# New Treatment for an Old Disease

A Major Qualifying Project

submitted to the Faculty of

WORCESTER POLYTECHNIC INSTITUTE

in partial fulfillment of requirements for the

degree of Bachelor of Science in Biology and Biotechnology

By


Megan V. Barter

Christine R. McCarron


April 27,2017

Approved by

Dr. Michael Buckholt, Advisor

Dr. Scarlet Shell, Advisor

# TABLE OF CONTENTS 2

# Abstract

Antibiotic resistance has increased for many different diseases including tuberculosis. Scientists have begun to seek ways and places to discover new antibiotics to combat this resistance. Studies over the last 60 years suggest that pathogen-inhibiting bacteria can be found in soil. A number of bacterial isolates potentially producing antibiotic compounds have been found in the soil by students in the Microbes to Molecules laboratory course at Worcester Polytechnic Institute (WPI). This project tested those isolates for the ability to inhibit the growth of *Mycobacterium smegmatis,* a model bacteria for *Mycobacterium tuberculosis*. The goal of the project described here was to identify isolates capable of inhibiting *M. smegmatis* and determine the mechanism(s) of inhibition.

# Introduction

Although tuberculosis (TB) is rare in the United States, it is thought to exist in its latent form in about one third of the world's population. 10.4 million new cases of TB were reported in 2015 (World Health Organization, 2015). The bacteria is known as a "crowd" pathogen because it can be easily spread from an infected person to an uninfected person through aerosols (Barbier, 2016). Once the bacteria enters the body, it enters the lungs and begins to grow and spread.

Drug resistance is a major problem for patients with TB. Drug resistance occurs when sensitive bacteria acquire mutations in drug targets, genes encoding enzymes required for prodrug activation, or genes controlling drug efflux (Calligaro, 2013). Prolonged use of antibiotics is associated with increased likelihood that drug-resistant bacteria will emerge (Calligaro, 2013). Resistance may occur if individuals fail to complete a course of antibiotics. This is particularly problematic in the case of TB, which requires a minimum of six months of multidrug treatment. Adherence to such a long regimen is challenging in the face of potentially serious side effects and inconsistent access to drugs in the resource-limited settings where TB is most prevalent. Other reasons for the rise in drug resistant forms include delays in treatment, inadequate space for doctors to treat people, and poor infection control (Calligaro, 2013).
TB is treated with antibiotic combinations in order to simultaneously target multiple bacterial functions, decreasing the probability of acquiring resistance. Ethambutol, isoniazid, rifampin, and pyrazinamide are the four first-line drugs used to treat drug-sensitive tuberculosis by targeting different cellular aspects of *M. tuberculosis.* Ethambutol obstructs the synthesis of the cell wall (Silve, 1993). Isoniazid also targets synthesis of the cell wall (Rozwarski, 1998). Rifampin targets RNA polymerase, blocking transcription (Wang, 2013). As resistance to these drugs becomes more prevalent, there is a need for new drugs with different targets.

*Mycobacterium smegmatis* is frequently used as a non-pathogenic model for *M. tuberculosis*. Like *M. tuberculosis*, it is aerobic, gram-variable, and nonmotile. *M. smegmatis* was first reported in 1884 by Lustgarten, who was studying syphilis chancres and found a bacillus with the staining appearance of tuberculosis bacilli. It is also present in soil around the

globe. Like *M. tuberculosis, M. smegmatis* protects itself with a lipid outer membrane that is distinct in composition from the outer membranes of gram-negative bacteria. It has very low permeability, conferring intrinsic resistance to many antibiotics. *M. smegmatis* grows much more quickly than *M. tuberculosis,* forming colonies on plates in three days (Akinola, 2013). The fast growth rate and non-pathogenic nature of *M. smegmatis* make it a useful model organism.

The purpose of this project was to find novel compounds produced by other bacteria that have potential to be used as antibiotics for tuberculosis. *M. smegmatis* was used as a safe model for identifying potential inhibitors of *M. tuberculosis* that are produced by bacteria isolated from soil. The bacterial isolates were found in the soil by students in the Microbes to Molecules course (part of the Small World initiative, which is trying to solve the worldwide health issue of antibiotic resistance by using crowdsourcing) during the spring terms of 2014-2016. The interactions between the soil isolates and *M. smegmatis* were studied to find which of the isolates had antimicrobial activity against *M. smegmatis*. We then attempted to identify a subset of the isolates through the use of two different PCR protocols and sequencing. Additionally, we found that an acellular extract of one of the isolates had antimicrobial activity against *M. smegmatis*. The ultimate goals for future research will be to identify the compound and discover the mechanism by which inhibition occurs. This will allow us to determine if we have isolated a bacteria producing an antibiotic that belongs to a new class and inhibits via a new mechanism.

# Materials and Methods

## General methods

We collected and cataloged all soil isolates given to us by an introductory biology lab course taught on campus known as "Microbes to Molecules". This lab was successful in isolating 75 bacterial isolates from local soils over the course of three years (2014-2016), which we used to begin our experiments. All isolates were previously stored in a -80°C freezer in 1 mL liquid aliquots.

Petri dishes containing luria broth (LB) agar gel were made (Appendix B) in order to grow and store the *M. smegmatis* and bacterial isolates. LB agar was chosen as the growth medium for *M. smegmatis* and the bacterial isolates because it is a nutrient rich media that supports the growth of a wide variety of bacteria.

## Bacterial Isolates culture

The soil isolates were previously patched on a gridded LB agar plate from the liquid aliquots by another group of students working with the isolates, allowed to grow into a master plate, and made available for use in experimentation. In order to have enough bacteria for experimentation, both the *M. smegmatis* and soil isolates were moved onto separate prepared LB plates and allowed to grow. A flame-sterilized inoculation loop was used to move small samples of each of the 75 soil isolates from the gridded agar plates to our prepared agar plates, and the loop was flame sterilized between each bacteria spread. These plates were incubated in a 37°C incubator for 3 days to allow for bacterial spread and growth, and then moved into a 4°C refrigerator until needed for experimentation. See Appendix C for a more detailed protocol.

## *Mycobacterium smegmatis* culture

*M. smegmatis* strain $mc^2155$ was provided by our advisor, Scarlet Shell. We grew a liquid culture to make frozen aliquots (Appendix D) for experimentation. We chose to use Middlebrook 7H9 Broth with Tween as the liquid growth medium, as several published protocols suggested that this combination of media allows mycobacteria, especially *M. smegmatis* and *M. tuberculosis*, to flourish and grow successfully. Liquid cultures were grown at 37°C with shaking and frozen at an optical density (OD) of 0.7. Aliquots were stored in a -80°C freezer.

## Isolate testing

To determine which of the 75 soil isolates had the ability to inhibit the growth of *M. smegmatis*, each isolate was plated together with *M. smegmatis* (Appendix E). LB agar plates were spread with 200µl each of the liquid *M. smegmatis* that was grown to an OD of 0.7 and allowed to dry for 5 minutes. Once dry, a flame sterilized inoculation loop was used to lift colonies of the soil isolates from the stock plates and spread on top of the dried *M. smegmatis*. These plates were then incubated at 37°C for 3 days.

Of the isolates that did inhibit the growth of *M. smegmatis*, four of them were chosen to continue research on. To discover if any of these four bacteria created secretions that have antimicrobial properties, a methanol extraction was performed (Appendix J). This was done by creating individual plates of each bacteria with thick lawns covering almost all of the plate. The bacteria-covered agar was then cut and placed in 50 mL conical tubes. The tubes were then placed in liquid nitrogen for 5 minutes. After the tubes had frozen, the 12 mL of methanol was added and then the tubes were left to shake at room temperature overnight. The liquid was then extracted from each tube using a pipette aid and placed in vials that were left open so that the methanol would evaporate off in a hood. Once ready to plate the extractions were redissolved in 1 mL of methanol and 30ul was applied to a filter disk and allowed to dry for 2 minutes before being placed on LB agar plates that had been spread with 200 μl of *M. smegmatis* at an OD of 0.7 and allowed to dry for five minutes.

## Isolate Identification

In order to identify these four bacteria, colony PCR was performed with forward primer 27F and reverse primer 1492R for all the isolates except 2014-3 where forward primer sss969 and reverse primer sss967 were used according to the detailed protocol outlined in Appendix F. The sequence for these four primers can be seen in table 1. For these bacterial isolates it was found that DNA extraction (Appendix G) followed by PCR (Appendix H) created better products for sequencing. PCR products were analyzed by gel electrophoresis (Appendix I) using 1% agarose gel, hyperladder marker, and SYBR green.

Table 1: sequence of primers used for PCR

| Primer | Primer sequence |
|---|---|
| 27F | AGAGTTTGATCMTGGCTCAG |
| 1492R | TACGGYTACCTTGTTACGACTT |
| sss967 | TACCTTGTTACGACTT |
| sss969 | AGGGTTCGATTCTGGCTCAG |

The PCR products that produced visible bands on the gels were sent to Eton Biosciences to be purified and sequenced. The sequence files were opened with a program called "4Peaks" and the ends with lower quality sequencing were trimmed. The remaining sequence was used as a query to search the NCBI nucleotide database using BLASTn. Matches with 99% identity were considered to be the most possible identities of the isolates. In cases where there was not 99% identity, the DNA was extracted again and a new PCR run to try to get better quality results.

## Results

To find soil isolates capable of inhibiting the growth of *M. smegmatis*, we patched 75 previously banked bacterial isolates (appendix A) on plates spread with liquid cultures of *M. smegmatis.* Fourteen of the bacteria inhibited *M. smegmatis* growth. Of the fourteen isolates, seven were from the year 2014, five were from 2015, and two were from 2016. These plates can be seen in figures 1 through 14 below with blue arrows pointing to the zones of inhibition surrounding the patch of soil isolate in the center of the plate. Four isolates that were distinct in color, colony growth pattern, and zone sizes were chosen for further study. Figures 3, 8, 9, and 13 show the different zone sizes, colors and colony growth patterns observed for the isolates that were chosen: 2014-3, 2015-1, 2015-27, and 2016-16.

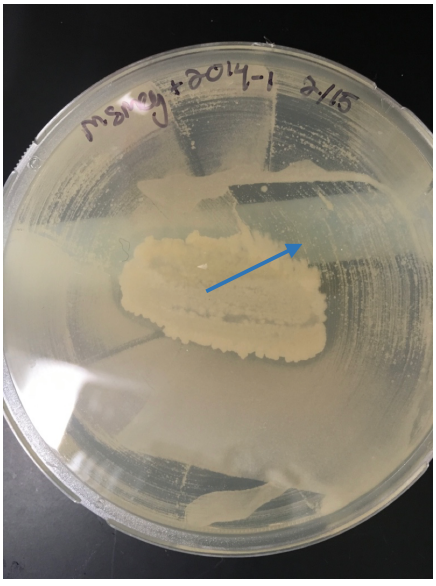

Figure 1: isolate 2014-1 plated on LB with *M. smegmatis*

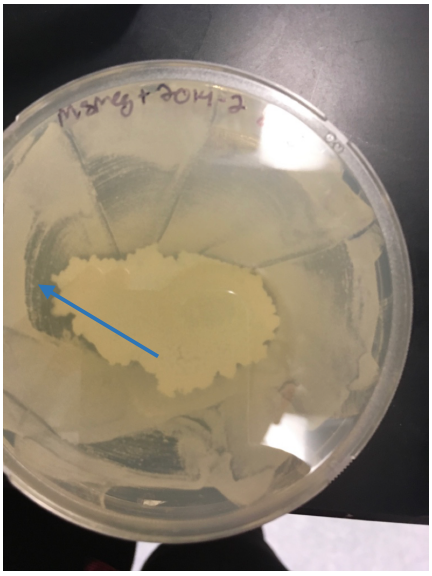

Figure 2: Isolate 2014-2 plated on LB with *M. smegmatis*

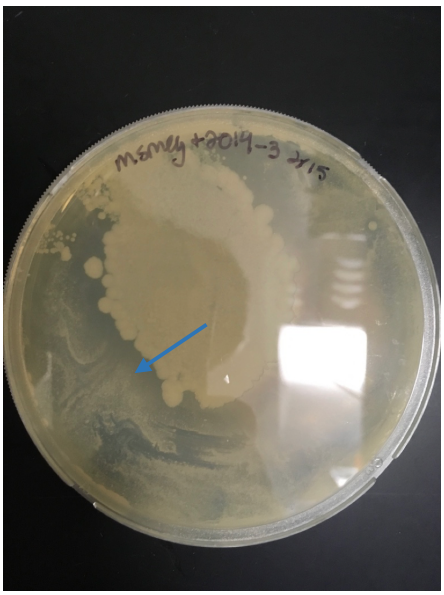

Figure 3: isolate 2014-3 plated on LB with *M. smegmatis*

Figure 4: Isolate 2014-14 plated on LB with *M. smegmatis*


Figure 5: isolate 2014-16 plated on LB with *M. smegmatis*

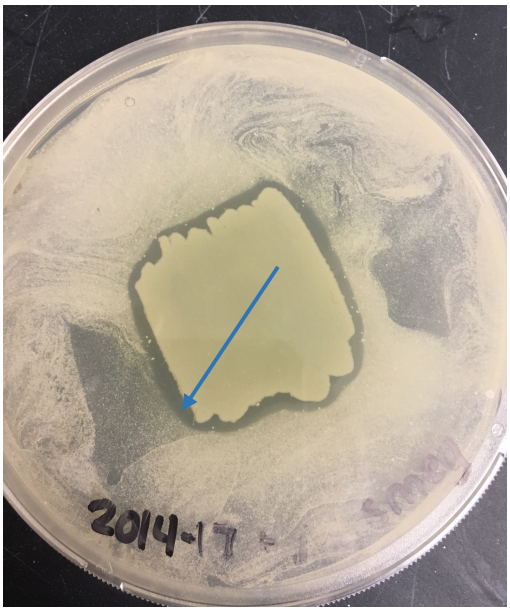


Figure 6: Isolate 2014-17 plated on LB with *M. smegmatis*

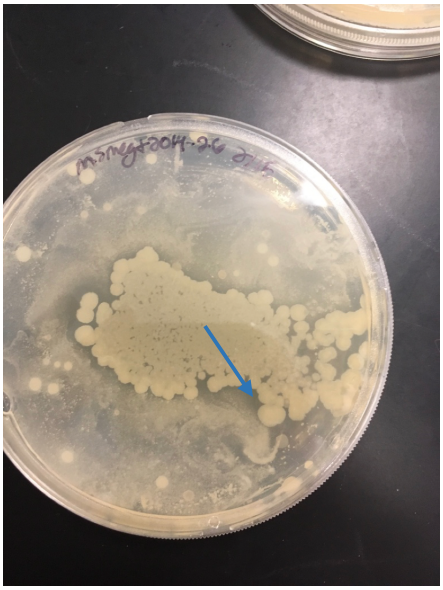

Figure 7: isolate 2014-26 plated on LB with *M. smegmatis*

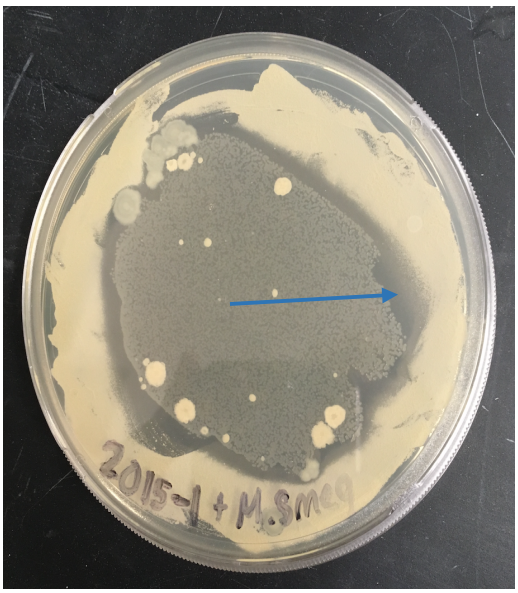


Figure 8: Isolate 2015-1 plated on LB with *M. smegmatis*

Figure 9: isolate 2015-27 plated on LB with *M. smegmatis*

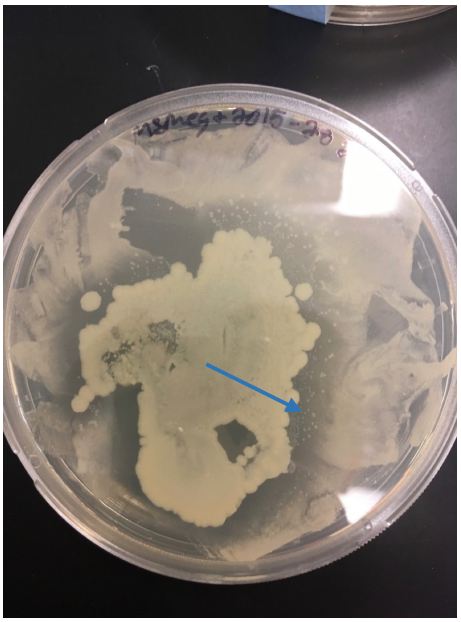

Figure 10: Isolate 2015-28 plated on LB with *M. smegmatis*

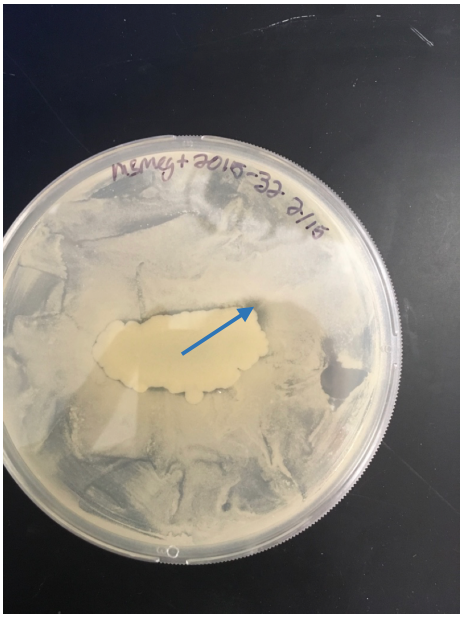

Figure 11: isolate 2015-32 plated on LB with *M. smegmatis*

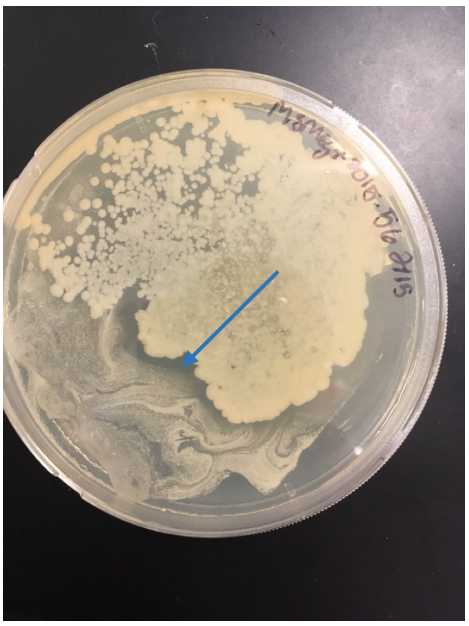

Figure 12: Isolate 2015-56 plated on LB with *M. smegmatis*

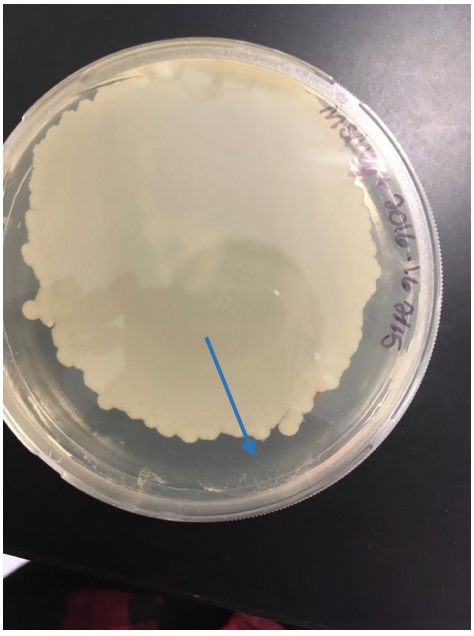

Figure 13: isolate 2016-16 plated on LB with *M. smegmatis*

Figure 14: Isolate 2016-22 plated on LB with *M. smegmatis*

We then attempted to identify isolates 2015-1, 2014-3, 2016-16, and 2015-27 by colony PCR using the 27F forward primer with 1492R reverse primer to amplify the 16S rRNA gene followed by sequencing. Two isolates, 2014-3 and 2016-16, produced bands between 1000 and 1500 base pairs which is expected as the product should be about 1500 base pairs, while the

other two isolates did not produce any visible bands (figure 15). The sequencing (done by Eton Bioscience) returned sequences of low quality which did not show homology to any known sequences in the database. This process was repeated twice with similar sequencing results that did not match any sequences in the 16S rRNA database.

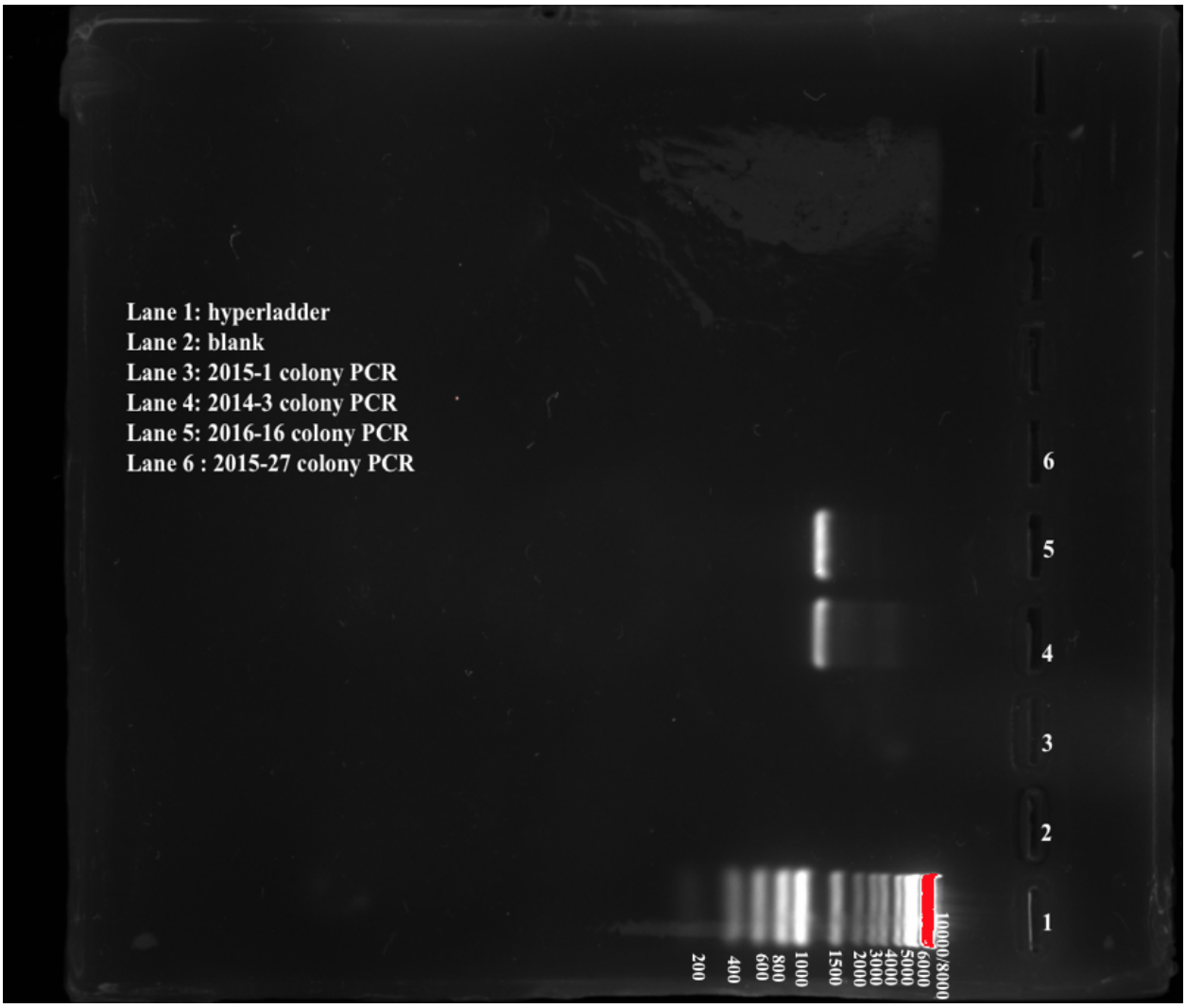

Figure 15: Analysis of colony PCR by gel electrophoresis.

As multiple attempts at colony PCR failed to produce bands and high quality sequencing data, DNA was extracted from colonies from streak plates of the bacteria and amplified using the 27F forward primer with 1492R reverse primer and sss969 forward primer with sss967 reverse primer. The second set of primers, sss969/sss967, was used as an attempt to get better sequencing results. Isolates 2015-1 and 2016-16 produced bands higher than the expected size of

approximately 1500 base pairs when primers 27F and 1492R were used, and 2014-3 also produced a band higher than the expected size when primers sss967 and sss969 were used (Figure 16).

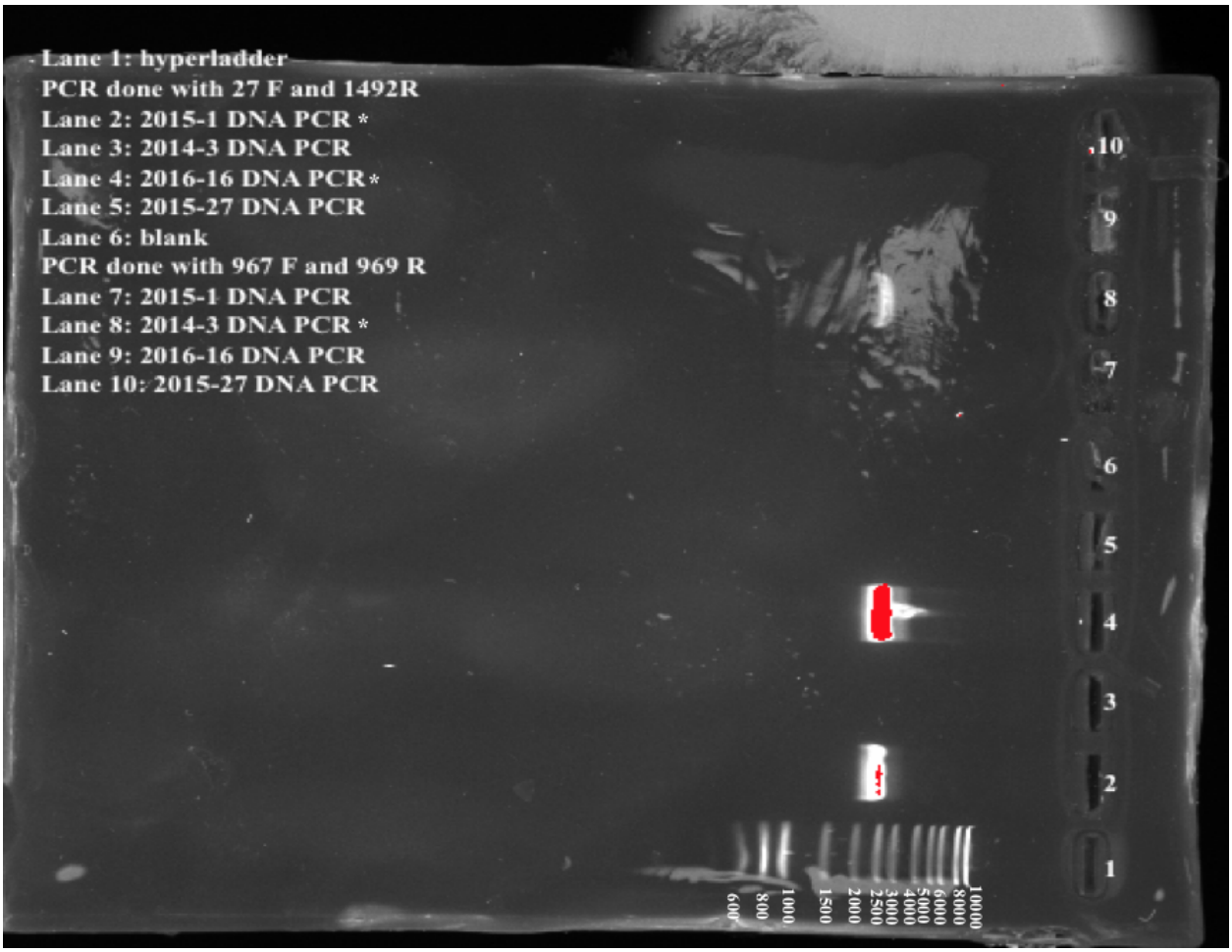


Figure 16: Gel electrophoresis of PCR performed on purified DNA with two sets of primers

In a further attempt to obtain a PCR product from isolate 2015-27, we repeated the DNA extraction and subject it to PCR with primer sets 27F/1492R and sss967/sss969. We also used these primer sets to re-amplify the product from the PCR shown in figure 16. The PCR using the newly extracted DNA with 27F/1492R produced the clearest band (Figure 17). The size of the band was unclear because the ladder was not clearly visualized.

The PCR products that produced the clearest bands on the gels in Figures 16 and 17 (indicated with stars) were then sequenced with primers 27F and 1492R for 2015-1, 2016-16, 2015-27 and primers sss967 and sss969 for 2014-3. NCBI's blast program revealed that both 2015-1 and 2014-3 show 99-98% identity over the longest stretch of sequencing with multiple

members of the genus *Bacillus*. Isolate 2016-16 appears to be either *Enterobacter* or *Raoultella* as both of these genera matched with 99% identity over long stretches of the sequence (95% and above). The 2015-27 sequencing data quality was too poor to obtain meaningful blast hits (Table 1). The sequences done with the primers 27F and sss967 can be seen in Appendix K.

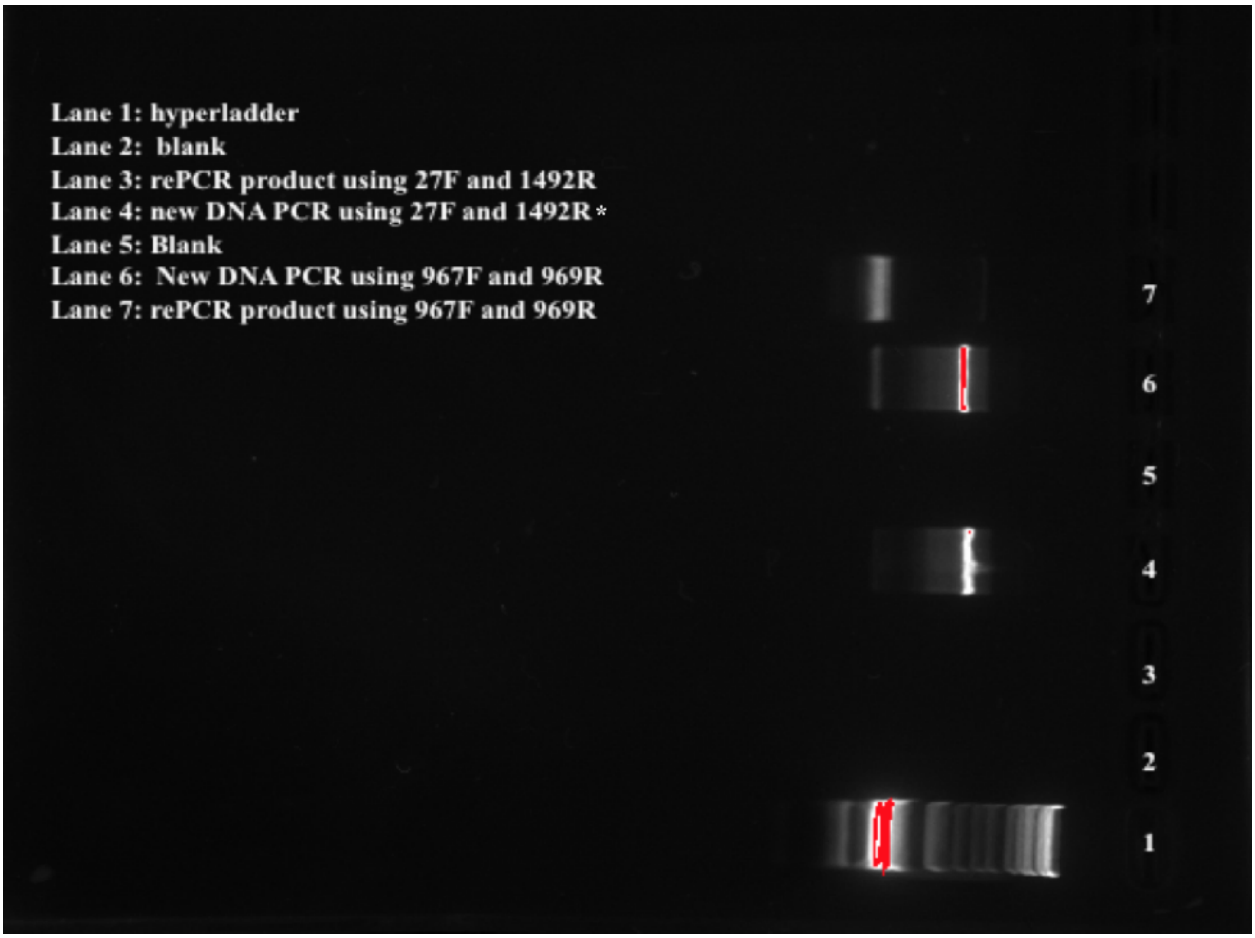


Figure 17: Analysis of PCR products obtained from isolate 2015-27.

Table 1: Isolate identities

| Isolate tag | 2014-3 | 2015-1 | 2015-27 | 2016-16 |
|---|---|---|---|---|
| Probable genus | *Bacillus* | *Bacillus* | Unknown | *Enterobacter* or *Raoultella* |

The initial goal of the project was to identify isolates that inhibited *M. smegmatis* by secretion of an inhibitory substance and not just by depletion of nutrients from the surrounding media. To assess the presence of inhibitory substances, we performed organic extractions on the

putative antibiotic producing isolates and tested the abilities of these extracts to inhibit growth of *M. smegmatis*. Extracts were applied to filter disks and overlaid on plates spread with *M. smegmatis.* As a positive control, rifampin diluted in DMSO and plated at three different concentrations inhibited growth of *M. smegmatis* as expected (Figure 18). The two negative controls DMSO (the solvent for rifampin) and methanol (the solvent for the extracts) failed to inhibit growth as expected (Figure 19). Extracts of isolates 2014-3, 2015-1, and 2016-16 showed no inhibition of *M. smegmatis* growth (Figures 20 and 21). However, an extract of isolate 2015-27 shows a clear zone of inhibition, suggesting that this organism either secretes or contains a methanol-soluble molecule capable of inhibiting the growth of *M. smegmatis* (Figure 21). Blue arrows point to the zones that were created by 2015-27 and rifampin. In several cases, including the DMSO control, a ring of bacterial growth was observed around the edge of the filter disk. This appears to be a contaminant. We therefore cannot exclude the possibility that inhibition observed for the isolate 2015-27 extract was due to the contaminant rather than the extract.

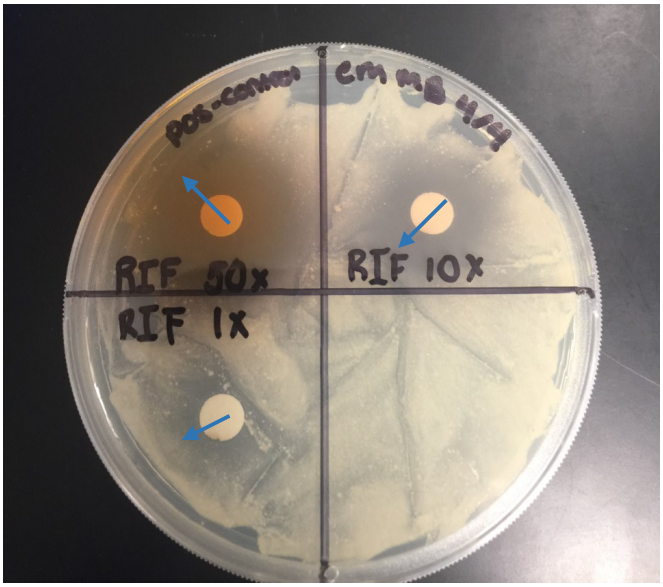


Figure 18: *M. smegmatis* spread on LB and overlaid with filter disks to which 30 ul of rifampin at concentrations of 50 mg/ml, 10 mg/ml, and 1 mg/ml were applied.

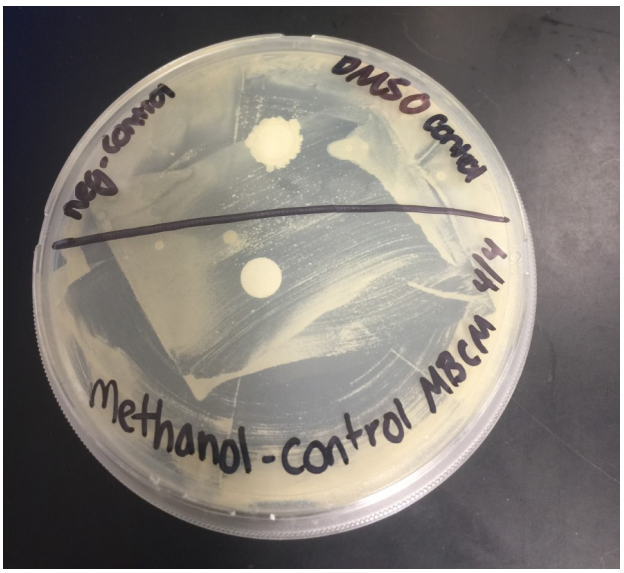


Figure 19: 30ul of DMSO or methanol was applied to filter disks that were overlaid on an LB plate spread with *M. smegmatis* as negative controls.


Figure 20: 30 ul of methanol extracts of isolates 2014-3 and 2016-16 where applied to filter disks and overlaid on LB plates spread with *M. smegmatis*.

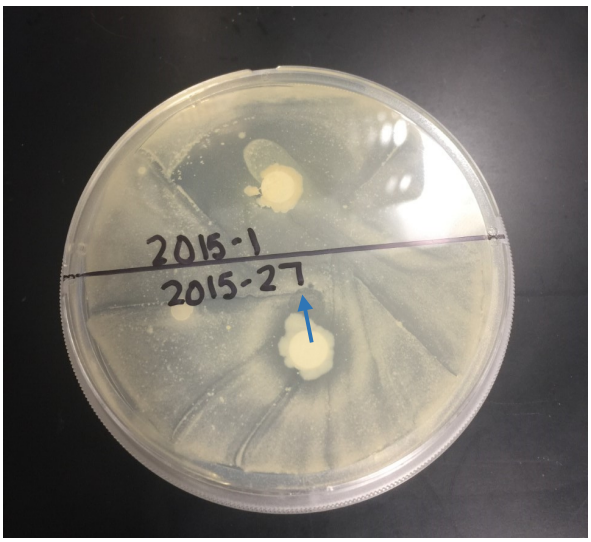


Figure 21: 30 ul of methanol extracts of isolates 2015-1 and 2015-27 where applied to filter disks and overlaid on LB plates spread with *M. smegmatis*.

## Discussion

The purpose of this project was to identify bacterial isolates from a pool of potential producers already isolated by the 2014-2016 Microbes to Molecules classes that show activity against *M. smegmatis*. Our plating of *M. smegmatis* in combination with the isolates taken from the "Microbes to Molecules" laboratory identified 14 isolates with inhibitory abilities. While this showed that these isolates could inhibit *M. smegmatis*, we then went a step further in order to identify these isolates and demonstrate that they were truly antimicrobial, and not just inhibiting growth by nutrient deprivation or similar mechanisms. To do this we chose four of the inhibiting isolates that seemed to have different properties from each other in order to ensure that we would not be testing the same species four times.

An extraction was performed on all four isolates in order to see if any contained or secreted substances capable of inhibiting *M. smegmatis* growth. Only 2015-27, the unidentified isolate, produced an extract that inhibited the growth of *M. smegmatis*. Extracts from the other three isolates did not produce any zone of inhibitions.

DNA extraction resulted in all four isolates producing PCR products that showed bands by gel electrophoresis. When sent out for sequencing, three out of the four isolates (2014-3, 2015-1, and 2016-16) returned high-quality sequences. 2014-3 and 2015-1 were concluded to be

species of *Bacillus*; 2016-16 was found to have an equal chance of being a species of *Enterobacter* or *Raoultella*, while 2015-27 returned only poor quality sequencing results. We speculate that 16S rRNA sequence of isolate 2015-27 differs at the locations where our primers anneal, resulting in poor annealing and poor quality sequence. Future efforts to identify this isolate could include designing and testing new primers for isolate 2015-27, perhaps based on the short stretches of sequence obtained that were of high enough quality to be interpretable. Biochemical tests, such as the triple sugar iron test or starch hydrolysis test, can be used to help find the best matching similar bacteria to guide creation of different primers. Identification of the 2015-27 bacteria would also be necessary to confirm that it is non-pathogenic and therefore safe enough to use for future research that involved liquid culture.

Future efforts should also focus on identifying the inhibitory substance produced by isolate 2015-27. These efforts could include fractionation of the extracts and identification of the components of inhibitory fractions by high performance liquid chromatography (HPLC) and mass spectrometry. The extract would also need to be tested against *M. tuberculosis* to see if its components have active antimicrobial behavior against it. Using the antimicrobial components of the extracts to select for resistant *M. smegmatis* would potentially allow for identification of the antibiotic target by using comparative sequencing of sensitive and resistant strains.

# Appendix A: tables of information on isolates

Table 1: isolate information from 2014

| Number | Genus | Description | Did it inhibit *M. smegmatis?* |
|---|---|---|---|
| 1 | Unknown | Medium yellow, small round colonies | yes |
| 2 | Unknown | Yellow/white, colony all together on plate, smooth | yes |
| 3 | Bacillus | White, medium sized round colonies, smooth | yes |
| 4 | Unknown | Medium yellow, medium round colonies | no |
| 5 | Unknown | Grows extremely quickly. Looks like a singular white/yellow colony but could be very large colonies grouped together | no |
| 6 | Unknown | none given | no |
| 8 | Unknown | none given | no |
| 11 | Unknown | none given | no |
| 12 | Unknown | none given | no |
| 13 | Unknown | none given | no |
| 14 | Unknown | white, small round colonies | yes |
| 15 | Unknown | Grows extremely quickly. Looks like a singular white/yellow colony but could be very large colonies grouped together | no |
| 16 | Unknown | White, small rod shaped colonies | yes |
| 17 | Unknown | White/yellow, small round colonies | yes |
| 19 | Unknown | White, medium round colonies | no |
| 20 | Unknown | White/yellow, looks like singular colony but might be small rod shaped colonies | no |
| 21 | Unknown | none given | no |
| 22 | Unknown | none given | no |
| 23 | Unknown | none given | no |
| 24 | Unknown | none given | no |
| 26 | Unknown | White round colonies | yes |
| 27 | Unknown | none given | no |
| 29 | Unknown | none given | no |

Table 2: Isolate information from 2015

| Number | Genus | Description | Did it inhibit *M. smegmatis?* |
|---|---|---|---|
| 1 | Bacillus | Medium yellow rod shaped colonies | yes |
| 2 | Unknown | White/yellow small round colonies | no |
| 3 | Unknown | White rod shaped colonies | no |
| 4 | Unknown | none given | no |
| 5 | Unknown | none given | no |
| 6 | Unknown | none given | no |
| 7 | Unknown | none given | no |
| 8 | Unknown | none given | no |
| 10 | Unknown | none given | no |
| 11 | Unknown | none given | no |
| 14 | Unknown | none given | no |
| 18 | Unknown | none given | no |
| 19 | Unknown | White medium round colonies | no |
| 20 | Unknown | none given | no |
| 21 | Unknown | none given | no |
| 24 | Unknown | none given | no |
| 25 | Unknown | none given | no |
| 26 | Unknown | none given | no |
| 27 | Unknown | Medium yellow, medium round colonies | yes |
| 28 | Unknown | white/yellow small round colonies | yes |
| 32 | Unknown | Medium yellow, medium round colonies | yes |
| 36 | Unknown | none given | no |
| 37 | Unknown | none given | no |
| 38 | Unknown | none given | no |
| 39 | Unknown | none given | no |
| 43 | Unknown | none given | no |
| 44 | Unknown | none given | no |

| | | | |
|---|---|---|---|
| 56 | Unknown | none given | yes |
| 68 | Unknown | none given | no |
| 83 | Unknown | none given | no |

Table 3: Isolate information from 2016

| Number | Genus | Description | Did it inhibit *M. smegmatis* |
|---|---|---|---|
| 1 | Unknown | None given | no |
| 2 | Unknown | None given | no |
| 3 | Unknown | None given | no |
| 4 | Unknown | None given | no |
| 5 | Unknown | None given | no |
| 6 | Unknown | None given | no |
| 7 | Unknown | None given | no |
| 8 | Unknown | None given | no |
| 10 | Unknown | None given | no |
| 11 | Unknown | None given | no |
| 12 | Unknown | None given | no |
| 13 | Unknown | None given | no |
| 14 | Unknown | None given | no |
| 15 | Unknown | None given | no |
| 16 | Enterobacter or Raoultella | small white colonies, grows in large clumps | yes |
| 17 | Unknown | None given | no |
| 18 | Unknown | None given | no |
| 19 | Unknown | None given | no |
| 20 | Unknown | None given | no |
| 21 | Unknown | None given | no |
| 22 | Unknown | white small round colonies | yes |
| 23 | Unknown | None given | no |

# Appendix B: Agar plate Protocol

1) 20 g dry LB agar powder
2) 500 ml distilled water
3) Stir rod
4) 500 ml beaker
5) Empty petri dishes

20 g of dry agar powder was weighed out and poured into a 500 ml beaker containing a stir rod. The beaker was then filled with 500 ml of distilled water, and contents were mixed using a stir plate until the mixture was smooth and contained no clumps. The mixture was then autoclaved for 30 minutes at 120°C to sanitize and destroy any bacteria. The hot agar mix was then poured into individual petri dishes, covered, and left for 1 hour at room temperature. After cooling, plates were stored in a 4°C refrigerator until needed.

# Appendix C: *M. smegmatis* and Bacterial Isolates Stock Plates Protocol

1) 200µl *M. smegmatis* aliquot
2) Bacterial isolates (previously plated) (Microbes to Molecules)
3) LB agar plates
4) Plate spreader
5) Ethanol
6) Inoculation loop

Stocks of *M. smegmatis* and the bacteria from the *Small World* lab were taken from previously prepared frozen aliquots to create personal stocks for use in this project. A sterilized metal bacteria spreader was dipped in 70% ethanol and dried with a kimwipe to sterilize. It was then used to spread 200µl of *M. smegmatis* onto a previously prepared agar plate, and the plate was set aside to dry. A flame sterilized inoculation loop was then used to lift bacteria from previously prepared *Small World* bacteria plates and then spread onto agar plates, which were sectioned and numbered on the outside with permanent marker. The loop was flame sterilized between each bacteria spread. These plates, including the *M. smegmatis* plate, were stored in a 37°C for 3 days and then moved into a 4°C refrigerator until needed.

# Appendix D: *M. smegmatis* Aliquot Stocks Protocol

1) *M. smegmatis* Plate
2) 20 ml 7H9 + Tween Medium
3) Inoculation loop
4) 1.5 ml eppendorf tubes

Liquid cultures of *M. smegmatis* was grown and stored for easier plating and experimentation. To do this, a flame sterilized inoculation loop was used to lift several colonies off the plate containing grown *M. smegmatis* and gently mixed into the 7H9 + Tween medium. This mixture was then stored in a 37°C shaker overnight until the optical density (OD) of the bacteria was about 0.7. Once this OD was reached the mixture was separated into 1.5 ml eppendorf tubes, labeled, and stored in a -80°C freezer.

# Appendix E: *M. smegmatis* and Bacterial Isolates Inhibition Plates protocol

1) *M. smegmatis* aliquots
2) Bacterial isolates plates (Microbes to Molecules)
3) LB agar plates
4) Plate spreader
5) Ethanol
6) Inoculation loop

To determine which of the mystery bacteria successfully inhibit the growth of *M. smegmatis*, the bacteria was plated together with the *M. smegmatis*. To do this, agar plates were spread with 200µl each of the aliquoted *M. smegmatis* using a plate spreader (sterilized with ethanol) and allowed to dry for 5 minutes. Once dry, a flame sterilized inoculation loop was used to lift colonies of the mystery bacteria from the stock plates and spread on top of the dry *M. smegmatis*. These plates were then stored in a 37°C incubator for 3 days and then viewed. If there was a distinct empty space between the mystery bacteria and the lawn of *M. smegmatis*, it indicated the bacteria was successful in inhibiting *M. smegmatis* growth.

# Appendix F: Colony PCR protocol

1) Soil isolate streak plates
2) Inoculation loop
3) 1.5 mL tubes
4) Pipet and pipet tips
5) Distilled water
6) TAQ master mix (QIAGEN)
7) 27 forward primer (IDT)
8) 1492 reverse primer (IDT)
9) Thermocycler

The first step for colony PCR was to scrape several colonies of the bacteria into 0.2 mL PCR tubes containing 100µL of distilled water. This mixture was then boiled in the thermocycler at 80℃for 10-15 minutes. 12.5µL of TAQ master mix was added to four 0.2mL PCR tubes. 2 µL of each boiled sample was placed in their labeled PCR tube. 1µL of forward primer and 1µL of reverse primer was added to each solution. 8.5µL of distilled water was then added and the solutions were mixed by pipetting up and down several times. The samples were then moved to the thermocycler. The thermocycler was set up to run the PCR protocol for 33 cycles at 95℃for 30 seconds, 54℃for 45 seconds, and 72℃for 90 seconds, and a final hold at 4℃until it was taken it out.

# Appendix G: DNA Extraction Protocol

10) Soil isolate streak plates
11) Inoculation loop
12) 1.5 mL eppendorf tubes
13) Pipet and pipet tips
14) Distilled water
15) Cell suspension liquid *
16) Cell lysis solution*
17) Protein precipitation solution*
18) Isopropanol
19) 70% ethanol
20) Heat block
21) Centrifuge
22) Room temperature shaker

*provided by QIAGEN

DNA extraction was completed following an adaptation of the protocol provided by the Gentra puregene kit produced by QIAGEN. Using a 200µL pipet tip several colonies of a bacteria was scraped and mixed, by pipetting up and down, into 300µL of Cell Suspension Solution in a clean 1.5 mL eppendorf tube. This solution was then centrifuged for 1 minute at 14,000 x g. The supernatant was discharged into a waste beaker. Then 300µL of Cell Lysis Solution was pipetted up and down to lyse the cells in the pellet. The sample was incubated at 80℃ for 5 minutes in the heat block. The sample was then put on ice for 1 minute to quickly cool the sample. 100µL of Protein Precipitation Solution was added then the solution was vortexed for 20 seconds at high speed. The sample was then centrifuged for 3 minutes at 14,000 x g. 300µL of isopropanol was pipetted into a new 1.5 mL eppendorf tube. The supernatant from the previous tube was added by carefully pipetting. This was mixed by inverting gently 50 times. Then the new tube was centrifuged for 1 minute at 14,000 x g. The supernatant was discarded and the pellet of DNA was washed with 300 µL of 70% ethanol. The sample was centrifuged again for 1 minute at 14,000 x g. The supernatant was discarded and the pellet was dried for 5 minutes then 100µL of distilled water was added. The sample was vortexed and incubated at 65℃ for 1 hour to dissolve the DNA. The dissolved DNA sample was left on the room temperature shaker overnight.

# Appendix H: DNA PCR Protocol

1) 0.2 mL PCR tubes
2) DNA extraction samples
3) TAQ master mix (QIAGEN)
4) 27 forward primer or sss969 forward primer (IDT)
5) 1492 reverse primer or sss967 reverse primer (IDT)
6) Distilled water
7) Thermocycler

In order to complete the PCR of the DNA that was extracted from the samples, the mixing proportions provided by QIAGEN was followed. 12.5µL of TAQ master mix was added to 4 0.2mL PCR tubes. 2 µL of each sample was placed in their labeled PCR tube. 1µL of forward primer and 1µL of reverse primer was added to the solution. 8.5µL of distilled water was then added and the solution was mixed by pipetting up and down several times. The samples were then moved to the thermocycler. The thermocycler was set up to run the PCR protocol for 33 cycles at 95℃for 30 seconds, 44℃for 45 seconds, and 72℃for 90 seconds, and a final hold at 4℃until it was taken out.

# Appendix I: Gel electrophoresis protocol

1) Agarose powder (provided by Michael Buckholt)
2) 1X TAE buffer
3) 150 ml Erlenmeyer flask
4) Gel tray
5) Well comb
6) Gel box
7) Electrodes
8) Voltage source
9) PCR samples
10) Hyperladder
11) SYBR green

In order to see if the PCR produced amplified DNA, gel electrophoresis was run on the samples. A 1% agarose gel was made by mixing 0.5 g of agarose powder with 50mL of 1X TAE Buffer in a 150 ml Erlenmeyer flask and microwaving until the powder dissolved. Once the flask cooled to the touch, the 1% agarose solution was poured into the gel tray with a well comb attached. The agarose solution was allowed to harden for 30 minutes at room temperature. The well comb was removed and the gel was repositioned in the gel box. Then 1X TAE buffer was poured into the gel box until it covered the agarose gel. The 6μL of the PCR sample was mixed with 1μL of SYBR green. The hyperladder was always loaded in lane 1 then the samples were loaded in the other lanes. The gel was run for 30 minutes at 100 volts.

# Appendix J: Secretion Extraction protocol

## Part 1

1) One plate of each soil isolate (4 total: 2014-3, 2015-1, 2015-27, 2016-16)
2) 100 mL glass bottle with cap (1 per isolate)
3) Microspatula
4) Liquid nitrogen OR -80°C freezer
5) Methanol
6) 20 mL glass bottle (1 per isolate)
7) Fume hood

An extraction was performed in order to determine if the isolates had secretions that were the cause of *M. smegmatis* inhibition. One plate of each type of soil isolate was used, grown previously on LB agar. Each plate had a dense lawn of isolates. A microspatula was used to cut the plate into small pieces (about 1 $Cm^2$). All pieces were scooped into labeled 100 mL glass bottle (a separate bottle for each isolate). The bottles were frozen in liquid nitrogen; alternatively, bottles can be frozen overnight in a -80°C freezer. After freezing, 12 mL of methanol was added to each bottle in a fume hood. The bottle was capped and placed on shaker at room temperature for 48 hours. The extract was then allowed to dry down via evaporation (uncapped bottle in fume hood).

## Part 2

1) Dried-down extract in glass bottle (1 per isolate)
2) Media plates (LB agar)
3) *M. smegmatis* culture
4) Methanol
5) Filter disks (autoclaved)
6) Rifampin (Concentrations of 50 mg/ml, 10 mg/ml and 1 mg/ml)
7) DMSO (negative control)
8) Sterile 15 mL test tube
9) Micropipette and tips (P20 and P200)

Dried-down extracts were resuspended in 80µL of methanol. Four agar plates were spread with 200µL of *M. smegmatis* and allowed to dry for 3-5 minutes. Plate one was divided into quarters; the first quarter was given a filter disk soaked with 30 ml Rifampin at 50 mg/ml concentration, the second quarter was given a filter disk soaked with 30 ml Rifampin at 10 mg/ml

concentration, the third quarter was given a filter disk soaked with 30 ml Rifampin at 1 mg/ml concentration, and the fourth quarter was left with only *M. smegmatis.* Plate two was divided in half; the first half was given a filter disk soaked with methanol, and the second half was given a filter disk soaked with DMSO. Plate three was divided in half; the first half was given a filter disk soaked with resuspended extract from 2014-3, and the second half was given a filter disk soaked with resuspended extract from 2016-16. Plate four was divided in half; the first half was given a filter disk soaked with resuspended extract from 2015-1, and the second half was given a filter disk soaked with resuspended extract from 2015-27. The plates were labeled and incubated at 37°C for 3 days. Plates were then observed for zones of inhibition.

# Appendix K: sequences of isolates

| Isolate | sequence |
|---|---|
| 2014-3 Sequencing primer: sss967 | TACCTATCCCTCTTTTAGCGGCTCATTTCTTACGCTGTAACCGGGCTGGCTGCCTGTCC<br>ACGACCGACGCCCATTGACGA<br>AAGACACTTATGAAGGTAATGTAACGTCGCAGTTCGATGCGTCCCATTTCCCATTATT<br>GACTATAGCGGACGGTGCCTGC<br>GATTGATATGACGCCTCCTTTATATAATTCCACCACTAAACCACGCCACTCAGTTAGA<br>TGTCCCCCAGTAATTACCTACG<br>CTATTTGGATTAAAAAAAGTTAATGTCAGTTAGGGGTTGGGGCGGGCGGTGCGGCGA<br>TTCTCGGGGCTGCAACATTAAAG<br>TTGTTTTGTATGCAAGACCAACTCGACCTCCCTTTGTAATATTTTATTAGTTAAAATGG<br>CTAATAGCCATGAGAAAACTT<br>TAGCCTTGTTTTAGCCCTAGA |
| 2015-1 Sequencing primer: 27F | NNATGGCGAGCGAGCATCATACATGCAGTCGAGCGAATGGATTGAGAGCTTGCTCTC<br>ATGAAGTTAGCGGCGGACGGGTG<br>AGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTA<br>ATACCGGATAACATTTTGAACTG<br>CATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGC<br>ATTAGCTAGTTGGTGAGGTAACG<br>GCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGAC<br>TGAGACACTACAAAAAACTTATA<br>CGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGC<br>CGCGTGAGTGATGAAGGCTTTCGG<br>GTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTT<br>GACGGTACCTAACCAGAAAGCCA<br>CGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAA<br>TTATTGGGCGTAAAGCGCGCGCA<br>GGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGA<br>AACTGGGAGACTTGAGTGCAGAA<br>GAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGGAGGAACA<br>CCAGTGGCGAAGGCGACTTTCTGG<br>TCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGGAGCAAACAGGATTAGATACCCT<br>GGTAGTCCACGCCGTAAACGATG<br>AGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACT<br>CCGCCTGGGGAGTACGGGCCGC<br>AAGGCTGAAACTCAAAGGGAATTGACGGGGGCCCGGCACAAGCGGTGGAGCATGTG<br>GTTTAAATCCGAAGCAACGCGAAG<br>AACCTTACCAGTCTTGACATCCTCTGAAACCTAGAGATAGGGCTTCTCTTCGGGAGCA<br>GAGGAAGGGGGGTGCATGGTGT<br>CGTCAGCTCGGGCCGGAGGAAGTTGGGATAGGTCCGCGAACGAGCGCCACCCTTTGA<br>TCTTATGCAATCTTAAATGGCAC<br>TNCTAAGAGGGACGGCGGGATG |
| 2015-27 | CGCTTTCAGCTTTTTTAGGGCATCGAAGTGTCGTCCCTCGACATGTGTTCTTTTGTCCA<br>CTCTTTTGATGCCTTTCCAGC |

| | |
|---|---|
| Sequencing primer: 27F | AGGAAAGTCTATCCGGTGTGTACTGGCCATAATGCCATGCATCCCCTTTAACGTAATT<br>GACGTCAATACGGTGGGTACTT<br>TTCATATGATAAACTAGATGTCCTGAAAATTTATCAGTTCACCTTGATACTCCACCCA<br>TTGACGTTTTGGAATGCCCTAT<br>TGCAGTGGTGTAGGATATACCTCCTTATTTTCGCCAATGGGCGGGGGTCGTCCTTCTT<br>CAGGAAAGCGCTCCATTTACCT<br>TATGCGATGTAACGGACTCTAACTCTAACCAACTCCATCCCCTGCTCATTGTGTGTTA<br>TGGAGAGGGGGCCTTTTTCATA<br>TGATTCCTTCATAGTTGTTTATTTC |
| 2016-16 Sequencing primer: 27F | NGNAGTGGCGGCAGGTCTACACATGCAGTCGAGCGGTAGCACAGAGAGCTTGCTCTC<br>GGGTGACGAGCGGCGGACGGGTG<br>AGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAA<br>TACCGCATAACGTCGCAAGACCA<br>AAGTGGGGGACCTTCGGGCCTCATGCCATCAGATGTGCCCAGATGGGATTAGCTAGT<br>AGGTGGGGTAATGGCTCACCTAG<br>GCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGG<br>TCAAAACACCTACGGGAGGCAGC<br>AGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAA<br>GAAGGCCTTCGGGTTGTAAAGTA<br>CTTTCAGCGAGGAGGAAGGCGTTAAGGTTAATAACCTTGGTGATTGACGTTACTCGC<br>AGAAGAAGCACCGGCTAACTCCG<br>TGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTA<br>AAGCGCACGCAGGCGGTCTGTCA<br>AGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCT<br>AGAGTCTTGTAGAGGGGGGTAGA<br>ATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGC<br>GGCCCCCTGGACAAAGACTGACG<br>CTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCG<br>TAAACGATGTCGACTTGGAGGTT<br>GTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGGAGT<br>ACGGCCGCAAGGTTAAAACTCAA<br>ATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACG<br>CGAAGAACCCTTACCTACTCTTG<br>ACATCCAGAGACTTAGCAGAGATGCTTTGGTGCCTTCGGGAACTCTGAGACAGGTGC<br>TGCATGGCTGTCGTCAGCTCGTG<br>TTGTGAAATGTGGGGTAAGTCCCGCAACGAGCGCCACTCTTATCTTTGTGGCAGCGGT<br>CGGCCGGACTCAAAGGAGACTG<br>CAGTGGATAACTGANAAGTGNTGACGTCAGGTCATCATGGCNTACGAGTAGGGCTAT<br>CACACGTGCTACATGCATTACAA<br>AGAAGGNNGC |

# Bibliography


Akinola RO. A Systems Level Comparison of Mycobacterium tuberculosis, Mycobacterium leprae and Mycobacterium smegmatis Based on Functional Interaction Network Analysis. Journal of Bacteriology & Parasitology. 2013;04(04).

Alam MA, Arora K, Gurrapu S, Jonnalagadda SK, Nelson GL, Kiprof P, Jonnalagadda SC, Mereddy VR. Synthesis and evaluation of functionalized benzoboroxoles as potential anti-tuberculosis agents. Tetrahedron. 2016;72(26):3795–3801.

Barbier M, Wirth T. The evolutionary history, demography, and spread of the mycobacterium tuberculosis complex. microbiolspec. 2016;4(4).

Calligaro GL. Drug-resistant tuberculosis. CME: Your SA Journal of CPD. 2013;31(9).

Drug-Resistant TB. Centers for Disease Control and Prevention. 2016 May . https://www.cdc.gov/tb/topic/drtb/

Handayuthapani S1, Zhang Y, Mudd MH. 1996. Oxidative stress response and its role in sensitivity to isoniazid in mycobacteria: characterization and inducibility of ahpC by peroxides in Mycobacterium smegmatis and lack of expression in M. aurum and M. tuberculosis.. PubMed (3641-9)

Henkel JS, Baldwin MR, Barbieri JT. Toxins from bacteria. Experientia Supplementum Molecular, Clinical and Environmental Toxicology. 2010:1–29.

Hibbing ME, Fuqua C, Parsek MR, Peterson SB. Bacterial competition: surviving and thriving in the microbial jungle. Nature Reviews Microbiology. 2009;8(1):15–25.

Kalab M. Mycobacterium smegmatis. Mycobacterium smegmatis. 2007 May [accessed 2016 Oct 3]. http://www.usu.edu/westcent/microstructure_food/myco-smegma.htm

Ling Lin P, Flynn JAL. Understanding Latent Tuberculosis: A Moving Target. The Journal of Immunology. 2010;185(1):15–22.

Lubran MM. Bacterial toxins. Annals of Clinical & Laboratory Science. 1988;18(1):58–71.

Olejníková P, Kurucová M, Švorc LU, Marchalín Š. Induction of resistance in Mycobacterium smegmatis. Canadian Journal of Microbiology. 2013;59(2):126–129.

Pawlowski A, et al. Tuberculosis and HIV co-infection. PLoS Pathogens. 2012;8(2).

Rozwarski DA, Grant GA, Barton DHR, Jacobs WIR, Sacchettini JC. Modification of the NADH of the Isoniazid Target (InhA) from Mycobacterium Tuberculosis. Science. 1998;279:98–102.

Sharma P, Kumar B, Gupta Y, Singhal N, Katoch VM, Venkatesan K, Bisht D. Proteomic Analysis of Streptomycin Resistant and Sensitive Clinical Isolates of Mycobacterium Tuberculosis. Proteome Science . 2010;8(1).
Silve G, Valero-Guillen P, Quemard A, Dupont MA, Daffe M, Laneelle G. Ethambutol inhibition of glucose metabolism in mycobacteria: a possible target of the drug. Antimicrobial Agents and Chemotherapy. 1993;37(7):1536–1538.

Wang X, Grace PM, Pham MN, Cheng K, Strand KA, Smith C, Li J, Watkins LR, Yin H. Rifampin inhibits Toll-like receptor 4 signaling by targeting myeloid differentiation protein 2 and attenuates neuropathic pain. The FASEB Journal. 2013;27(7):2713–2722.

World Health Organization. Global Tuberculosis report. 20th ed. Geneva, Switzerland: World Health Organization; 2015.

Zumla A1 A1, Chakaya  J2, Centis R3, D'Ambrosio L3, Mwaba P4, Bates M4, Kapata N5, Nyirenda T6, Chanda D4, Mfinanga S7, Hoelscher M8, Maeurer M9, Migliori GB3.. 2015 Mar 9. Tuberculosis treatment and management--an update on treatment regimens, trials, new drugs, and adjunct therapies. 220-34. U.S National Library of Medicine:PubMed; [cited 2016 Sept 28] .