The Hunt for TB Antibiotics from the Soil

A Major Qualifying Project
submitted to the Faculty of
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degree of Bachelor of Science in Biology and Biotechnology

By

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Abstract
In recent years, antibiotic resistance in pathogens has forced scientists to find and develop new treatments to tackle the latest iterations of familiar diseases. Tuberculosis (TB) is known to be notoriously difficult pathogen to rid from of the patient. In a bid to find alternative ways to produce new antibiotics, some biologists have turned to the soil to find microbes that could produce molecules that could be developed into new antibiotic drug treatments. The goal of this project was to test microbes isolated from the soil for antibiotic activity against *Mycobacterium smegmatis*, a non-pathogenic relative of the bacterium that causes tuberculosis. Soil isolates that produced zones of inhibition were identified by 16S rRNA sequencing. Acetone and ethyl acetate extracts were made from these isolates, and several extracts inhibited the growth of *M. smegmatis*. HPLC was used to compare the chemical profiles of several extracts. In total, 6 of the 21 tested isolates were found to have antibiotic activity against *M. smegmatis*.

Introduction

One third of the world's population currently has a form of tuberculosis (TB), either in latent or active form (Kim, 2017). Even more concerning, strains of the causative agent *Mycobacterium tuberculosis* are becoming resistant to multiple antibiotics, preventing patients from being cured and increasing deaths due to TB (CDC, 2016). Scientists around the world are searching for more antibiotics to combat the rising number of antibiotic resistant diseases like TB, and they are now turning to the soil for more solutions.

The discovery of antibiotics was vital in preventing various bacteria from killing millions of people, but what does an antibiotic do to stop bacterial disease? There are a number of processes that antibiotics target to kill bacteria or inhibit bacterial growth, including inhibition of DNA replication (e.g., quinolones), RNA synthesis (e.g. rifamycin), cell wall synthesis, and protein synthesis (Clardy, 2009). An antibiotic can either kill a wide range of bacterial species (broad spectrum) or a narrow range (narrow spectrum); they can be administered orally or via injection; and they can either kill the bacterium outright or inhibit growth. In nature, most antibiotics are found in the soil. Actinomycetes, a bacterial family with 80 known genera across all soil types, are the primary source of antibiotics and secondary metabolites (Clardy, 2009; Chaudhary, 2013). In fact, more than 70% of antibiotics can trace their lineage back to this family (Bizuye, 2013). With the many types of antibiotics, scientists have been able to synthesize these secondary metabolites, meaning that some of the natural compounds in the antibiotics can now be synthesized chemically, and that the search doesn't have to be restricted to the soil (Bhattacharjee, 2016).

However, almost as quickly as antibiotics were put into production in the 1940s, many bacteria including *M. tuberculosis* adapted and became antibiotic resistant. Reasons for the current TB antibiotic resistance crisis include misuse of the drugs, issues with drug availability in certain areas in the world, and a rise in HIV cases (Smith, 2014; CDC, 2017; Schmidt, 2008). Tuberculosis patients are required to take a minimum of 6 months of antibiotics, usually a combination of 4 drugs under medical supervision, with quarantine not always required as the TB becomes non-contagious within a few days of initial treatment (WHO Media Center, 2017). Due to the intensive treatment, some people will sometimes forget to take their antibiotics or will stop taking them when they feel better. Discontinued use of antibiotics provides the opportunity for surviving bacteria to grow and acquire mutations that confer resistance (WHO Media Center, 2017). In cases where dormant bacteria survive and the patient relapses, they can usually be cured with another round of treatment, but if they acquire mutations that confer resistance, the disease will relapse and the patient will not be cured by another course of treatment with those same drugs because the bacteria are now resistant to them. In that case second-line drugs (a completely different and more toxic drug regime with a longer course of administration) are required (Conolly, 2007). Areas with limited access to medical treatment and overcrowding that leads to the faster spread of TB are where some of the multidrug resistant strains of *M. tuberculosis* are persisting. Not only is access to drugs important to curing the disease, but having the lab infrastructure to diagnose TB quickly is equally as important to treating the patients and preventing spread of the disease. According to WHO, the regions of the world with rising cases of drug-sensitive TB as well as multidrug resistant TB include Africa, the Middle East, and Southeastern Asia (WHO, 2017). Also, a person is more susceptible to getting other disease when they have an immunosuppressant disease such as HIV/AIDS, which highly prevalent in Africa and Southeastern Asia (Schmidt, 2008). As for the Middle East, lack of

medical treatment and poor environmental conditions due to war have led to the increased number of multidrug resistant TB cases (WHO, 2017).

Antibiotic resistance in TB is not only caused by human factors, but also by adaptations in the bacterium itself. *M. tuberculosis* is intrinsically resistant to many antibiotics due to a number of mechanisms. One mechanism includes the multi-layer cell wall, which is difficult for most antibiotics to penetrate (Smith, 2014). *M. tuberculosis* can also enter non-growing states in which it is temporarily insensitive to antibiotics, because most antibiotic targets are proteins required for replication (Conolly, 2007). Genetic antibiotic resistance arises frequently, making multidrug 6-month long treatments necessary (Connolly, 2007). *M. tuberculosis* is a difficult bacterium to treat, but modern methods of finding new antibiotics are working to ease overall treatment of the disease.

One recent example of a newly discovered antibiotic with potential for clinical use against TB is Teixobactin, found in 2016. Teixobactin is synthesized by a newly discovered species of beta proteobacteria and has been found to kill bacteria that cause pneumonia, MRSA, and tuberculosis (Piddock, 2015). The antibiotic was discovered through isolation from soil via a tool called iChip, which allowed the bacterium to grow and be isolated from the produced product. The iChip is a type of assembly made up of plastic plates and membranes to filter out and concentrate microorganisms found in the soil (Piddock, 2015). The big limitations of this tool are that it is unsuccessful in identifying gram-negative bacteria and that this device can only find small quantities of the microorganisms (Piddock, 2015). With those limitations considered, iChip shows promise as a new method to use in identifying new isolates to treat tuberculosis and other bacterial diseases.

The discovery of oxazolidinones has allowed for the synthesis of additional new antibiotics. Scientists from EI DuPont de Nemours & Co. Inc synthesized an initial variant of an oxazolidinone antibiotic that unfortunately was modified to the point where it was too toxic for human use (Bozdogan, 2004). Nine years later in 1996, Upjohn Laboratories (now known as Pfizer), were able synthesize less toxic variants of oxazolidinones that led to the creation of the drugs linezolid and eperezolid, with linezolid currently an option for use in the treatment of tuberculosis (Bozdogan, 2004). Linezolid was shown, in combination with other drugs and under close supervision for adverse reactions, to cause a negative sputum culture in 79% of patients with multidrug-resistant TB within 4 months (Lee, 2015). Modifying known antibiotics is therefore a viable path to improved drugs for TB (Coates, 2007). There is also promise of new antibiotics for tuberculosis and other bacterial diseases from the soil. In the Badia region of Jordan, 4 *Streptomyces* isolates were able to cause inhibition against *Staphylococcus aureus* , *Escherichia coli* and *Klebsiella pneumoniae* (Saadoun, 2003). The antibiotic activity of the 4 isolates was attributed by the arid environment and the lack of nutrients in the soil causing the *Streptomyces* samples to be a threat to antibiotic-resistant bacteria (Saadoun, 2003). Antibiotics can also be found in microbes growing on plants. An endophyte of *Grevillea pteridifolia*, *Streptomyces* sp. NRRL 30566, was collected in Australia from the stems of the snakevine plant (Castillo, 2003). The organism was then isolated from the plant and put through a series of analytical assays (Castillo, 2003). The antibiotic produced by the streptomycete was kakadumycin A, a broad spectrum antibiotic that targets gram-positive bacteria such as *Bacillus anthracis* (Castillo, 2003). Antibiotics can come from many sources, which is vital for resolving the TB antibiotic resistance crisis.

Other new TB antibiotics include bedaquiline, delamanid, pretomanid, and lassomycin. Bedaquiline fumarate, known as Sirturo™, is an FDA-approved drug used to treat multidrug-

resistant (MDR) TB (CDC, 2016). Bedaquiline has a shelf life of 4-5 months, is only recommended to be used for 24 weeks, and must be in conjunction with at least 3 other TB drugs for maximal efficacy (CDC, 2016). Delamanid is also an FDA-approved drug, is used in conjunction with several other TB antibiotics to treat MDR TB, and acts by inhibiting the synthesis of methoxy mycolic acid and ketomycolic acid, TB cell wall components (Matsumoto et al., 2006). Pretomanid is a potential TB antibiotic and while little is known about the mechanism of action, it has been shown to be a safe and effective TB drug in combination with moxifloxacin and pyrazinamide (Dawson, 2015). Lassomycin is new potential TB antibiotic not in clinical use yet, but the mechanism of action is the uncoupling of ATPase from proteolytic activity (Gavrish, 2014).

The goal of our project is to find another anti-mycobacterial antibiotic from microbes found in the soil. We used *M. smegmatis* for our project in place of *M. tuberculosis* because it is safer and grows more rapidly. We attempted to complete this goal through testing the inhibitory activities of banked microbes from the WPI course Microbes to Molecules, in which students take soil samples and culture isolates that have the potential for producing antibiotic products. From the initial 21 isolates grown, 10 of them had zones of inhibition against *M. smegmatis*. Of those 10 isolates, 9 were identified and 6 had secondary metabolites that could create potential antibiotics against TB after organic extraction and filtration.

Methods and Materials

Soil-derived Microbial Isolates

The list of isolates tested are as follows: 2014-01, 2014-14, 2014-17, 2014-26, 2015-28, 2015-56, 2016-16, 2017-37, 2017-38, 2017-50, SS-X_0020, SS-X_0036, SS-X_0037, SS-X_0038, and SS-X_0039. These isolates were obtained from various soil samples that were purified during the Microbes to Molecules class from 2014-2017 and stored in frozen glycerol stocks. The isolates were labeled by year they were collected from the soil, then by sample number from the class. The intention of the course was to collect microbes from the soil around Worcester and determine their potential use as an antibiotic. All 12 isolates were streaked on LB plates from frozen stocks. Isolates SS-X_0037 and SS-X_0039 were also plated on TSA plates to see if further growth occurred since the samples had difficulty growing on LB plates. Plates were incubated for three days in a 37°C. From there, master plates were created using a colony from each plate and the other plates were placed in the 4°C refrigerator for further use. The master plates were plates that were used to grow a singular isolate and take samples from for further experimentation. They were made from the frozen stocks or from another colony form earlier master plates, and were made at least 3 times in order to keep the microbes fresh.

M. smegmatis Inhibition Assay

Testing the isolates involved the use of the *M. smegmatis*, colony samples from the master plates, and LB plates. The *M. smegmatis* was dispensed (200 µL at an OD of 0.8 and wavelength of 600nm) on each LB plate and dried. From there, each microbe colony was put on the plate using a flame-sterilized metal loop and the pick and patch method to spread the microbes. The Pick and Patch method is where multiple isolates can be tested and grown on the same plate (Sanders, 2012). A patch grid is drawn on a petri dish and a colony is picked and streaked in one of the patches, not the whole plates (Figure 1; Sanders, 2012).

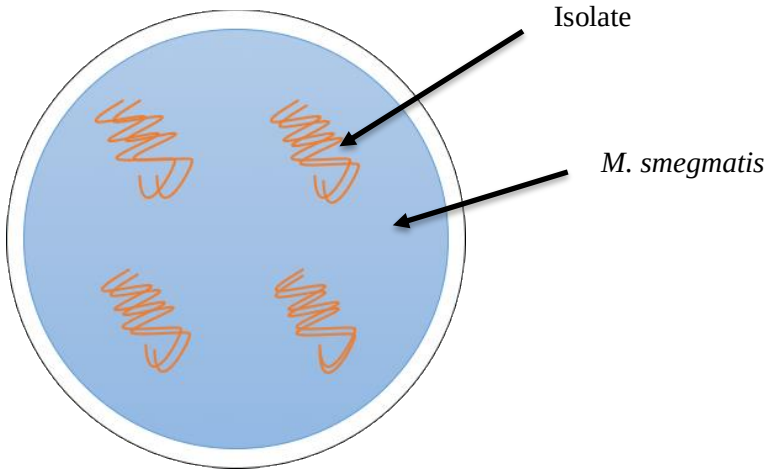


Figure 1: Pick and Patch Method used for the Inhibition Assay

Each microbe was tested against *M. smegmatis* three times. The criteria for a positive result is a zone of inhibition, or a ring around the microbe in which *M. smegmatis* growth was not observed.

Mycobacterium smegmatis Liquid Culture
Frozen stocks of *M. smegmatis* strain mc^2155 were provided by our advisor, Professor Scarlet Shell. M. smegmatis was grown in Middlebrook 7H9 broth supplemented with glycerol (final concentration 0.2%), dextrose (final concentration 2 g/L), Tween 80 (final concentration 0.05%), bovine serum albumin fraction V (final concentration 5 g/L), and sodium chloride (850 g/L). OD was then measured in a spectrophotometer at 600 nm. We diluted the culture to an OD between 0.83-0.9 and stored aliquots at -80°C for subsequent use in the inhibition and activity assays.

PCR & Gel electrophoresis to produce amplicons for 16S rRNA sequencing

To amplify the gene encoding the 16S rRNA for sequencing, a colony was sterilely put into a microtube with 100 µL sterile water. The tubes were placed in a Thermal Cycler and were incubated for 10 minutes at 95°C. PCR mixes contained 25 µL of 2X OneTaq (New England BioLabs), 2 µL of 5 µM 27F Primer (AGAGTTTGATCMTGGCTCAG), 2 µL of 5 µM 1492R Primer (GGTTACCTTGTTACGACTT), and 21 µL of the boiled colony liquid in a total reaction volume of 50 µL. The alternative primers that were used were 8F (AGAGTTTGATCCTGGCTCAG) and 1391R (GACGGGCGGTGTGTRCA). The PCR reaction is first set to warm up to 95°C for two minutes. Then a cycle of 95°C for 30 seconds, 49°C for 45 seconds and 72°C for two minutes is set to run for 30 continuous times. After the cycles are done, the PCR conditions is set to 72°C for 10 minutes and then ends. Following that, the PCR products were analyzed by gel electrophoresis in TAE buffer using 1% agarose gels. Each individual gel had 0.5 grams of agarose powder in 50 mL of 1X TAE buffer and 20 µL of 1000X (0.02M) ethidium bromide added in its aqueous state before being poured. Unless otherwise specified, 10 µL each of HyperLadder (Bioline) and the PCR reactions were run at 100 volts for 45 minutes. Once the gel was run, it was removed and taken to the gel image machine to view and take pictures of the results.

16S rRNA sequencing analysis

The PCR products were cleaned up by the company Eton Bioscience, a biotech company that sequences DNA. The PCR products, which were at least 1 kb long, were sequenced from both ends by primers 27F, 8F, 1391R, and 1492R, all of which were sent to Eton. After the isolates were sequenced, we took the sequences and used the program 4Peaks, a DNA sequence trace viewer application, to observe our confidence in the sequence being correct. The criteria we used for a high-confidence sequence was that each nucleotide needed to have a clear, singular peak; if there were multiple peaks that were small, then the sequence would be low confidence. Figure 5 is an excellent example of high confidence data, as the peaks for each nucleotide were singular and larger. The low-confidence regions were then deleted from the ends of sequence. From there, the edited, high-confidence sequences were then put in BLAST, or the Basic Local Alignment Tool from the National Center for Biotechnology Information. When the sequences were entered into the database, the top identities were listed based on the percentage of how identical the

sequences were to each other. The identity for an isolate was determined based on the highest percentage identity to the sequence (needed to be at least 98% for a likely identification), but for the isolates that had multiple identities at the same percentage, the correct identity could not be determined, as there were no further analytic tests performed to differentiate. For example, isolate 2014-01 had both *Enterobacter aerogenes* and *Klebsiella aerogenes* 99% identical, and since both bacteria are very similar, the isolate could not be identified as one over the other.

Organic Extraction

Isolates to be extracted were spread on the surfaces of LB plates to grow thick lawns. After three days, the agar and lawn on each plate was cut into pieces and moved into 100 mL bottles, which were then placed in the -20℃ freezer for three days. Next, 12 mL of methanol was added to each bottle and each bottle was placed on a rotary shaker at 25℃ and 220 rpm overnight. For the ethyl acetate, 4 mL of DI water with 6 mL of ethyl acetate was added to a bottle with half a plate of the grown isolates. For the acetone, 6 mL of acetone was added to a different bottle with half a plate of the grown isolates. The next day, the bottles were then removed from the shaker and the methanol-extract liquid was removed from the mixture using a Pasteur pipet, then in their own vials. The vials were then placed uncapped in a fume hood until all visible liquid was evaporated. The extracts took a minimum of 2 weeks to evaporate with the methanol, but only 1 day with the acetone, and two days with the ethyl acetate. Following the evaporation, the extracts were then re-suspended in 2-2.5 mL of methanol. We then used a 3 mL syringe and a syringe filter (0.2 μm pores) to filter-sterilize the re-suspended extracts.

Organic Extraction Activity Assay
We set up an assay for the extracts in order to test for antibiotic activity and all of the isolate extracts were tested alongside a negative control of methanol. 200 μL of freshly thawed *M. smegmatis* freezer stock (OD 0.8) was plated on each of several LB plates and spread using a glass hockey stick. Filter discs that had absorbed either 30 μL or 60 μL of the extracts were placed on those plates after the bacteria dried. The filter discs for this assay were made using a hole puncher and large sheets of filter paper. The discs are the 1 mm in diameter and were placed in a glass petri dish and autoclaved for 20 minutes on a dry cycle. The plates were incubated at 37ºC for three days and all isolate extracts and controls were tested in duplicate. The positive results had a zone of inhibition around the filter disc indicating that *M. smegmatis* growth was inhibited. The negative controls for this assay were the filter disc without any liquid and a methanol soaked filter disc, which test the sterility of both the filter discs and the methanol used.

HPLC
The HPLC was used to observe the chemical components of the extracts from the isolates that showed zones of inhibition in the extract activity assay. The HPLC was made by Agilent Technologies, the software used to run the HPLC was OpenLab and a 0.5 μM C18 reverse phase silica column was used to seperate the samples. . The program was set to run on the C18 column in the HPLC. The instrument was turned on and allowed to run for 20 minutes prior to the start of running the sample. For each sample used, 500 μL was placed in each vial to prevent air bubbles being run through the column. Each sample ran for 58 minutes at a flow rate of 1 mL/min, and 100 μL of each isolate was loaded into the column. The gradient of the HPLC started with 90% of 0.1% Formic Acid in distilled water and 10% of 0.1% Formic Acid in

Acetonitrile and ended with 90% of 0.1% Formic Acid in acetonitrile and 10% of 0.1% Formic Acid in distilled water in a continuous gradient (Table 1). This was set up to move the samples from a polar to a non-polar gradient to separate the molecules. The graphs of the peaks were collected and compared to the control sample and to other samples for similarities and differences.

Table 1: Gradient of the HPLC that was used in the experiment, going from polar to non-polar.

Time (min)	% In Water	% In Acetyl Nitrile
0	90	10
4.25	90	10
29.25	80	20
37.55	80	20
37.56	50	50
45.85	50	50
45.86	20	80
54.15	20	80
54.16	10	90
58.15	10	90

Results

In this project, isolates collected from various soil samples were plated on top of *M. smegmatis* to see if there are any zones of inhibition. If yes, the colonies of the isolates went through the PCR and went through gel electrophoresis. If the isolates showed on the gel, they were then sent for sequencing and subsequently identified through BLAST. Then the isolates went through an organic extract and activity assay. Finally, the molecules in the isolates were analyzed using HPLC.

Inhibition and Morphology Assay

The goal of this assay is to screen for soil isolates capable of inhibiting the growth of *M. smegmatis* from a panel of soil isolates that were collected and banked in the WPI course Microbes to Molecules. The isolates were plated on LB agar plates to determine if they could grow in that environment. The selected microbes were thawed and plated in a streaking fashion and were placed in an incubator at 37℃ and were documented over the course of three days. Ten isolates were able to grow on LB plates: 2014-01, 2014-14, 2014-17, 2014-26, 2015-28, 2015-56, 2016-16, SSX-20, SSX-36, and SSX-38 (See Appendix A). The isolates exhibited a variety of morphologies, colors, and colony sizes. Isolates SSX-37 and SSX-39 had very little to no growth on LB plates. We tried TSA plates for both of the isolates but did not observe growth after 3 days at 37℃.

Figure 2 demonstrates the varied growth of the isolates, and morphology is described in Table 2. The best example for the morphology can be seen in part A from Figure 2, as it shows white, spotty, and wispy growth. The rest of the isolate morphologies can be found in Appendix B.

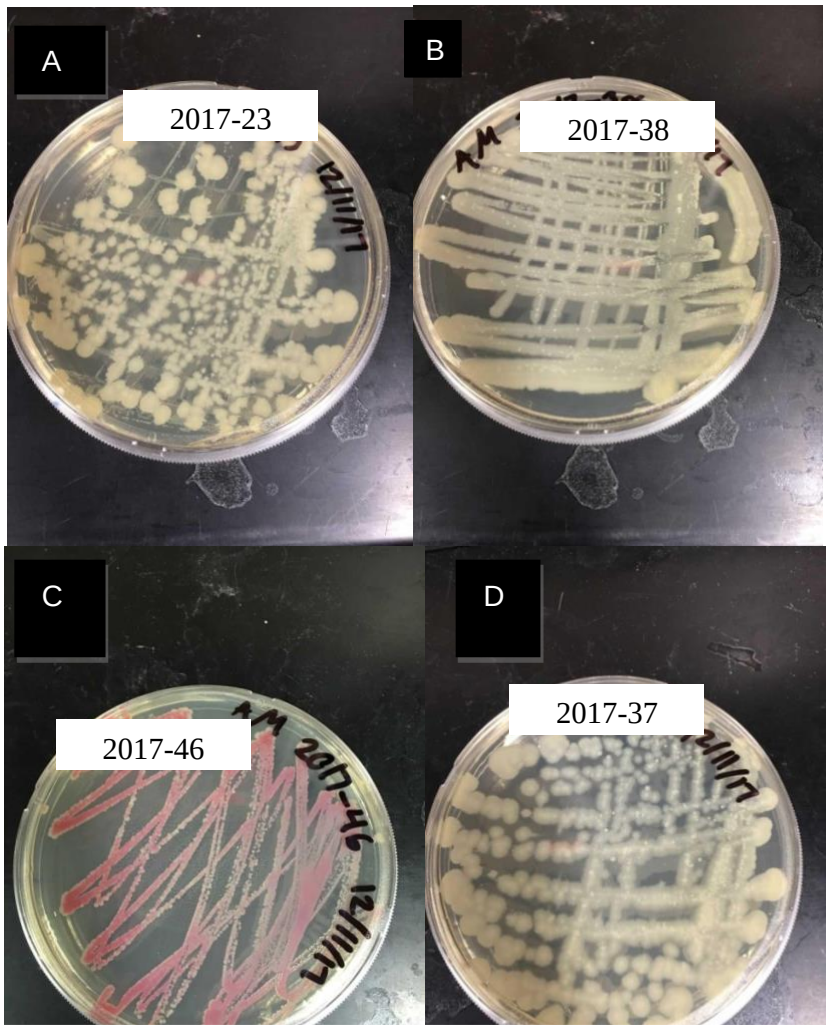


Figure 2: Growth of Isolates on LB plates. All observations were made after 3 days in a 37ºC incubator.

To test the isolates for ability to inhibit mycobacterial growth, each isolate was patched onto an LB agar plate freshly spread with liquid *M. smegmatis* culture. Results were observed after 3 days at 37℃ (Figure 2). The zone of inhibition is defined as a clear space surrounding the isolate that is preventing the growth of the *M. smegmatis*. This zone will be evident if an isolate has any potential antibiotic properties against *M. smegmatis*. Inhibition of *M. smegmatis* means that it could potentially be effective against *M. tuberculosis* as well, as they are similar organisms. Ten of the 21 isolates tested had a zones of inhibition against the *M. smegmatis* (Figure 3 and Appendix B).

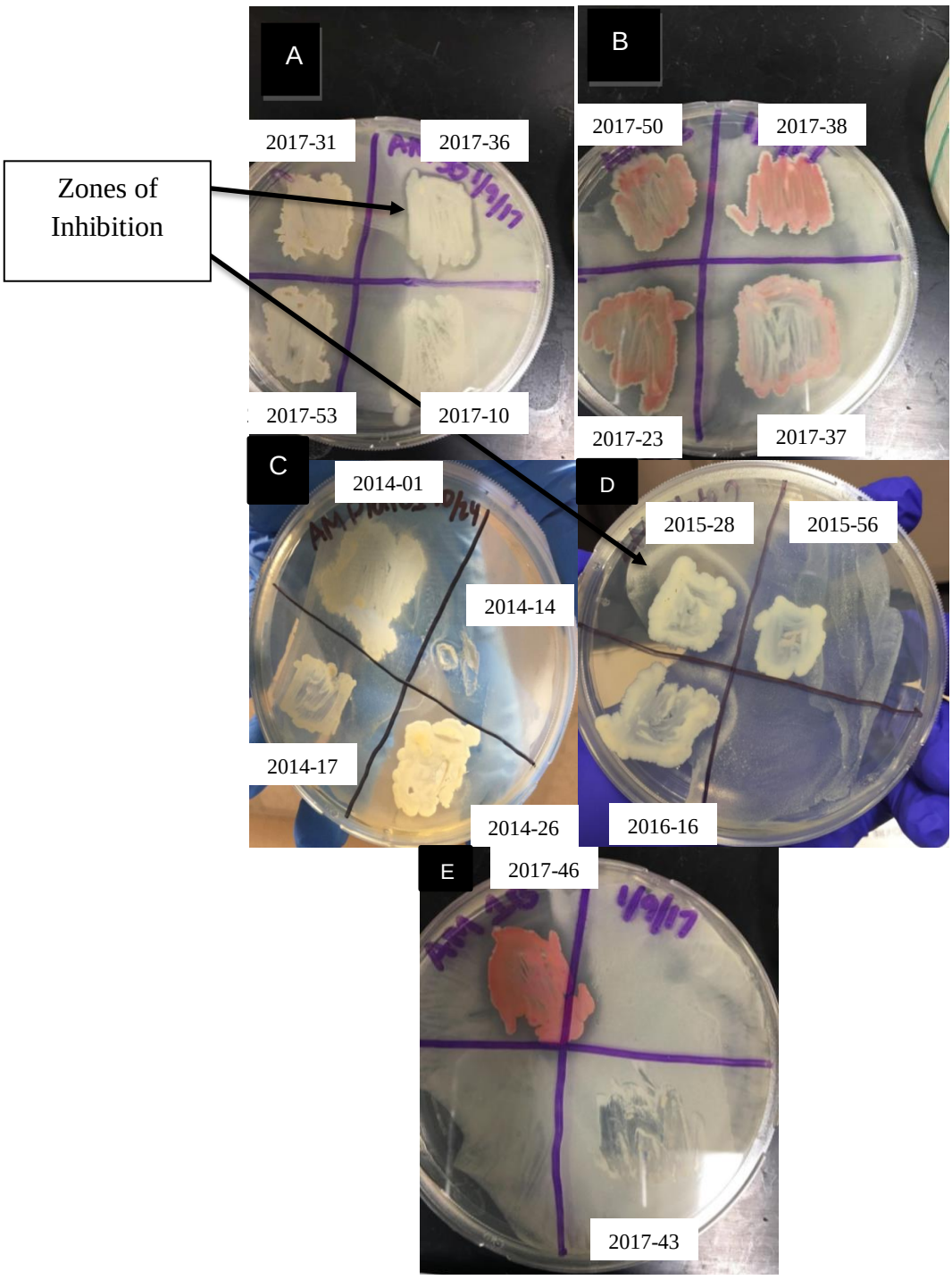


Figure 3: Zones of Inhibition on LB Agar Plates on an M. smegmatis lawn

Table 2: Observations of the Inhibition Assay and Morphology of the Isolates

Isolate Name	Morphology	Zone of Inhibition
2014-01	white, fluffy, spotty	Yes
2014-14	light, white, wispy	None
2014-17	small, wispy, white	None
2014-26	white, fluffy, spotty	Yes
2015-28	white, fluffy, spotty, wispy	Yes
2015-56	white, fluffy, spotty, wispy	None
2016-16	white, fluffy, spotty	Yes
SS-X_0020	white, fluffy, spotty	None
SS-X_0036	larger, white, spotty, fluffy	None
SS-X_0037	spotty, white, fluffy, small	None
SS-X_0038	White, hardened, spotty	None
SS-X_0039	N/A	N/A
2017-010	spotty, fluffy, white	None
2017-23	spotty, fluffy, white	Yes
2017-31	white, smooth	None
2017-36	large spotty, fluffy, white	Yes
2017-37	spotty, fluffy, white	Yes
2017-38	smaller dots, white, fluffy	Yes
2017-46	pink, smaller dots, wispy	Yes
2017-50	white, fluffy, larger spots	Yes
2017-53	smaller dots, white, fluffy	None

Identification of soil isolates

In order to determine the identities of the soil isolates, those that produced zones of inhibition against *M. smegmatis* were subject to 16S rRNA sequencing. Colony PCR was used to amplify the 16S rRNA gene from selected isolates that displayed zones of inhibition (see Figure 3). PCR products were visualized by gel electrophoresis (Figure 4).

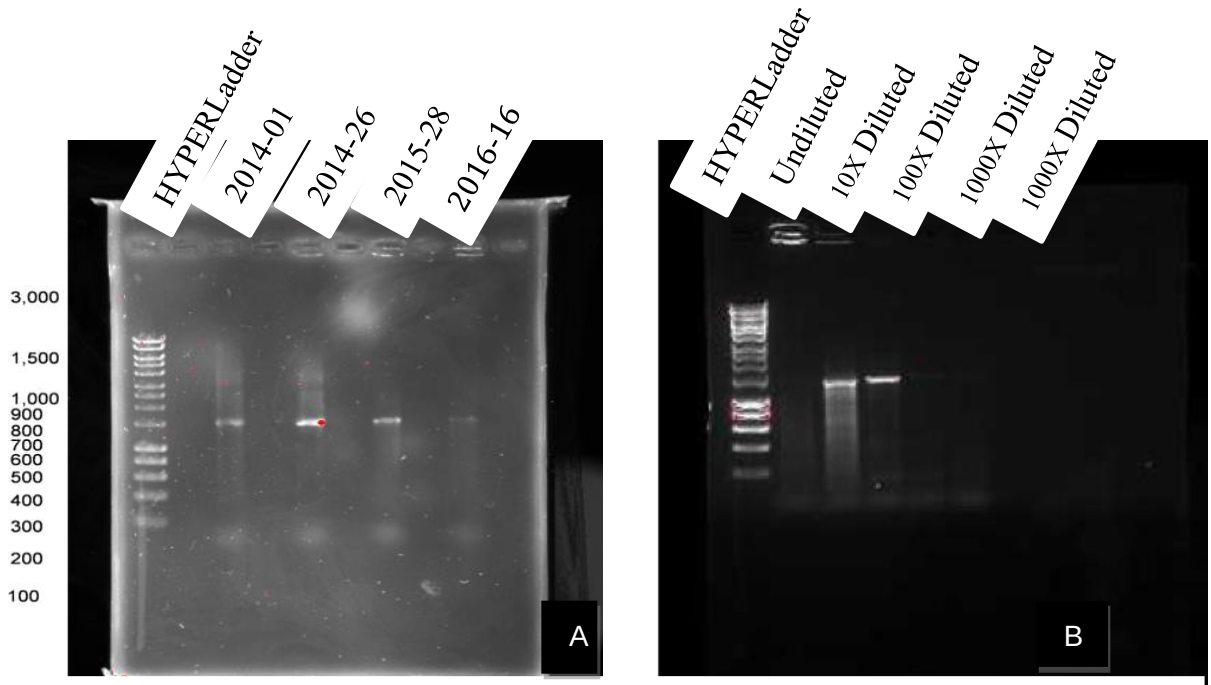


Figure 4: Agarose Gels of Isolates (A) 2014-01, 2014-26, 2015-28, and 2016-16 using primers 1492R and 27F; (B) 2017-38 was amplified using primers 1391R, 1492R, and 27 F with 10X-decreasing dilutions of template.

Figure 4 (A) shows that all of the isolates that produced PCR products of approximately 1500 base pairs. For isolate 2017-38, several dilutions of the template were tested to optimize the reaction (Figure 4B). Figure 4 (B) indicated that the first and second template dilutions, columns C and D, displayed bands with the combination of the three primers at almost the same band size. Not all of the isolates worked with the Microbes to Molecules primers, 1492R and 27F, so 8F and 1391R were used, sometimes in combination with the other primers, to allow us to identify 9 isolates. The isolates that didn't produce the visible product of the 16S rRNA gene with the 1492R and 27F primers were 2017-23, 2017-37, 2017-38, and 2017-50.

Table 3: List of Isolates and BLAST Identifications

Isolate Name	BLAST Top Hit and Percentage
2014-01	*Enterobacter/Klebsiella aerogenes 99%*
2014-26	*Enterobacter/Klebsiella aerogenes 98%*
2015-28	*Enterobacter/Klebsiella aerogenes 99%*
2016-16	*Enterobacter/Klebsiella aerogenes 100%*
2017-23	*Uncultured Bacteria Clone 98%*
2017-36	*Klebsiella pneumoniae 98%*
2017-37	*Uncultured Bacteria Clone 98%*
2017-38	*Uncultured Bacterium DGGE 100%* *Serratia nematodiphila 100%* *Lelliottia amnigena 100%*
2017-50	*Bacillus cerus 99%*

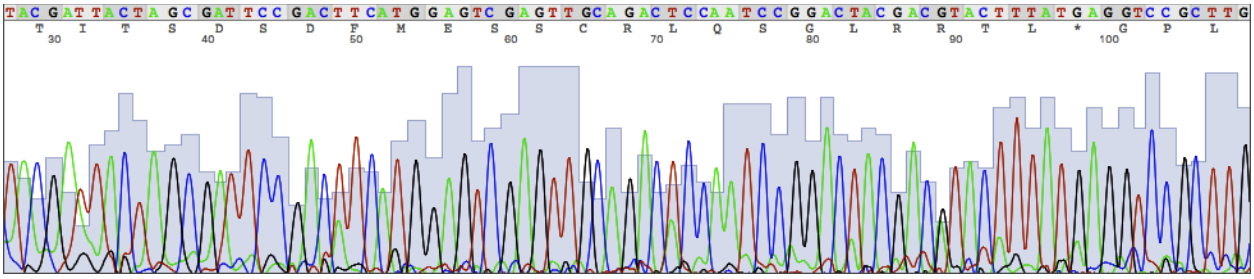


Figure 5: 4Peaks Analysis of isolate 2017-38. The primer used for the sequencing reaction was 1391R. The PCR was done with a mix of 27F, 1391R, and 1492R.

Each PCR product was sequenced, and low-quality regions of the sequencing trace were trimmed using 4Peaks. The results for each sequence are shown in Figure 5 and in Appendix D. 2017-38 is shown as an example of excellent quality sequence. From there, the sequences were put in NCBI nucleotide BLAST and the top hit(s) for identification was placed in Table 3. For only one of the PCR products, (isolate 2017-46), we were unable to get high-quality sequence data and were therefore unable to make an identification. The isolates listed in Table 3 had sequences of high enough quality where each was able to be identified. However, there was some uncertainty to the specific identification of some of the isolates because the isolate sequence was deemed equally similar to multiple identities and there was no discernable way to tell which identity was correct for the same percentage. For example, 2017-38 had 100% sequence similarity with Uncultured Bacterium DGGE, *Serratia nematodiphila,* and *Lelliottia amnigena* (Table 3). Isolates 2014-01, 2014-26, 2015-28, and 2016-16 were all identified as the same two bacteria with equal identity percentages (see Table 3).

Organic Extraction and Activity Assays

The goal of these assays was to determine if secondary metabolites with inhibitory activity could be extracted from the isolates by organic solvents. Following the organic extraction of each of the isolates separately in methanol, acetone, and ethyl acetate, extracts derived from each of the isolates were tested for antibiotic activity. Once the extraction solvents had evaporated, the extracts were re-suspended in methanol and absorbed on filter discs, and in the similar manner to the inhibition assay, the filter discs were placed on top of freshly spread *M. smegmatis* liquid culture, incubated for 3 days at 37ºC, and observed for zones of inhibition.

Figure 6: Methanol Extractions (A) Methanol-soaked filter discs on an LB Agar Plate grown with M. smegmatis with no isolates; (B) 2014-01 Extract re-suspended in Methanol on a filter disc grown with M. smegmatis on LB Agar Plate

The methanol extraction for all for the isolates took a total of 3 weeks to completely evaporate. All of the inhibition assays using methanol extractions had similar results to that of 2014-01, which was inconclusive (Figure 6). These results are inconclusive because there are no zones of inhibition and in some cases there is additional microbial growth around the disks, likely because the extractions were not filtered, meaning that contamination was the likely reason for antibiotic activity. There was a small zone of inhibition with the methanol, likely due to incomplete evaporation of the methanol before placing the disc on the plate.

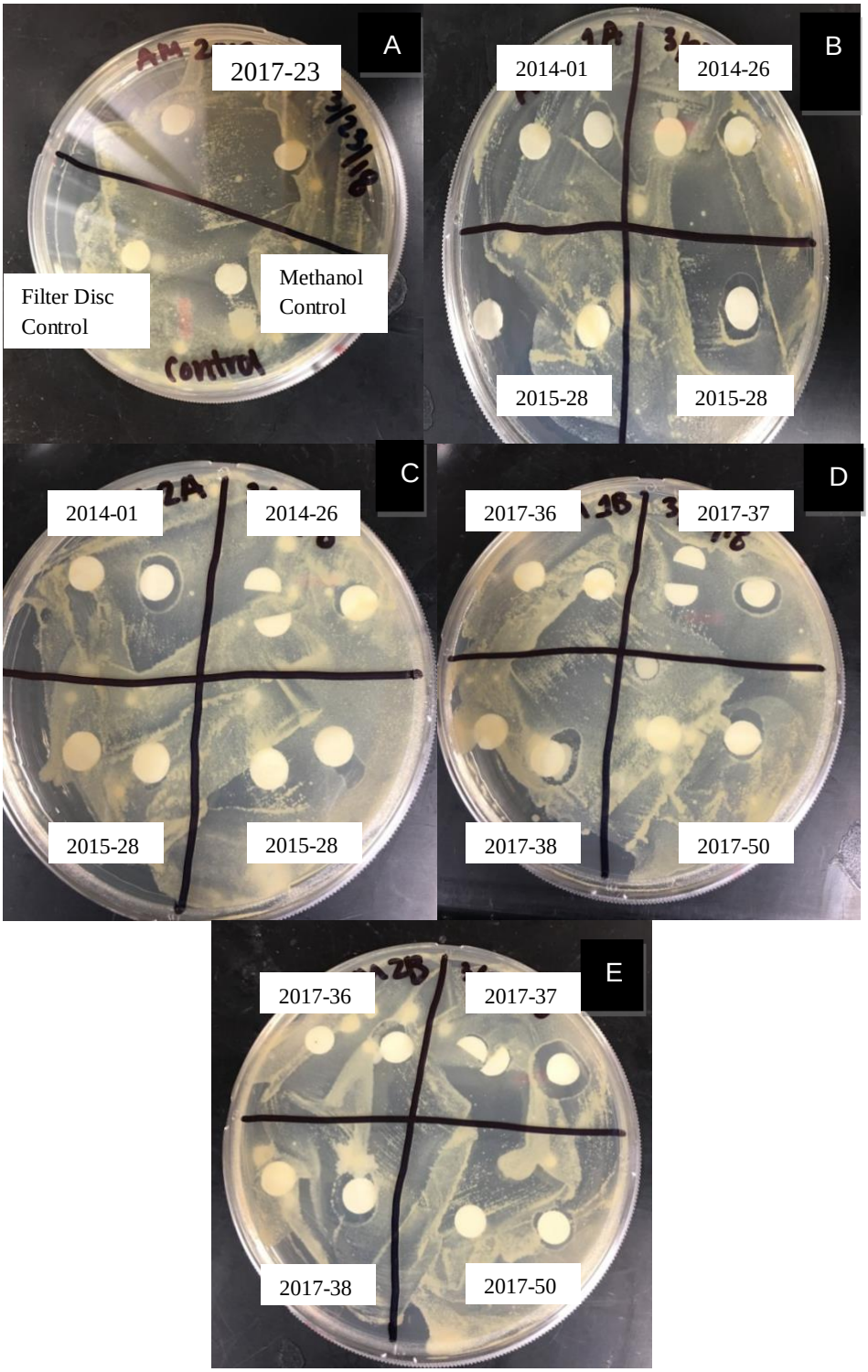


Figure 7: Organic Extraction Activity Assays; in each quadrant, right filter discs have 60 µL of extract and left filter discs have 30 µL of extract from the same isolate.

Following three attempts of the Organic Extraction Activity Assay, the attempt shown in Figure 7 was successful in showing the isolates with zones of inhibition. B and D are Ethyl Acetate Extractions, and C and E are Acetone Extractions (Figure 7). Isolate 2017-23 only had an

extraction with acetone, since its extraction with ethyl acetate solidified and formation to a liquid was therefore difficult. The other two attempts did not have enough extract on the filter discs to warrant a conclusive zone of inhibition. Figure 7 shows the left filter discs with 30 µL of extract having minimal, if any, zones of inhibition while the right filter discs had 60 µL of extract and showed the only definite zones of inhibition. A zone of inhibition is more conclusive evidence of antibiotic activity for the isolates. Extracts from isolates 2014-26, 2015-28, 2016-16, 2017-37, 2017-38, and 2017-50 re-suspended in methanol all produced clear zones of inhibition when 60 µL of extract was applied to the filter.

HPLC

HPLC was done on extracts from selected isolates in order to determine if there are similar or different peaks found in each sample that could indicate the chemical components of the isolates. The seven samples that displayed the best inhibition zones (2014-26, 2015-28, 2016-16, 2017-37, 2017-38 and 2017-50) were made into ethyl acetate and acetone extractions. All of the isolates used for the HPLC analysis were re-suspended in methanol. The ethyl acetate extractions of isolates 2014-26, 2016-16 and 2017-37, and the acetone extractions of isolates 2015-28, 2017-38 and 2017-50 were chosen.

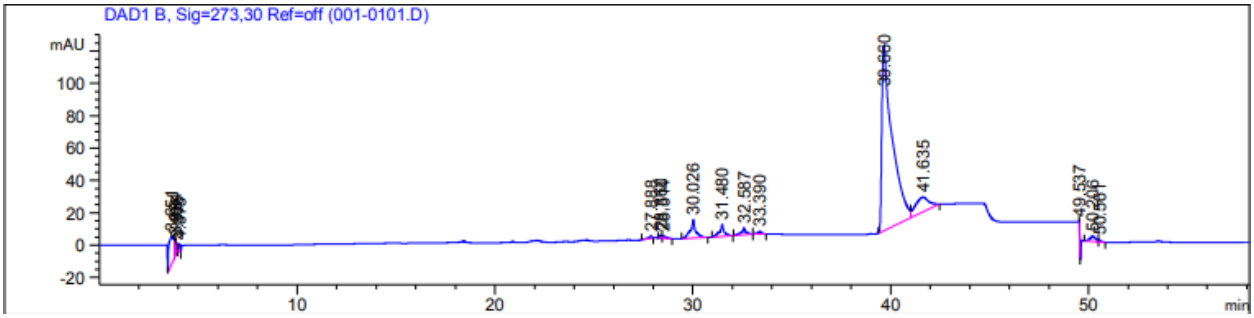


Figure 8: Graph highlighting the two peaks of the Methanol Control sample

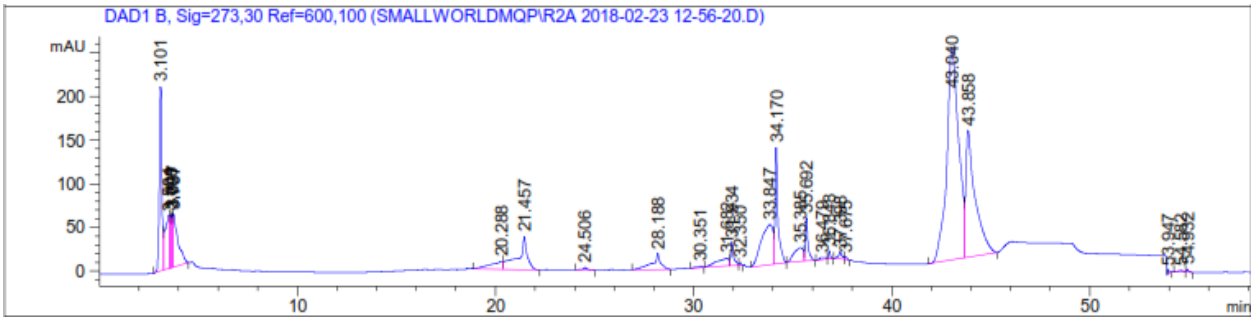


Figure 9: Graph highlighting the peaks of the ethyl acetate extract from Isolate 2014-26

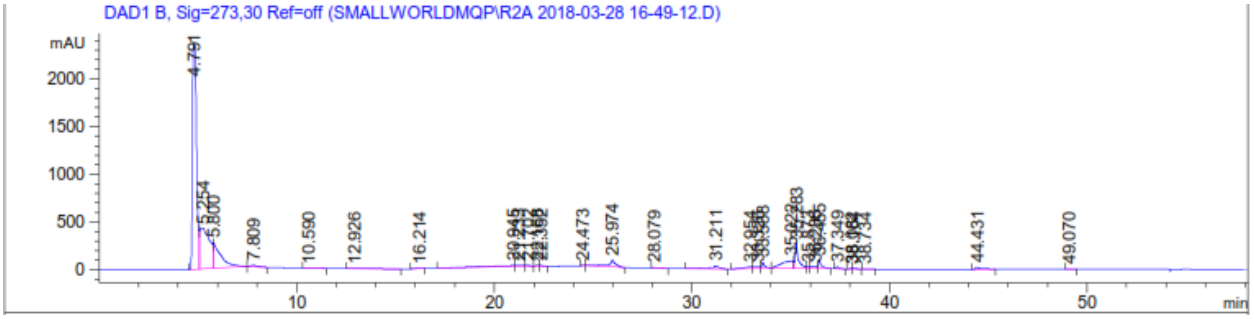


Figure 10: Graph highlighting the peaks of the acetone extract from Isolate 2015-28

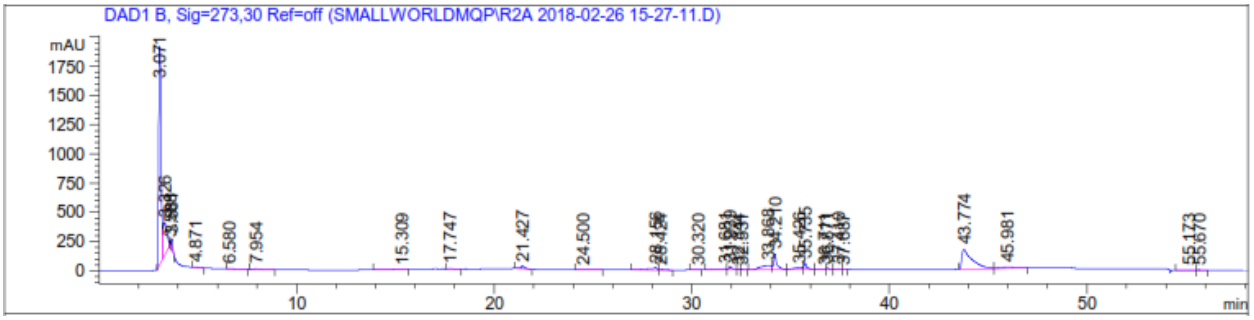


Figure 11: Graph highlighting the peaks of the ethyl acetate extract from Isolate 2016-16

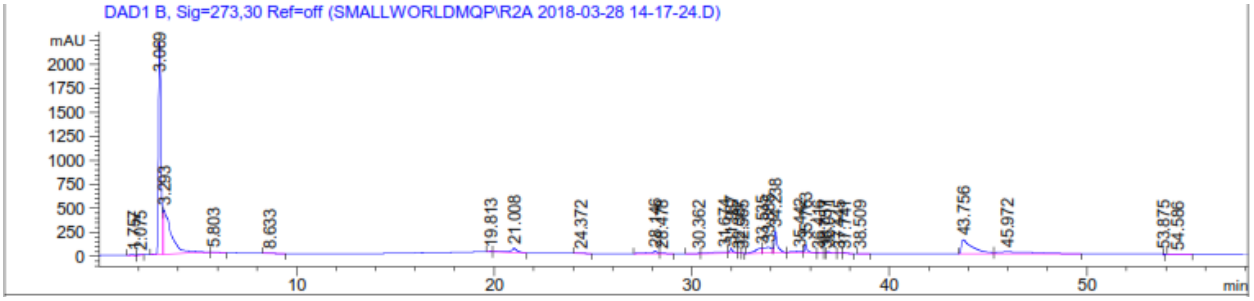


Figure 12: Graph highlighting the peaks of the ethyl acetate extract from Isolate 2017-37

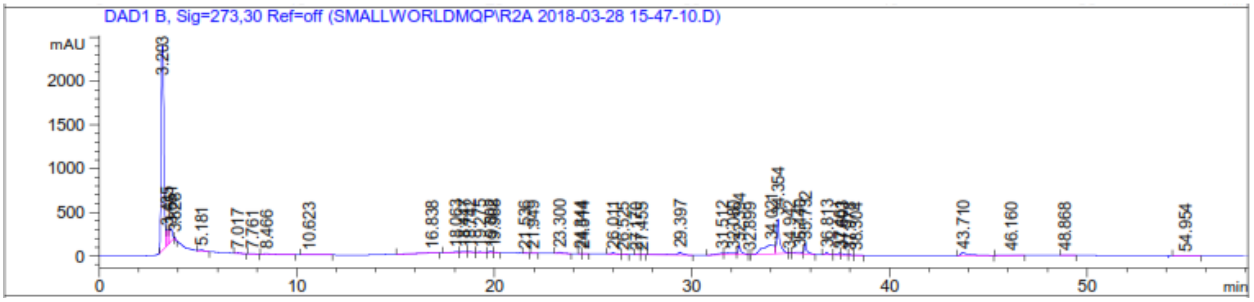


Figure 13: Graph highlighting the peaks of the acetone extract from Isolate 2017-38

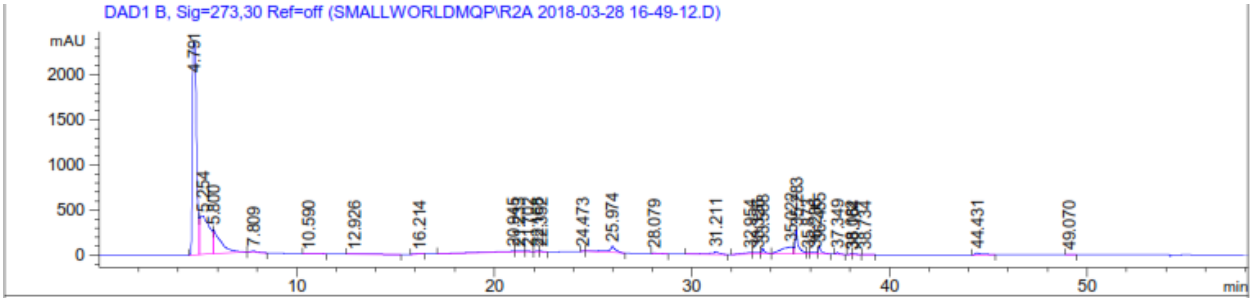


Figure 14: Graph highlighting the peaks of the acetone extract from Isolate 2017-50

All of the chromatograms indicate a larger presence of a peak at the beginning of the graphs, with the isolate extracts showing peaks at 5 minutes going past 2000 mAU (Figures 10-14). Extracts from isolates 2015-28 and 2016-16 are nearly identical in the number of peaks, including two small peaks located at the 55 minute marker, which is not surprising since they are the same microbe (Figures 10 and 11). 2016-16 has smaller peak markers highlighted before the 20 minute mark than the 2024-26 chromatogram. Isolates 2015-28, 2016-16, and 2017-37 all have a singular, smaller peak at the 44 minute mark, which indicates that there is a presence of a similar chemical component besides the methanol peak that is present in the majority of the samples. (Figures 10-12). Isolates 2014-26, 2016-16, and 2017-37 all have tall peaks at the 3.0-3.1 minute mark, and the latter two isolates also have a smaller peak at the 43.7 minute mark (Figure 9, 11, and 12). The chromatograms for 2015-28 and 2017-50 have a tall peak at the 4.791 minute mark (Figures 10 and 14). Isolates 2015-28 and 2017-38 have multiple small peaks within the 20-30 minute mark (Figures 10 and 13). All of the figures have multiple smaller peaks within the 30-40 minute mark and no other peaks with an absorbance greater than 300 mAU occurred besides at the start of the data collection.

Discussion

Using the methods provided by the Small World Initiative, we were able to analyze soil isolates whose secondary metabolites could potentially be used as antibiotics against TB. We selected 21 isolates from the Microbes to Molecules course from the years 2014-2017 that were tested for antibiotic activity. Of those, the first 11 isolates were selected from a previous MQP that were tested against *M. smegmatis* and zones of inhibition were seen (Barter et al., 2017). The other ten isolates were randomly selected from the 2017 class. From the total 21 isolates, only 10 isolates had any zones of inhibition when plated with *M. smegmatis*. Isolate SS-X_0039 had no growth at all, either because it was lost in the process of preserving it, or it required nutrients that TSA and LB plates could not provide. The isolates 2014-01, 2014-26, 2015-28, and 2016-16 were expected to be the same isolate based on the morphology, and the results from the BLAST sequences proved that to be correct. Of the 10 isolates that initially showed zones of inhibition, only one isolate (2017-46) did not produce a PCR product adequate for sequencing. After several attempts at PCR including dilution of the template, 2017-46 failed to appear on the gel, presumably due to lack of PCR product.

For the organic extraction activity assay, the methanol-only extractions of the 2014, 2015, and 2016 samples did not produce extracts with inhibitory activity. While after any organic extraction the extracted material has to be re-suspended in methanol, we speculate the reason why the methanol only did not work is because methanol is a very polar molecule and had difficulty interacting with the secondary metabolites. Ethyl acetate and acetone are far less polar than methanol and were successful in interacting with/preserving the secondary metabolites. The isolates that produced acetone or ethyl acetate extracts with antibiotic activity following filtration were 2014-26, 2015-28, 2016-16, 2017-37, 2017-28, 2017-50, which indicates these isolates produced soluble molecules with inhibitory activity. We were surprised that extracts from isolate 2014-01 did not have inhibitory activity despite this isolate having the same identity as 2014-26, 2015-28, and 2016-15. We speculate that 2014-01 was a different strain than the other isolates. Isolates 2017-23 and 2017-36 may not have had secondary metabolites with antibiotic activity based on the activity assay following the organic extraction and filtration.

For the HPLC data, the results displayed similar looking graphs with minor differences between a few isolates. All of the traces besides the methanol-only control had a tall peak at either near the 3 minute mark (Isolates 2014-26, 2016-16, 2017-37, and 2017-38) or the 4.7 minute mark (Isolates 2015-28 and 2017-50). While the methanol control had a peak right on the 40 minute mark, none of the other results collected had a peak right on the 40 minute mark; it is unclear why this was the case. Based on the evidence found in Figures 9-11, there could be some evidence in differentiating between *Enterobacter aerogenes* and *Klebsiella aerogenes*. Figure 9 had much larger peaks than was seen in Figure 10 and 11, which could indicate that 2014-26 is not the same species as the other two isolates. Despite not knowing the exact similar chemical components amongst the results, there is evidence that the isolates have similar chemical components that could reflect molecules with antibiotic activities or simply other shared chemical components of these organisms.

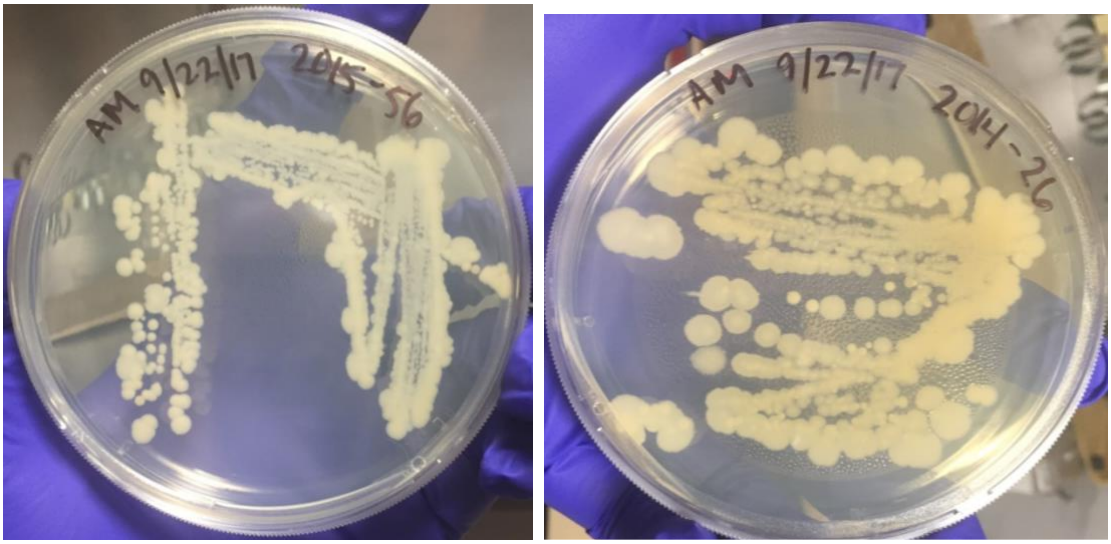
Future research can go into further isolate identification, more activity assay using fractions from the HPLC samples, and further testing of the other isolates with different primers or media. Most of the isolates were not able to be unambiguously identified at the species level, so further testing using more specific primers could help better with identification, especially defining the difference between *Enterobacter aerogenes* and *Klebsiella aerogenes* that are

especially similar. If fractions are taken from an isolate extraction while undergoing HPLC analysis, then those fractions can be tested against *M. smegmatis* to see what molecule(s) in the microbes produces the antibiotic activity. Testing out alternative primers for at least the isolate that did not produce a product with our 16S primers could allow for better DNA synthesis such that the isolate could be identified. For an isolate like SS-X_0039, different media for plates could be used to see if the microbe can grow, because in order for the isolate to have been preserved in the Microbes to Molecules class, the isolate needed to grow. Ultimately, we were able to find 7 isolates that had definite antibiotic activity (Appendix C), indicating that further study of these isolates and the molecules they produce may be warranted.

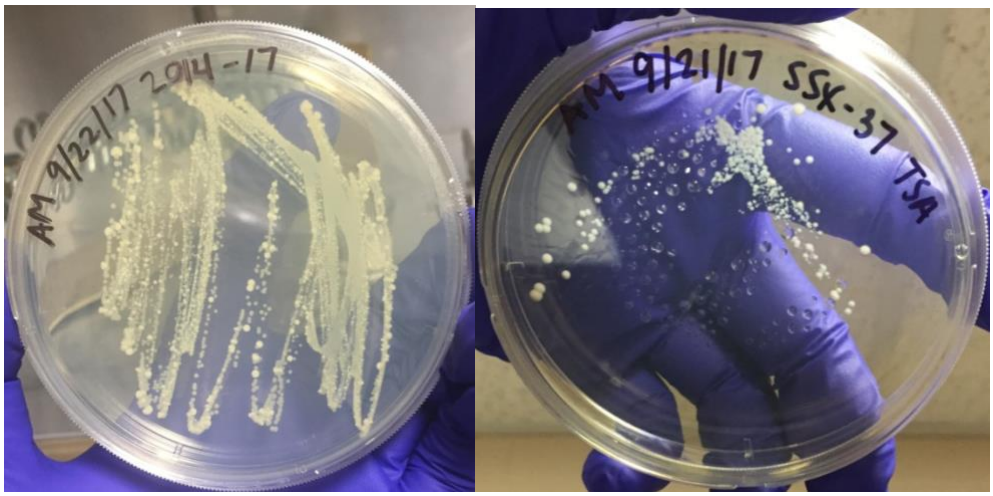
Appendix

Appendix A: LB Plates of Isolates growth

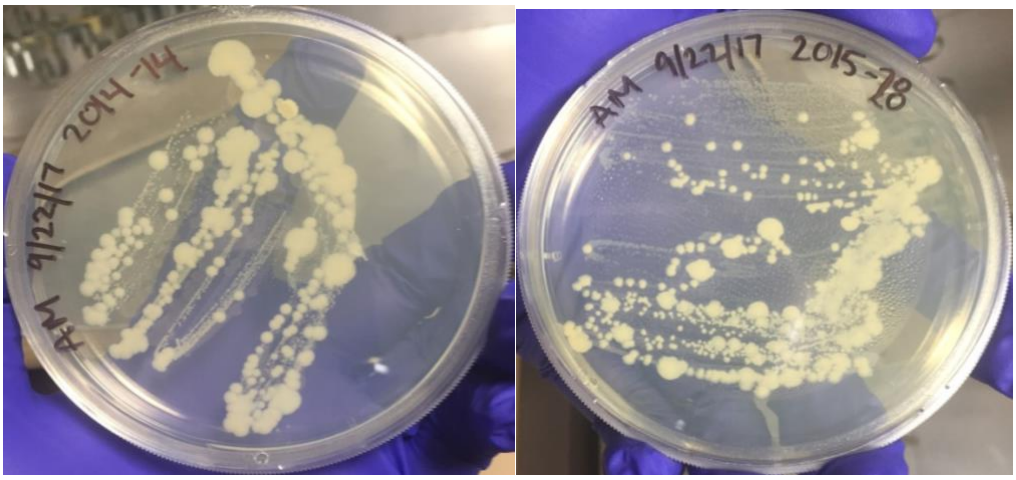
Left is 2014-01 on an LB plate, Right is 2016-16 on an LB plate



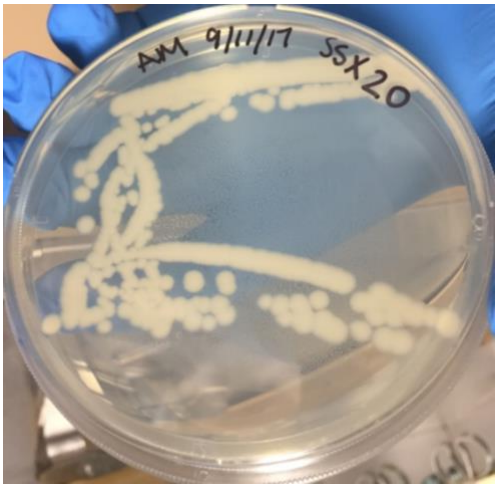
Left is 2015-56 on an LB plate, Right is 2-14-26 on an LB plate



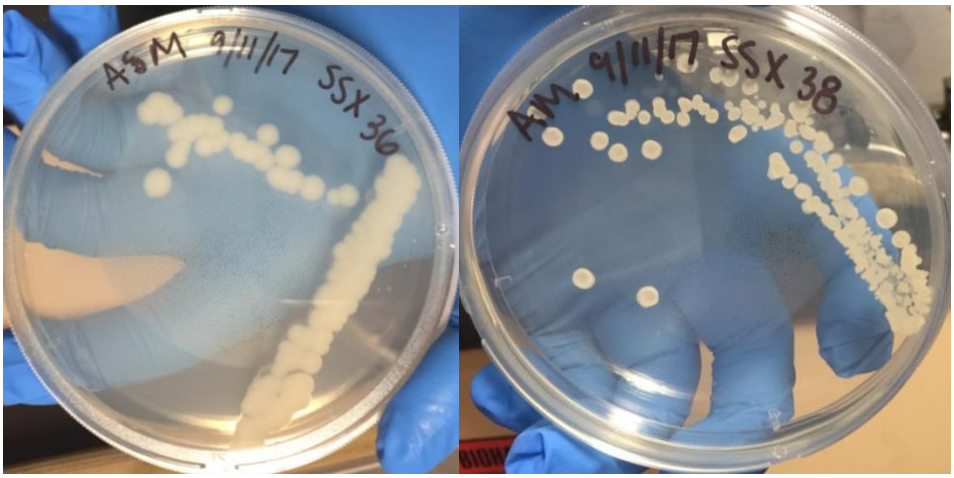
Left is 2014-17 on an LB plate, Right is SSX_037 on a TSA plate



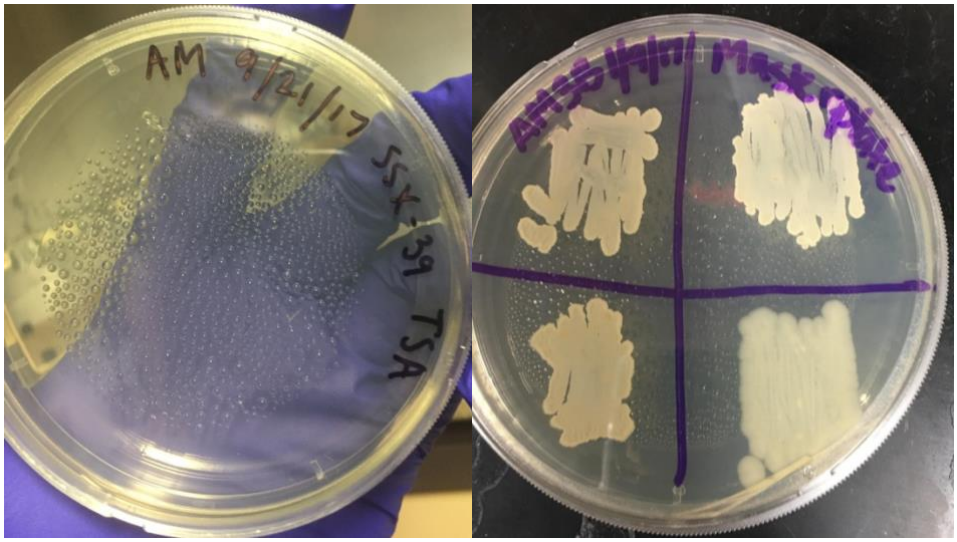
Left is 2014-14 on an LB plate, Right is 2015-28 on an LB plate



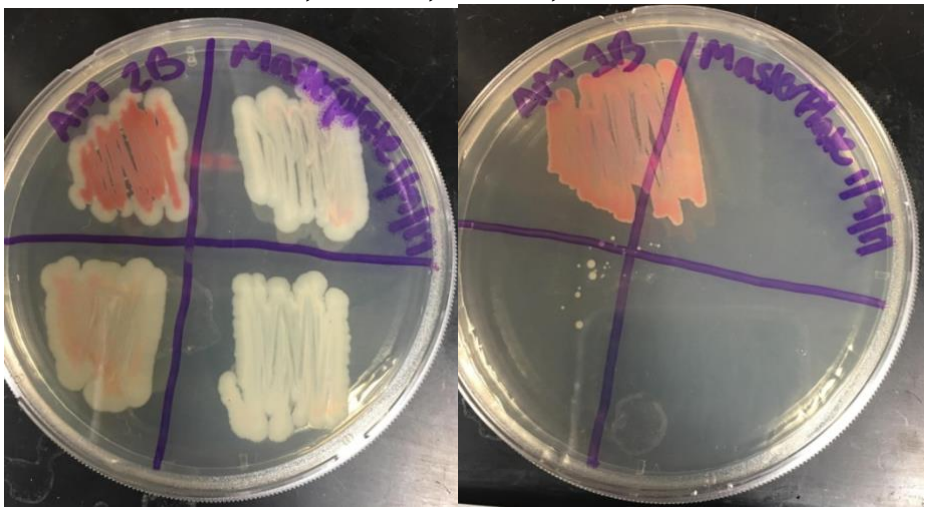
SSX_020 on an LB plate



Left is SSX_036 on an LB plate, Right is SSX_038 on an LB plate



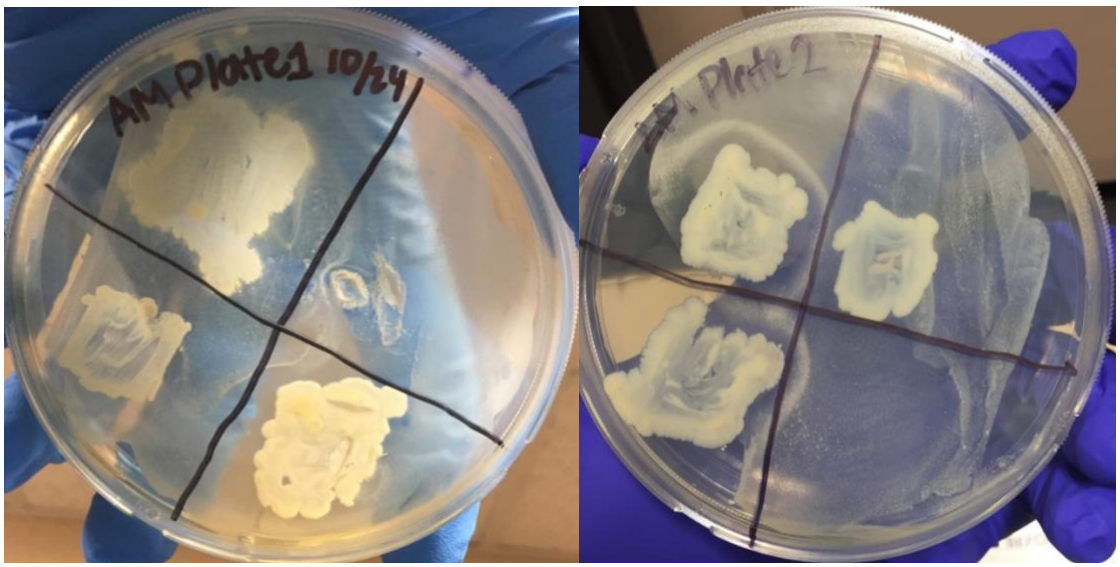
Left is SSX_039 on an LB plate, Right is 2017 Master Plate; from top left to bottom right is 2017-31, 2017-36, 2017-53, and 2017-10



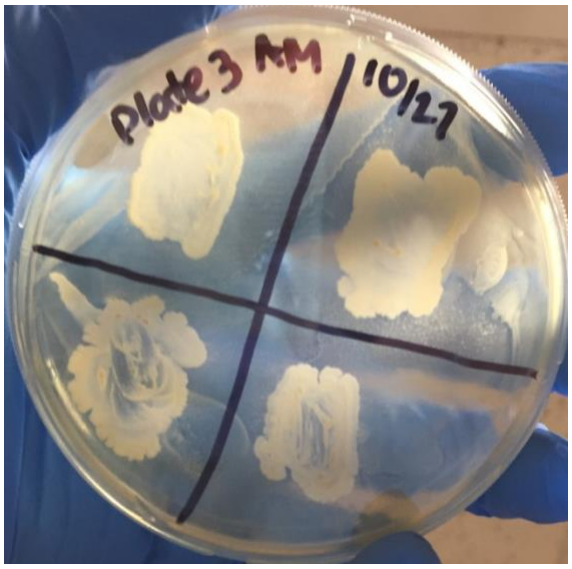
Left is (from top left to bottom right) 2017-38, 2017-50, 2017-37, and 2017-23 on an LB plate, Right is 2017-46 (top) and 2017-43 (bottom) on an LB plate

Appendix B: Isolates with *M. smegmatis* displaying zones of inhibition

Left is 2017-46 (top) and 2017-43 (bottom) on an LB plate with a lawn of M. smegmatis with patches of isolates, Right is LB in-between plate with M. smegmatis; from top left to bottom right is 2017-31, 2017-36, 2017-53, and 2017-10



Left is an LB plate with M. smegmatis lawn and isolates, from top left to bottom right: 2014-01, 2014-14, 2014-17, and 2014-26; Right is an LB in-between plate, from top left to bottom left: 2015-28, 2015-56, and 2016-16



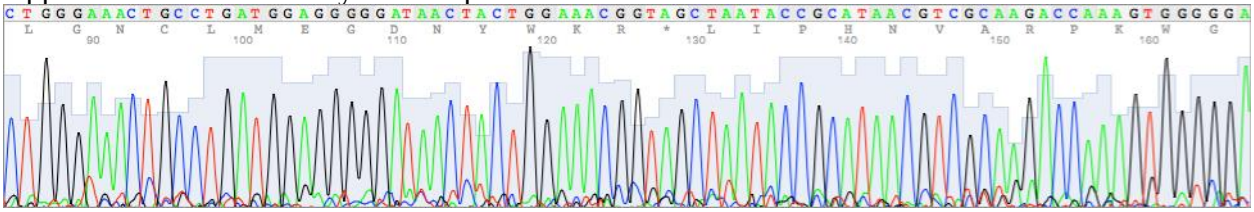
LB plate with M. smegmatis lawn and isolates, from top left to bottom right: SSX_020, SSX_036, SSX_037, and SSX_38

Appendix C: Table of Isolates and Assay Results

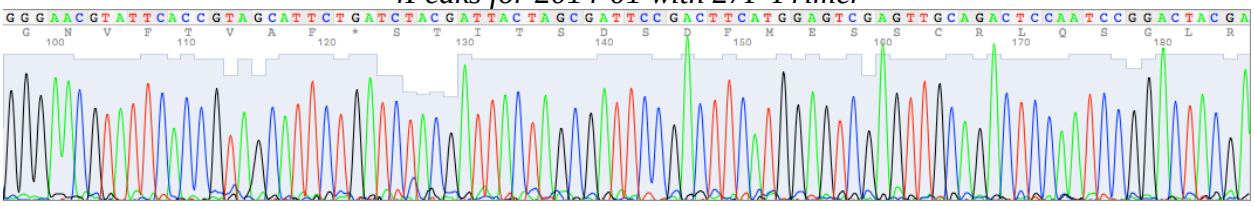
Isolate Name	Morphology	Zone in Inhibition	Primers Used	BLAST Top Hit/%	Extraction Results 1	Extraction Results 2	Extraction Results 3	Extraction Results 4	Extraction Results 5
2014-001	white, fluffy, spotty	Yes	27F, 1492R	*Enterobacter /Klebsiella aerogenes* 99%	Inconclusive	Inconclusive	Inconclusive	Some	Yes
2014-14	light, white, wispy	None	N/A	N/A	N/A	N/A	N/A	N/A	N/A
2014-17	small, wispy, white	None	N/A	N/A	N/A	N/A	N/A	N/A	N/A
2014-26	white, fluffy, spotty	Yes	27F, 1492R	*Enterobacter /Klebsiella aerogenes* 98%	Some with Acetone	Yes	Yes	Some	Some
2015-28	white, fluffy, spotty, wispy	Yes	27F, 1492R	*Enterobacter /Klebsiella aerogenes* 99%	Yes, 7/8 filter discs	Mostly (was it zone of inhibition or lack of *M.Smeg*?)	Yes	Some	Inconclusive
2015-56	white, fluffy, spotty, wispy	None	N/A	N/A	N/A	N/A	N/A	N/A	N/A
2016-16	white, fluffy, spotty	Yes	27F, 1492R	*Enterobacter /Klebsiella aerogenes* 100%	Only with Acetone (re-test ethyl acetate)	Yes with Acetone	Yes with Acetone	Inconclusive	Some
SS-X_0020	white, fluffy, spotty	None	N/A	N/A	N/A	N/A	N/A	N/A	N/A

SS-X_0036	larger, white, spotty, fluffy	None	N/A	N/A	N/A	N/A	N/A	N/A	N/A
SS-X_0037	spotty, white, fluffy, small	None	N/A	N/A	N/A	N/A	N/A	N/A	N/A
SS-X_0038	White, hardened, spotty	None	N/A	N/A	N/A	N/A	N/A	N/A	N/A
SS-X_0039	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
2017-010	spotty, fluffy, white	None	N/A	N/A	N/A	N/A	N/A	N/A	N/A
2017-23	spotty, fluffy, white	Yes	8F, 1391R	Unculture Bacteria Clone 98%	Inconclusive	Yes	Yes	Inconclusive	Yes
2017-31	white, smooth	None	N/A	N/A	N/A	N/A	N/A	N/A	N/A
2017-36	large spotty, fluffy, white	Yes	27F, 1492 R	*Klebsiella pneumoniae* 98%	Minimal (small with half of the filter discs)	Mostly	Some	Inconclusive	Some
2017-37	spotty, fluffy, white	Yes	8F, 1391R*	Uncultured Bacteria Clone 98%	Inconclusive	Inconclusive	Inconclusive	Some	Yes
2017-38	smaller dots, white, fluffy	Yes	27F, 1391 R, 1492 R	*Serretia sp*. 96%	N/A	N/A	N/A	N/A	N/A
2017-46	pink, smaller dots, wispy	Yes	N/A	N/A	N/A	N/A	N/A	N/A	N/A
2017-50	white, fluffy, larger spots	Yes	8F, 1391R	*B. cerus* 99%	Some (2 filter dics)	Inconclusive	Inconclusive	Some	Some
2017-53	smaller dots, white, fluffy	None	N/A	N/A	N/A	N/A	N/A	N/A	N/A

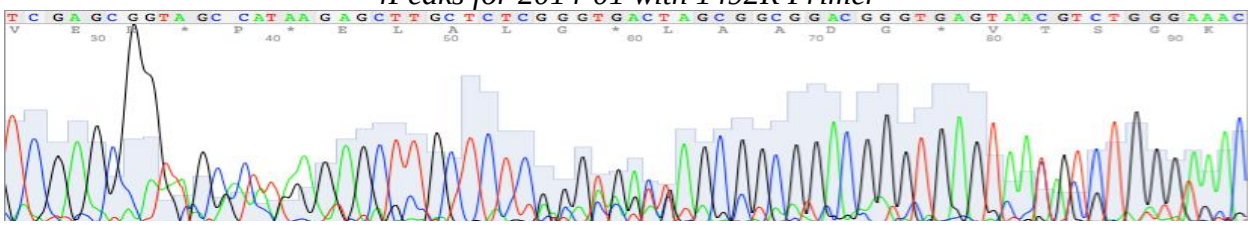
Appendix D: 4Peaks Analytical Graphs for all Identified Isolates



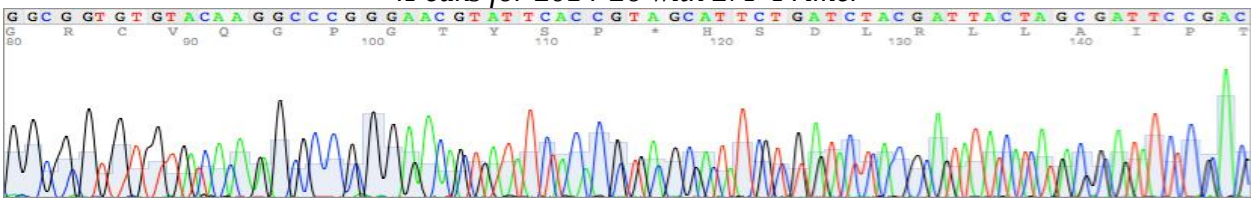
4Peaks for 2014-01 with 27F Primer



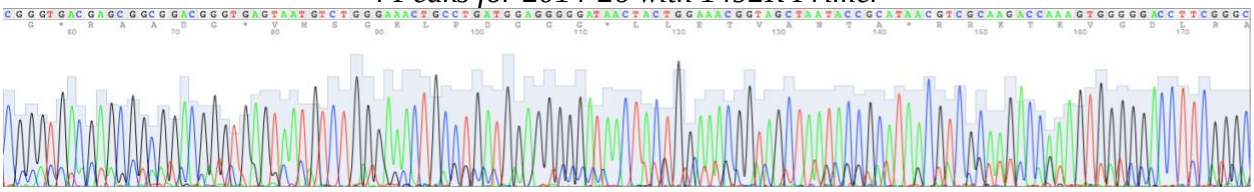
4Peaks for 2014-01 with 1492R Primer



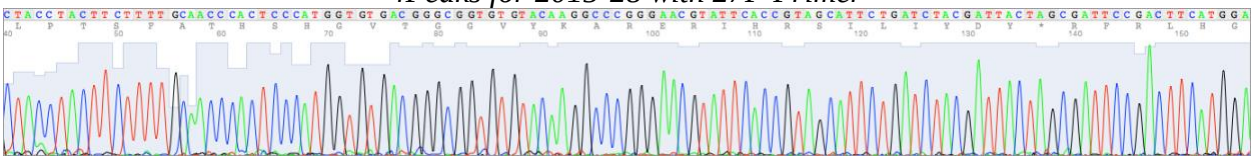
4Peaks for 2014-26 with 27F Primer



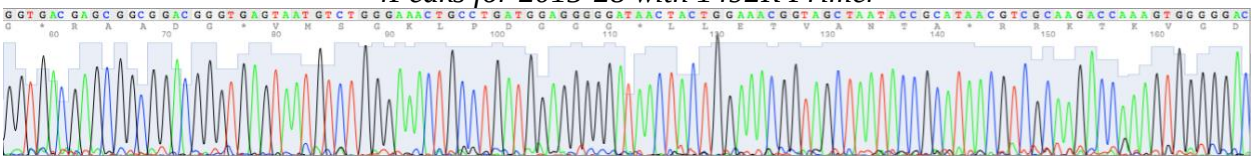
4 Peaks for 2014-26 with 1492R Primer



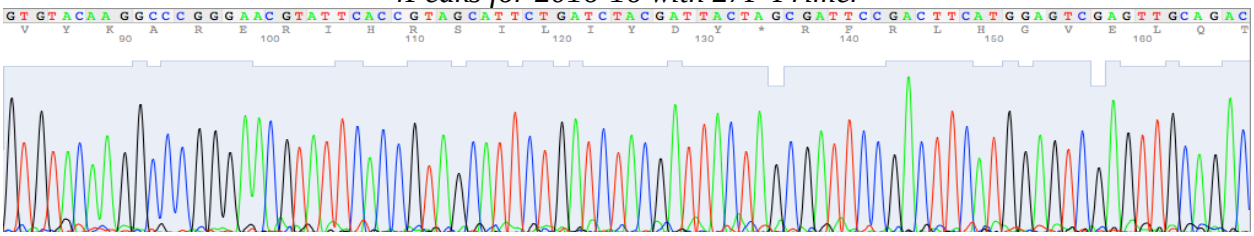
4Peaks for 2015-28 with 27F Primer



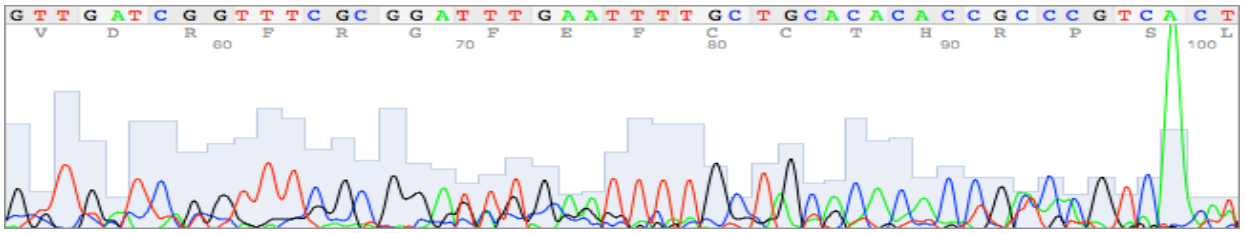
4Peaks for 2015-28 with 1492R Primer



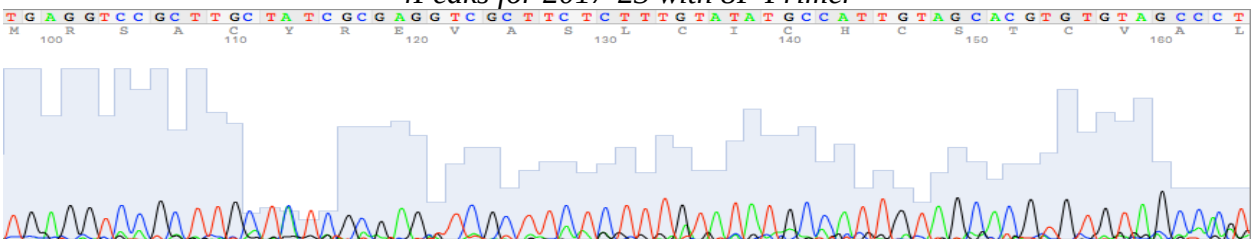
4Peaks for 2016-16 with 27F Primer



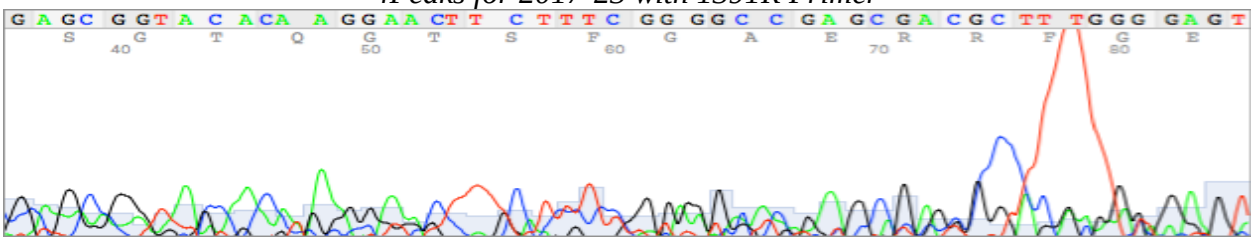
4Peaks for 2016-16 with 1492R Primer



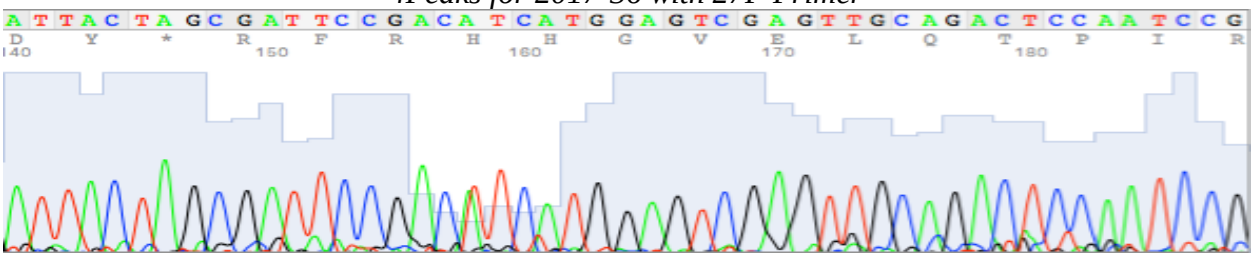
4Peaks for 2017-23 with 8F Primer



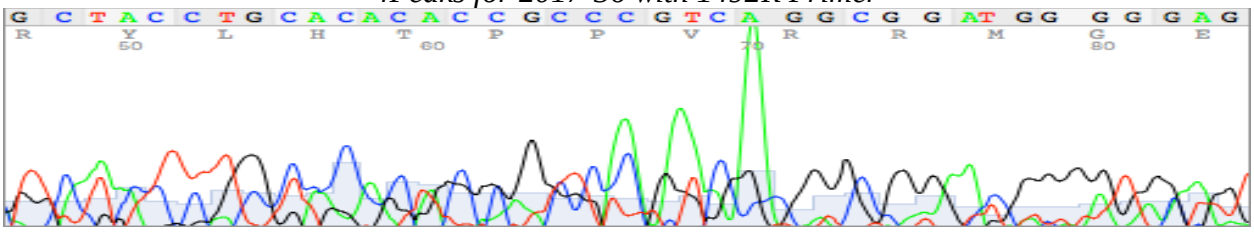
4Peaks for 2017-23 with 1391R Primer



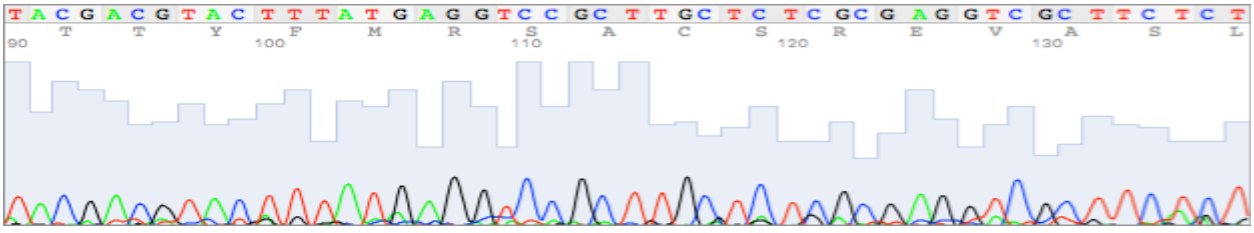
4Peaks for 2017-36 with 27F Primer



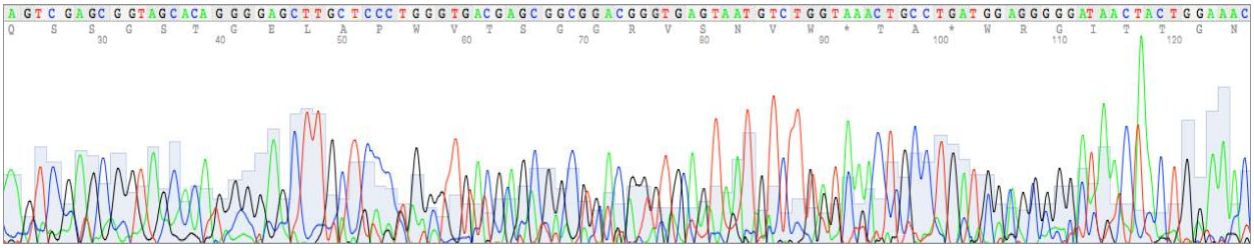
4Peaks for 2017-36 with 1492R Primer



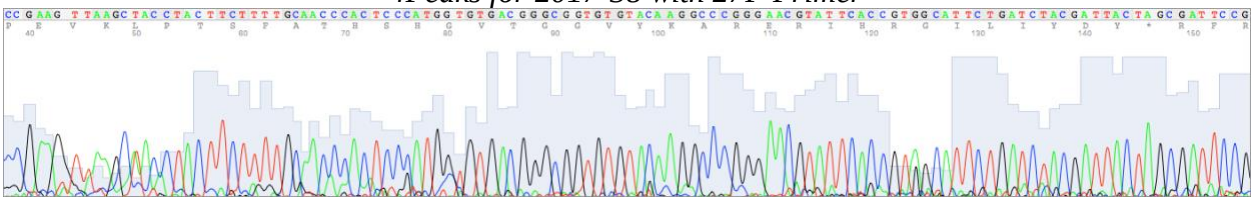
4Peaks for 2017-37 with 8F Primer



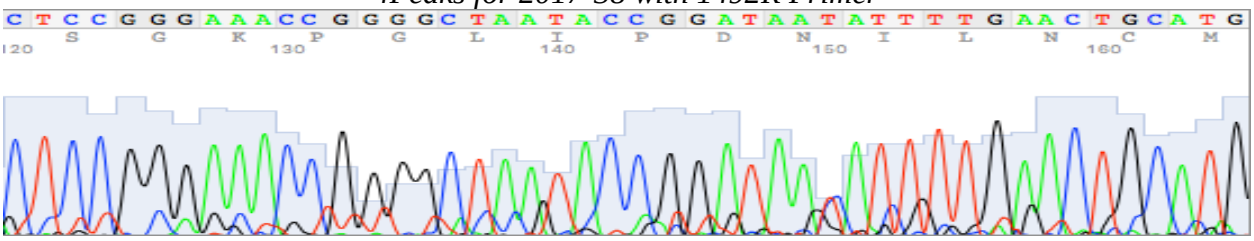
4Peaks for 2017-37 with 1391R Primer



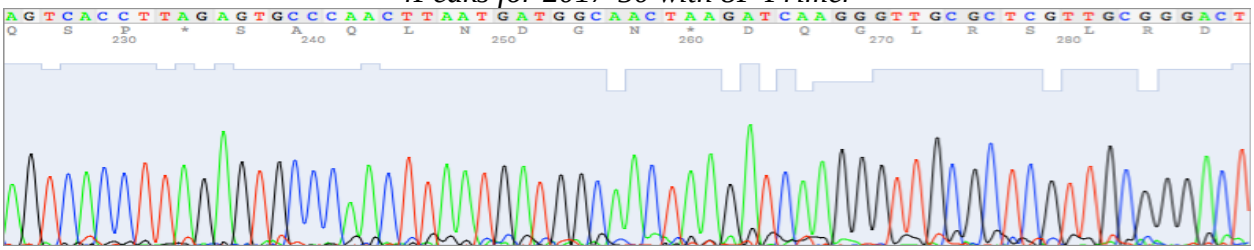
4Peaks for 2017-38 with 27F Primer



4Peaks for 2017-38 with 1492R Primer



4Peaks for 2017-50 with 8F Primer



4Peaks for 2017-50 with 1391R Primer

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