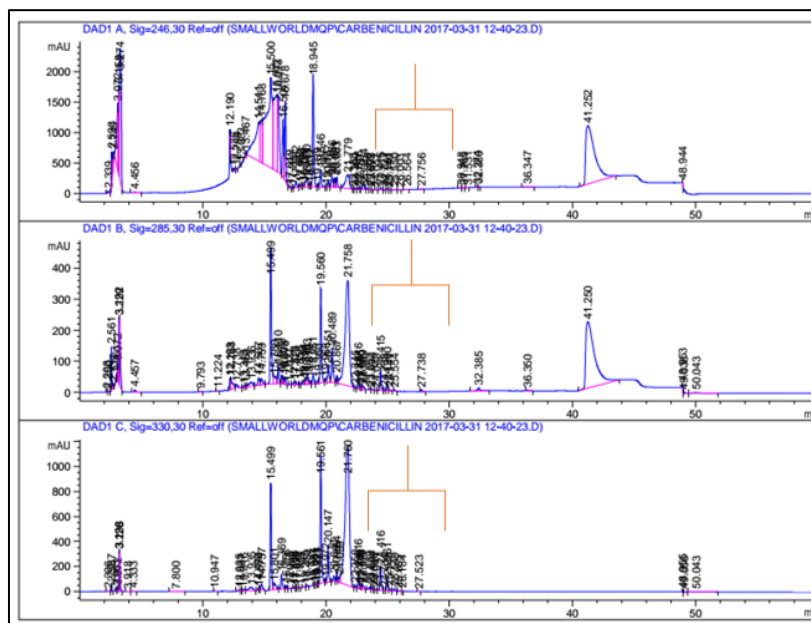
Figure 47: HPLC chromatogram of carbenicillin

The HPLC chromatogram (*Figure 47*) of carbenicillin is characterized by one sharp peak at approximately three minutes and three sharp peaks from approximately 15-22 minutes. A large peak is also present from approximately 41-49 minutes on the 246 nm and 285 nm wavelength chromatograms.
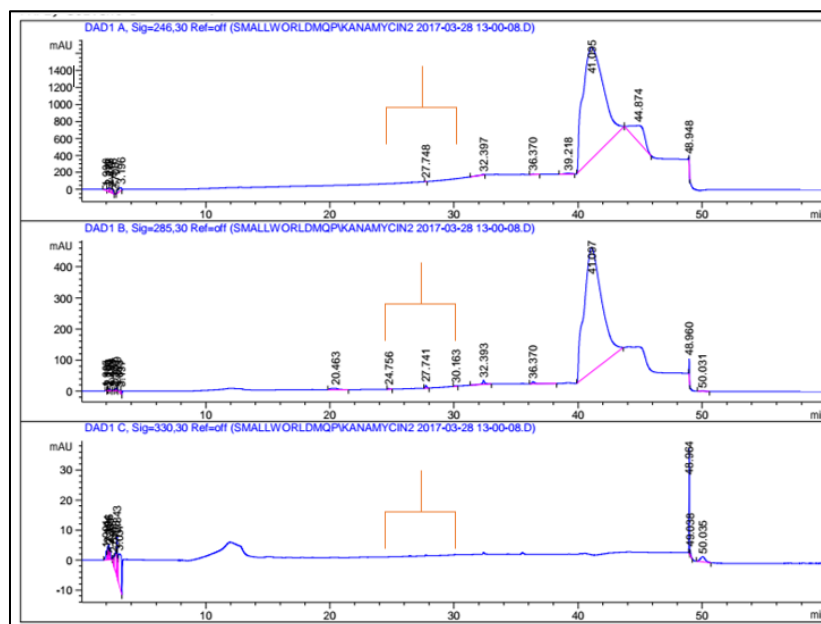
Figure 48: HPLC chromatogram of kanamycin

The HPLC chromatogram (*Figure 48*) of kanamycin is characterized by a small broad peak

from approximately 10-13 minutes on the 330 nm chromatogram and a large broad peak from 40-50 minutes.

Figure 49: HPLC chromatogram of gentamicin

The HPLC chromatogram (*Figure 49*) of gentamicin is characterized by two sharp peaks at 2 minutes and 3.5 minutes respectively as well as a large broad peak from 40-50 minutes.

Figure 50: HPLC chromatogram of ampicillin

The HPLC chromatogram (*Figure 50*) of ampicillin is characterized by several small peaks ranging from approximately 15-24 minutes and a large broad peak from approximately 40-50

minutes.

Figure 51: HPLC chromatogram of Amphotericin B

The HPLC chromatogram (*Figure 51*) of amphotericin B is characterized by a peak at 3 minutes and a dense cluster of peaks ranging from 10 minutes to 40 minutes. This standard like all the others tested shares the peak from 40-50 minutes. This can be assumed to be the solvent they are suspended in as this consistent with the methanol chromatogram in *Figure 41.*

4. Discussion

The rising threat of antibiotic resistant bacteria is a problem that science must address. Many new strains of what were once easily treated diseases have emerged and threaten the world with catastrophic epidemics. The current arsenal of effective antibiotics available to combat such pathogens has started to dwindle to stronger antibiotics that have negative consequences for the patient when used to treat the bacterial pathogen. However, groups such as the Small World Initiative have formed to help. Worcester Polytechnic Institute has begun their own contributions for the initiative in the form of the Microbes and Molecules BB2905 laboratory course. The goal of this project was to take the preexisting methods and identified producers from past BB2905 classes to create an optimized pipeline system for the identification of soil microbial isolates, selection of microbial isolates that produce antibacterial products, isolate said products, and characterize them. The hope of this pipeline was to allow students to identify new sources of antibiotics and new classes of antibacterial

products that could be used to combat antibiotic resistant bacteria.

The first step of the pipeline was to test which of the microbe samples from previous years of the BB2905 Lab course were still viable after cryopreservation and which of those produced antibacterial compounds. All samples were successfully grown on the LB media, shown in *Figure 13*, confirming that all samples were still viable and that the cryopreservation protocols of the BB2905 course worked as intended. All samples were then tested using a top agar assay against *E.coli* and *B.subtilis* bacteria. *E.coli* and *B.subtilis* were selected as they were well established lab cultures of gram negative (*E.coli*) and gram positive (*B.subtilis*) bacteria. Other bacteria types were used to determine which bacteria standards should be used, such as *S.epidermis, E.aerogenes, A.baylyi,* and *K.pneumonia*. However, the other bacteria were harder to grow and showed inconsistent results for basic testing. *E.coli* and *B.subtilis* strains, conversely, showed more consistent results, and were then used for the other experiments in this project. If the BB2905 microbes produced an antibiotic product effective against *E.coli* or *B.subtilis*, a visible zone of inhibition would have been observed. After they were incubated, numerous samples, shown in *Figure 14*, created zones of inhibition on the *E.coli* and *B.subtilis* top agar assay plates. With a variety of samples to choose from, samples that showed to have antibacterial activity against both *E.coli* and *B.subtilis* were selected for further testing.

The selected samples were then identified, as many groups in the BB2905 course did not have success sequence identifying their samples. This was mostly due to the inconsistent success of attempts to PCR amplify the 16s ribosomal subunit of microbial DNA. Most often, the PCR did yield sufficient amounts of DNA or long enough strands of useable for sequencing. The protocols for the 16s PCR were altered and tested to determine which kinds of changes yielded enough DNA to be sequenced without becoming too inaccurate. This was done through gradient PCR and using a new PCR mix (OneTaq, instead of Q5 High Fidelity Master Mix). The gradient PCR indicated that using the provided 16s primers (27F and 1492R) with an annealing temperature of 44°C, five degrees below its original annealing temperature, produced better results. The new protocol yielded more DNA product that was sufficient in length to be properly sequenced. Additionally, repeat tests of this protocol produced consistent sequencing results, indicating that the DNA was not too random to potentially misidentify a sample (a prominent concern due to the very low annealing

temperature). However, the resulting DNA sequences were only able to identify the genus of each sample, showing multiple species to have similar probability of being the sequenced sample. The most notable example of this was with samples identified to be in the *Bacillus* genus. These samples were highly similar and individual species could not be distinguished on the basis of the 16S ribosomal sequences alone.

PCR protocols and primers that targeted the gyrase gene were tested on the *Bacillus* samples as an attempt to identify them further. Unfortunately, most of the samples failed to produce any PCR product and those that did, when sequenced, produced sequences that were identified as being from either completely different genera of bacteria or from different genes all together. Due to the many potentially pathogenic species within the *Bacillus* genus that were among those identified by sequencing, samples identified as such were not selected for further testing. Further testing of the PCR protocol for the gyrase gene was not pursued due to time constraints. Just as the PCR protocol for the 16s subunit was optimized for use on soil bacteria, the PCR protocol for the gyrase gene should be tested and optimized by future projects. Additionally, alternate gene targets for PCR should be researched and tested to help increase the identification accuracy of collected soil microbes. The samples that were selected were 15-6 (identified to be in the *Streptomyces* genus) and 14-29 (identified to be in the *Brevibacillus* genus). The level of accuracy obtained from these tests was sufficient for the purposes of this project as no notable pathogenic strains were shown to be the likely identity of these samples.

Alternate non-PCR methods for identifying soil microbe samples were also tested. Focusing on the metabolism of the organisms, different growth media was used to determine if the organisms could metabolize different sugar sources. Other tests focused on the cellular structure of the microbes such as the presence of a cell wall. Using readily available media, the different tests did yield information regarding each sample. The problem with this data is that, in order to use it, it would need to be compared to previously compiled information on specific bacteria. This is a much more time consuming method and it can run into the same problem as the 16s target sequencing. Many organisms share similar metabolic and cellular processes and for any certainty, sequencing would be needed for any confirmation. As an educational tool, having students conduct these tests would be useful. That being said, the primary method for identifying the soil samples should be sequencing techniques.

Additionally, experimenting with which types of media the microbes grow on more efficiently should be conducted. This project only tested the 15-6 sample using multiple agar media (LB, R2A, PDA, THA, and TSA) due to limited resources and time. When tested, potato dextrose agar was shown to be a much more viable media to grow the sample on than the previously used LB agar.

The problem with growing soil microbes on agar is that optimum growing conditions have to be determined for each sample. Even then, the optimum conditions may take a considerable amount of time. The solid cultures for the 15-6 samples took at least five days to grow and produce significant levels of antibacterial product. The 14-29 sample took less time to grow, but did not produce as concentrated antibacterial product. When 15-6 was identified, the creation of liquid cultures in LB media was attempted. However, the cultures needed to be thoroughly aerated to grow significant amounts of cells and this was difficult with the available materials. Additionally, 15-6 was observed to clump together at the bottom of the tubes, given its filamentous cellular structure. These samples, just like the agar media cultures, required up to a week to grow sufficiently and had to be grown at room temperature. The challenge with growing soil microbes to produce desired antibacterial products is a balance between stress and growth. Certain media may provide the bacteria with the required nutrients to replicate to higher colony counts or concentrations, but might not stress them enough or give them the right conditions to produce their product. Growing the cultures on low nutrient media may stress the organism, but the media may lack the required materials for the bacteria to make its product. Co-culturing the bacteria with another established strain of bacteria (such as *E.coli*) may help produce the product. However the presence of the other bacteria may make extracting the antibacterial product harder as it may produce its own molecules that could be similar to or block the desired product. Further experimentation into growing protocols for more efficient antibacterial molecule production should be investigated. Such experiments could entail, testing selected samples on a wider variety of rich and low nutrient media as well as different temperatures. This data would allow for much more efficient sample growth protocols and should be thoroughly documented to aid future experimentation on selected soil bacterial samples.

The grown 15-6 and 14-29 LB cultures were then extracted using different extraction techniques to separate out any bacterial products from the cells and media. Two extraction

protocols were used: ethyl acetate extraction and methanol extraction. The ethyl acetate extraction technique yielded clean and positive results for the 15-6 sample. Additionally the bacterial products suspended in ethyl acetate were much easier to dry down for resuspension in set volumes, due to the volatile nature of ethyl acetate. The other extraction technique utilized methanol for its suspension solvent. It was primarily used for sample 14-29 because the sample was observed to only be successfully extracted using the methanol extraction protocol. However, the methanol extracts contained significant amounts of particulate. These samples had to be filtered using 0.2 micron filters before they could be analyzed using HPLC to prevent them from clogging up the column. The results of the extraction protocols showed that the success of the extraction depended on the target product. Ethyl acetate extraction yielded a cleaner product, but not all molecules were soluble in it. Methanol, comparatively, was able to extract out a wider variety of products than ethyl acetate. More products being soluble in methanol resulted in messier samples that required additional filtering and testing to ensure the antibacterial product was still present. Even though the two samples were successfully extracted using ethyl acetate and methanol techniques, there is no guarantee that all antibacterial products created by soil microbes will be. Because of this, other extraction techniques such as dichloromethane based extraction or solid phase extraction methods should be investigated. These new techniques could help extract different antibacterial samples and could potentially show what types of chemicals unknown antibacterial products are soluble in. This data could then be used to help analyze the chemical makeup, such as presence of chlorine or amine groups, of the unknown antibiotic compounds.

The extracts from samples 15-6 and 14-29 were then analyzed and purified using a C18 HPLC column. Using a solvent gradient of water with 0.1% formic acid and acetonitrile with 0.1% formic acid, the samples were separated into twelve equal factions over the course of one hour. Each of these fractions was then tested to determine at what time point the antibacterial product passed through the column. This information was then matched to the graphs created by the HPLC machine. The 15-6 sample was observed to elute through the column into fraction six, at the 25-30 minute time period, due to the clear zone of inhibition when tested against *B.subtilis* and *E.coli*. This was matched to the single peak recorded by the HPLC machine within the 25-30 minute time period. The 14-29 sample showed a more complicated result. When the fractions were tested using a zone of inhibition test, fractions

four, five, and six showed signs of containing antibacterial products. When the HPLC graph was analyzed, three peaks were observed within the three adjacent time periods collected in fractions four, five, and six. This indicated that the 14-29 sample may have produced three separate antibacterial products. In order to help analyze these HPLC results, known antibiotics (shown in *Figures 44-51*) were run through the HPLC column to create a standard for comparison. When compared, neither samples 15-6 or 14-29 matched up enough with the created standards to allow for a confident identification. The fact that neither sample matched with any standards could have been several factors. The first was that the antibiotics used for the creation of the standards were stocks taken from what was available in the laboratory. Normally, companies specifically make antibiotic standards for use in HPLC and these standards may run differently than the stocks used for these experiments. The next factor is that only nine antibiotics were used to create the standards. These nine did not cover every class and type of antibiotic available to modern medicine. More types of antibiotics, including standards specifically for the protocol used for the HPLC column, should be used to create standard HPLC graphs. These standards would allow for the potential identification of a wider variety of antibiotics. In future experiments when these standards are created, samples 14-29 and 15-9 should be compared again. If neither sample matches with any known antibiotic standard, it could indicate that the samples may be a new type of antibiotic.

The characterization of the antibacterial samples utilizing more chemical techniques was then investigated for the 15-6 sample. The 15-6 sample was selected due it only having a single fraction that showed strong antibacterial activity. The most efficient method of compound identification was a combination of nuclear magnetic resonance spectroscopy (NMR) and mass spectroscopy. The purified products of 15-6, unfortunately, were not tested using this method. The limiting factor that prevented the use of the combination of NMR and mass spectroscopy was that it required 25 mg of sample and only 0.04 mg of 15-6 was obtained. This was due to the lack efficient methods for the mass production of antibacterial product from the 15-6 sample. Liquid cultures of 15-6 took too long to create and were observed to not produce significant concentrations of antibacterial products compared to the solid media cultures. More optimal growing protocols for each soil microbe sample should be established to allow for larger quantities of antibacterial product to be isolated. Additionally, tests for more efficient growing conditions that produce more concentrated samples of

antibiotic product should be conducted. A wider variety of rich and minimal media cultures as well as differential and selective media should be used to grow the bacterial cultures. Once grown, any products should be extracted and analyzed to determine concentration. Other alternate techniques such as attempting co-cultures of antibiotic producing bacteria against *E.coli* or *B.subtilis* could also be analyzed. The bacterial culture used for the co-culture should be tested to determine if it makes any antibacterial products beforehand, to prevent any potential false positives.

Other potential identification methods should be investigated that allow for accurate identification of complex molecules that require smaller samples. Future experiments should consider investigating the use of tests such as UV and IR spectroscopy would be useful for gathering additional data on the properties of isolated antibiotic samples (especially if they are observed to produce fluorescence). Other tests such as paper chromatography could also be useful for analytical purposes. Paper chromatography is not as specific as HPLC, NMR, or mass spectroscopy when it comes to analyzing the chemical makeup of a compound. However, paper chromatography may be useful in an undergraduate laboratory course setting. The test is more simplistic to use and can be compared to standards created from well documented antibiotics. This kind of test should be used by BB2905 and the results documented to provide a jumping off point for further analysis.

Each step of the antibacterial identification pipeline was then summarized to key protocol changes for use by the BB2905 laboratory course. The later steps of the pipeline were noted to potentially be too time consuming and complicated for a seven week undergraduate laboratory class to thoroughly complete. Common antibiotics are known to be highly variable in terms of chemical structure and mechanistic class. An undergraduate biology laboratory course would most likely be able to reach the purification of antibacterial products into HPLC separated fractions. From here the creation of a connected chemistry or biochemistry based laboratory course that focuses on characterizing and identifying unknown antibacterial fractions from the biology class would be very advantageous. This course could perform numerous experiments to test the chemical properties of the isolated samples. Even if the second laboratory course is unsuccessful in fully identifying the unknown antibiotic, the data gathered would still be valuable for the groups such as the Small World Initiative.

The purpose of this project was the creation of an antibiotics identification pipeline.

Utilizing techniques and antibacterial compound producing bacteria samples from the BB2905 course, each step of the proposed pipeline was created and optimized. This pipeline was designed to identify soil bacteria, detect if the identified bacteria produced any antibacterial compounds, extract these antibacterial compounds, purify the extracts, and characterize them. The protocols improved in this process allowed for the identification of bacterial samples 15-6 and 14-29 to be in the *Streptomyces* and *Brevibacillus* genuses respectively. This also allowed in the optimization of the 16s subunit PCR protocols, enabling a higher sequencing success rate for future laboratory courses. 15-6 and 14-29 were also confirmed to produce antibacterial compounds effective against *E.coli* and *B.subtilis* cultures. The created pipeline was also able to extract 15-6 products using acetyl acetate extraction and 14-29 products using methanol extraction. These samples were then successfully purified using an HPLC column to create isolated fractions of antibacterial products. Unfortunately, the characterization of the purified fractions was not accomplished. NMR and mass spectroscopy were to be used to characterize the fractions, but not enough product was available for such testing. Future project groups and BB2905 courses should expand upon the techniques tested by this project. A crucial component that should be investigated further is the methods for producing larger quantities of antibacterial product from selected soil microbes. Alternate characterization tests should also be investigated, such as UV spectroscopy, to try to characterize purified samples while requiring less material to work with. Other more basic chemical tests such as pH, polarity, and the separation of potential products using paper chromatography should also be investigated. These tests might not be as data rich as NMR or mass spectroscopy, but could be more practically implemented into undergraduate biology, biochemistry, and chemistry laboratory courses. Additional input from biochemistry and chemistry departments should be obtained to potentially create a new course to tackle the challenge of identifying the many purified antibiotic samples that will be created utilizing the pipeline created by this project.

References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. J Mol Biol 215(3):403-10.

American Chemical Society (ACS) and the Royal Society of Chemistry (RSC). 1999. The discovery and development of penicillin [Internet]. [cited Oct. 6, 2016]. Available from: https://www.acs.org/content/dam/acsorg/education/whatischemistry/landmarks/flemingpenicillin/the-discovery-and-development-of-penicillin-commemorative-booklet.pdf

Buckholt MA. 2014. Amylase Test. BB 290X: Microbes to Molecules Protocols.

Center for Disease Control and Prevention. 2016. Antibiotic/Antimicrobial Resistance [Internet]. [cited Oct. 7, 2016]. Available from: https://www.cdc.gov/drugresistance/

Chopra, I., Roberts, M. 2001. Tetracycline Antibiotics: Mode of Action, Applications, Molecular Biology, and Epidemiology of Bacterial Resistance. *Microbiology and Molecular Biology Reviews: American Society for Microbiology*. 65(2):232-260

Citron, K., Emerson, P.A., Joyce., R.B., *et al.*1968. Comparison of Side-Effects of Tetracycline and Tetracycline Plus Nystatin. *British Medical Journal*. 4(5628): 411-415.

Clardy J, Fischbach MA, Currie CR. 2009. The natural history of antibiotics. *Current Biology* 19(11):R437-41.

Cunha, B.A. 2001. Antibiotic Side Effects. *Medical Clinics of North America*. 85(1):149-85

DeLong EF and Pace NR. 2001. Environmental diversity of bacteria and archaea. *Systematic*

Biology. 50(4):470-8.

Eisenstein BI, Oleson FB,Jr, Baltz RH. 2010. Daptomycin: From the mountain to the clinic, with essential help from francis tally, MD. *Clin Infect Dis* 50 Suppl 1:S10-5.

Fierer N, Breitbart M, Nulton J, Salamon P, Lozupone C, Jones R, Robeson M, Edwards RA, Felts B, Rayhawk S, *et al.* 2007. Metagenomic and small-subunit rRNA analyses reveal the genetic diversity of bacteria, archaea, fungi, and viruses in soil. *Applied and Environmental Microbiology.* 73(21):7059-66.

Finch, R.G., Eliopoulos, G.M. 2005. Safety and efficacy of glycopeptide antibiotics. *Journal of Antimicrobial Chemotherapy.* 55, Suppl. S2:ii5-ii13

Floss HG and Yu T. 2005. Rifamycin mode of action, resistance, and biosynthesis. *Chem Rev* 105(2):621-32.

Fumo, G., Akin, C., Metcalfe, D.D., Neckers, L. 2003. 17-Allylamino-17-demethoxygeldanamycin (17-AAG) is effective in down-regulating mutated, constitutively activated KIT protein in human mast cells. *Blood.* 103(3):1078-84

Handelsman J, Rondon MR, Brady SF, Clardy J, Goodman RM. 1998. Molecular biological access to the chemistry of unknown soil microbes: A new frontier for natural products. *Chemistry & Biology*. 5(10):R245-9.

Hermann T. 2007. Aminoglycoside antibiotics: Old drugs and new therapeutic approaches. *Cellular and Molecular Life Sciences*. 64(14):1841-52.

Hernandez S, Tsang T, Handelsman J. 2015. Small World Initiative: Research Protocols.

Hof, H. 1994. Macrolides, a group of antibiotics with broad spectrum of activity. *Immunität und Infektion*. 22(2):66-71

Holten, K.B., Onusko, E.M.2000. Appropriate Prescribing of Oral Beta-Lactams Antibiotics. *American Family Physician*. 1;62(3):611-20

Ling LL, Schneider T, Peoples AJ, Spoering AL, Engels I, Conlon BP, Mueller A, Schäberle TF, Hughes DE, Epstein S. 2015. A new antibiotic kills pathogens without detectable resistance. *Nature*. 517(7535):455-9.

Macrolides. 2016. [cited Oct. 6, 2016]. Available from: http://dhh.louisiana.gov/assets/oph/Center-PHCH/Center-CH/infectious-epi/VetInfo/VetAntibioResSen/LADDL/AntimicrobialClasses/macrolides/Macrolides.pdf

Malabarba A, Nicas TI, Thompson RC. 1997. Structural modifications of glycopeptide antibiotics. *Med Res Rev*. 17(1):69-137.

Mingeot-Leclercq MP, Glupczynski Y, Tulkens PM. 1999. Aminoglycosides: activity and resistance. *Antimicrobial Agents and Chemotherapy*. 43(4):727-37.

National Center for Biotechnology Information. PubChem Compound Database [Internet]; Penicillin G CID=5904, [cited Oct. 6, 2016]. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/5904

National Center for Biotechnology Information. PubChem Compound Database [Internet]; Streptomycin CID=19649, [cited Oct. 6, 2016]. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/19649

National Center for Biotechnology Information. PubChem Compound Database [Internet]; Vancomycin CID=14969, [cited Oct. 6, 2016]. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/14969

National Center for Biotechnology Information. PubChem Compound Database [Internet]; Erythromycin CID=12560, [cited Oct. 6, 2016]. Available from:

https://pubchem.ncbi.nlm.nih.gov/compound/12560

National Center for Biotechnology Information. PubChem Compound Database [Internet];
Daptomycin CID=16129629, [cited Oct. 6, 2016]. Available from:
https://pubchem.ncbi.nlm.nih.gov/compound/16129629

National Center for Biotechnology Information. PubChem Compound Database [Internet];
Polymyxin B CID=49800004, [cited Oct. 6, 2016]. Available from:
https://pubchem.ncbi.nlm.nih.gov/compound/49800004

National Center for Biotechnology Information. PubChem Compound Database [Internet];
Chloramphenicol CID=5959, [cited Oct. 6, 2016]. Available from:
https://pubchem.ncbi.nlm.nih.gov/compound/5959

National Center for Biotechnology Information. PubChem Compound Database [Internet];
Tetracycline CID=54675776, [cited Oct. 6, 2016]. Available from:
https://pubchem.ncbi.nlm.nih.gov/compound/54675776

National Center for Biotechnology Information. PubChem Compound Database [Internet];
Teixobactin CID=86341926, [cited Dec. 12, 2016]. Available from:
https://pubchem.ncbi.nlm.nih.gov/compound/86341926

Pankey, G.A., Sabath, L.D. 2004. Clinical Relevance of Bacteriostatic versus Bactericidal
Mechanisms of Action in the Treatment of Gram-Positive Bacterial Infections. Oxford
Journals. 38(6):864-870

Power DA and Johnson JA. 2009. Difco™ & BBL™ manual.

Prayle, A., Watson, A., Fortnum, H., Smyth, A. 2010. Side effects of aminoglycosides on the
kidney, ear and balance in cystic fibrosis. *Thorax.*, 65(7):654-58

Small World Initiative. 2015. Our Approach [Internet]. [cited Oct. 6, 2016]. Available from:

http://www.smallworldinitiative.org/about/

Spellberg B and Gilbert DN. 2014. The future of antibiotics and resistance: A tribute to a career of leadership by John Bartlett. *Clinical Infectious Diseases.* 59(suppl 2):S71-5.

Steenbergen, J.N., Alder, J., Thorne, G.M., Tally, F.P. 2005. Daptomycin: a lipopeptide antibiotic for the treatment of serious Gram-positive infections. *Journal of Antimicrobial Chemotherapy*. 55(3):238-288

Tomasz A. 1979. The mechanism of the irreversible antimicrobial effects of penicillins: How the beta-lactam antibiotics kill and lyse bacteria. *Annual Reviews in Microbiology*. 33(1):113-37.

U.S. National Library of Medicine. 2016. Chloramphenicol Injection. Retrieved Dec 2, 2016 from: https://medlineplus.gov/druginfo/meds/a608008.html

U.S. National Library of Medicine. 2016.Rifampin. Retrieved Dec 2, 2016 from: https://medlineplus.gov/druginfo/meds/a682403.html

Wehrli W. 1977. Ansamycins chemistry, biosynthesis and biological activity. In: Medicinal chemistry. Springer. 21 p.

Wobbe CR. 2015. Methods for purifying and analyzing antibacterial compounds from soil microbes in an undergraduate laboratory course. Department of chemistry and biochemistry, Worcester Polytechnic Institute.

Yamamoto, S., Harayama S. 1995. PCR amplification and direct sequencing of gyrB genes with universal primers and their application to the detection of taxonomic analysis of Pseudomonas putida strains. *Applied and Environmental Microbiology*, 61(3):1104-1109

Yim G, Wang HH, Davies J. 2007. Antibiotics as signalling molecules. *Philosophical*

Transactions of the Royal Society of London Series B Biological Sciences. 362(1483):1195-200.

Yu Z, Qin W, Lin J, Fang S, Qiu J. 2015. Antibacterial mechanisms of polymyxin and bacterial resistance. *BioMed Research International* 2015.

Appendix I: Microbes to Molecules Freezer Stock Isolates

Year	ID number	Student initials	Writing on tube
2014	14-1	DA	DA & RH
	14-2	ARB	Baker
	14-3	HMB	col.1/col.3 HB 5/5/14
	14-4	CAB	CB Col 1/2
	14-5	AHB	AB IS 1/2
	14-6	PDB	SW6 Iso9 ; Iso20; A,B,C
	14-8	REC	N/A
	14-10	KMD	KMD 5/5/14
	14-11	RMG	Rebecca German #18 SW11
	14-12	RSG	Rhiannon Goddard SW12
	14-13	CHH	CH 23, CH 8
	14-14	WJH	WH RM SW14 SW23
	14-15	RDH	N/A
	14-16	JLH	EP JH SW26 SW16
	14-17	AK	AK SA
	14-18	CBK	N/A
	14-19	GK	G.K. 11 SW19A; G.K. 24 SW19B

	14-20	SNL	SL1; SL2
	14-21	CLL	SW21A 5/5/14 CL, SW21B 5/5/14
	14-22	KEL	KL CT 5/5/14
	14-23	RAM	WH RM SW14 SW23
	14-24	DMM	DM
	14-25	BNS	N/A
	14-26	EJP	EP JH SW26 SW16
	14-29	ER	SW29A-D CR+ER
2015	15-1	LG	N/A
	15-2	LG	N/A
	15-3	JD/GS	N/A
	15-4	JD/GS	N/A
	15-5	KK	N/A
	15-6	KK	N/A
	15-7	VS	N/A
	15-8	VS	N/A
	15-11	EB	N/A
	15-14	KK	N/A
	15-18	BH	N/A
	15-19	BH	N/A

	15-20	BH	N/A
	15-21	BH	N/A
	15-24	SE	N/A
	15-25	SE	N/A
	15-26	KC	N/A
	15-27	KC	N/A
	15-28	CB/EP	N/A
	15-32	SW/CM	N/A
	15-36	AP	N/A
	15-37	AP	N/A
	15-38	LG	N/A
	15-39	LG	N/A
	15-43	SL	N/A
	15-44	SL	N/A
	15-56	NF	N/A
2016	16-1	NB	N/A
	16-2	JB	N/A
	16-3	JB	N/A
	16-4	MH	N/A
	16-5	AM	N/A

	16-6	NS	N/A
	16-7	DC	N/A
	16-8	DC	N/A
	16-10	MH	N/A
	16-11	TN	N/A
	16-12	HM	N/A
	16-13	LM	N/A
	16-14	LM	N/A
	16-15	AW	N/A
	16-16	HN	N/A
	16-17	AH-M	N/A
	16-18	AH-M	N/A
	16-19	HM	N/A
	16-20	-	N/A
	16-21	AS	N/A
	16-22	AS	N/A
	16-23	HN	N/A

Appendix II: Indole Assay Procedure

Goal:
To determine if tryptophanase is present in the bacterial isolate culture to give insight into the metabolism of the isolate.

Materials:
- Test tubes
- Tryptone broth
- Bacterial isolates
- Kovac's reagent

Methods:

The culture was grown in tubes containing tryptone broth (a broth high in tryptophan) at room temperature for four days. After the incubation period a drop of Kovac's reagent was added to the culture tube and the tube was observed for a color change. A negative test will result in a orange ring at the top of the liquid and a positive test will result in a purple ring at the top of the liquid.