A Novel Investigation of the Interactions between Caenorhabditis elegans and Mycobacterium smegmatis.

Mycobacterium tuberculosis (Mtb) is acquiring resistance to known antibiotics due to its lipid-rich cell wall consisting of mycolic acids. The research investigated *C. elegans* as a source of antibiotics to inhibit the *Mtb* cell wall. *Mycobacterium smegmatis (Msm)* was used instead of *Mtb* because *Msm* is non-pathogenic and faster growing. *C. elegans* were grown in *Msm* cultures in ten different experimental set-ups, organized into three phases, to study the interactions between the *C. elegans* and *Msm*. The research identified four new phenomena that support *C. elegans* as a source for antibiotics. Phase 1 results demonstrated that *C. elegans* were able to convert *Msm* into a food source and thrive in the presence of *Msm*. Phase 2 results supported inhibition of *Msm* by *C. elegans*. Zones of inhibition were observed along with a change in the morphology of *Msm* colonies. Phase 3 results demonstrated that *C. elegans* damaged the *Msm* cell wall. Some trials showed *Msm* colonies adopting a wet texture, indicating degradation of *Msm* lipids. Additionally, staining of worms and PCR tests supported a new phenomenon: *Msm* appeared to be binding to the lipid cuticle of *C. elegans*. The results demonstrate that this novel investigation is a step forward in understanding how to inhibit mycobacterial growth.

Introduction:

Mycobacterium tuberculosis (Mtb) is the causative agent of tuberculosis (TB). Its cell wall is uniquely rich in lipids, namely mycolic acids and it is believed that this property contributes to the resistance of *Mtb* (2). The alpha and beta fatty acids of the mycolic acids make the cell wall impermeable to chemical compounds (9). Current antibiotics, such as Isoniazid target mycolic acid synthesis (19). However, most current antibiotics are ineffective in many TB patients because *Mtb* is able to adapt and circumvent the treatment. Consequently, there is a need for new antibiotics or even new sources of antibiotics to combat the prolific disease.

Our research focuses on determining if *Caenorhabditis elegans* are a potential source for new antibiotics. The inspiration for the research came from an experiment completed in the 1950's where an unknown microbiologist decided to expose *Mtb* bacilli to earthworms (14). Although the results seemed inconclusive, the scientist believed that the worms were inhibiting *Mtb*. However, the study was discontinued as other research was prioritized (14).

We decided to pursue the experiment. However, earthworms would be impractical to use because of their size, slow reproduction, and the inability to see inside the worm. Additionally, *Mtb* would be difficult to use because of its slow reproduction rate and BSL-3 rating. Therefore, the research had to replace earthworms and *Mtb* with better suited organisms.

Caenorhabditis elegans replaced earthworms. *C. elegans* are non-parasitic, transparent nematodes that live in temperate soil environments, similar to earthworms (15). *C. elegans* are 1mm long and rapidly reproduce in large numbers. Because the nematode is transparent, it is easier to use genetic markers to identify bacteria inside the worm without the need of an electron microscope.

Mycobacterium smegmatis (Msm) replaced *Mtb* in the research. The *Msm* cell wall shares over 2,000 common homologs with the *Mtb* cell wall. Additionally, *Msm* reproduces every 2-3 days, making it feasible to perform many trials (9)**.** *Msm* is classified as BSl-2, which made it feasible to use for our research. Some may be skeptical of using a less virulent isolate of *Mtb* to find an antibiotic for *Mtb*. However, the research focuses on the cell wall, which means it is irrelevant if the bacteria used have the same pathogenic properties as *Mtb*.

The focal point of the research is to investigate the interactions between *C. elegans* and *Msm.* This novel research hopes to study variables such as survivability, reproduction, and cell wall structure of *Msm* when exposed to *C. elegans*. By understanding the interactions between the two organisms, it can be determined if *C. elegans* are a source for antibiotics.

The literature investigating the interactions between *C. elegans* and *Msm* is very sparse. One study examined whether or not *C. elegans* moved towards *Msm*, but it does not answer whether or not *C. elegans* are a source for antibiotics (18). Another study investigated fruit flies as models for *Mycobacterium*-host interactions (13). Similarly, other studies that focused on *C. elegans* did not study *Msm* in conjunction with *C. elegans*. In addition, very few TB research labs are looking at organisms as potential source for antibiotics, making the approach of this research novel.

The methodology of this project lies in three phases; each phase was dedicated to answering a specific question. The answers to all three phases would allow us to answer the nexus question: Are *C. elegans* a potential source of *Mtb* antibiotics?

Phase 1: Do *C. elegans* thrive in the presence of *Msm*? If so, are *C. elegans* eating *Msm*? By determining if *C. elegans* thrive in the presence of *Msm* by eating the bacteria, we can determine both a novel food source for *C. elegans* and whether or not the digestive tract is playing a role in

cell wall degradation. The hypothesis for phase 1 is: If exposed to *Msm, C. elegans* will thrive by converting the *Msm* to a food source.

Phase 2: Does *Msm* thrive in the presence of *C. elegans*? By determining that *Msm* is being inhibited by *C. elegans*, we can confidently assert that *C. elegans* may harbor an antibiotic that inhibits *Mtb*. Hypothesis for phase 2 is: If exposed to *C. elegans*, *Msm* will not thrive and instead will be inhibited by *C. elegans*.

Phase 3: Are *C. elegans* damaging the cell wall of *Msm*? The main question to determine whether or not *C. elegans* are a source for an antibiotic is to determine if *C. elegans* cause cell wall degradation. Hypothesis for phase 3 is: *C. elegans* are damaging the cell wall of *Msm* by either eating the lipids found in the cell wall or secreting a chemical compound.

Three important terms to define in the context of the research are "thrive", "damage", and "inhibit". "Thrive" is defined as being able to reproduce and develop similar to the control group. "Damage" is defined as harming or breaking down. "Inhibit" is defined as preventing growth.

By understanding and documenting the interactions between the two organisms, it can be determined whether or not *C. elegans* are a potential source for antibiotics. The answer to the above questions will allow further research to potentially identify novel antibiotics found in *C. elegans* to combat tuberculosis.

Materials & Methods:

Note: The protocols below are used to explain the general experimental setup. Discussion of the procedure in our own words is included below these protocols.

Preparing *C. elegan* growth media - *C. elegans* provided by Carolina Biological

1. *Preparation of Food Source*
    a. Obtain a starter culture of *E.coli* OP50.

b. With a loop, inoculate an air tight LB nutrient broth tube with the *E. coli*

c. Allow the inoculated culture to grow overnight at room temperature (37 C)

2. *Preparation of NGM plates*

a. Obtain NGM (nematode growth medium) agar and petri plates- Fill petri plates ⅔ full with NGM agar

b. Apply 0.05 mL of E. coli OP50 to small plate of prepared NGM agar or 0.1 mL of E. coli to large plate of prepared NGM agar. Spread the *E. coli* with sterile spreaders.

Preparing *M. smegmatis* culture

1. *Making 7H10 Agar Medium (for 1 L)*

a. Place 19g 7H10 agar base, 12.5mL 40% glycerol, and 890mLMillipore water in 1L bottle with loose lid

b. Autoclave at 121 degrees Celsius, 25 minutes, liquid cycle

c. Before pouring the plates, cool the solution down in a water bath to 50 degrees

d. (Optional) Add 100mL OADC or kanamycin to solution after cooling

e. Pour 25 mL per plate and let dry. Avoid air bubbles.

2. *Preparing M. smegmatis culture plates.*

a. Pipette 30-50 uL of bacteria culture from an inoculated tube onto the agar plate.

b. Use an inoculator loop or L-spatula to spread *M. smegmatis* around the plate in the desired areas. For example, full lawn, or place specific areas of colonies in four different corners, etc.

c. Parafilm and place in incubator at 37 degrees Celsius for 2-3 days

d. Keep *M. smegmatis* bacteria culture plates at 4 degrees Celsius for later use

Cleaning *C. elegans*:

1. With autoclaved water, pipette worms from a plate into an Eppendorf tube containing PBS buffer.
2. Centrifuge at 2000 RPM for 5 minutes. Repeat 3 more times. Pipette out the supernatant.

Transferring *C. elegans* to *M. smegmatis* plate (15):

1. Note- When transferring the *C. elegans* to the *M. smegmatis* plate, make sure no *E. coli* is placed on the *M. smegmatis* plate.
2. *Chunking:*
    a. Use a sterilized scalpel to move a chunk of agar to a new plate
    b. Place agar on the plate and let the worms crawl off the agar
    c. Remove the chunk of agar once all worms have crawled off of it
3. *Pipetting:*
    a. Under a bacteria hood, pipette autoclaved water (~10-30 ul) onto the plate containing *C. elegans.* <u>*Note:*</u> If you are using *C. elegans* cleaned in an Eppendorf tube, you may skip this step.
    b. Pipette the water on the plate back up. The pipette tip should now contain *C. elegans*
    c. Pipette back out onto desired plate

Once the *C. elegans* were on the desired plates, we generally kept them between room temperature to 28 degrees Celsius. Some were kept at 37 C, however *C. elegans* do not reproduce at this temperature, as was observed in the experiment.

Counting *C. elegan* populations:

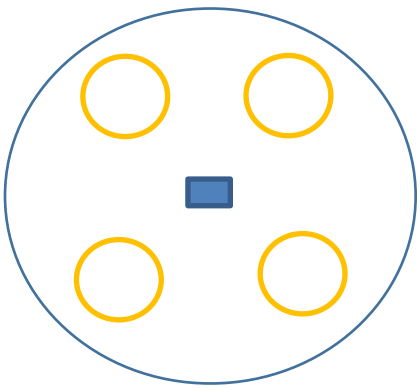
1. Set up a grid on the bottom of a plate containing *C. elegans*.
2. Once a day, go through each grid square and count the population. Add all the grid square populations together to get a rough estimate of population.

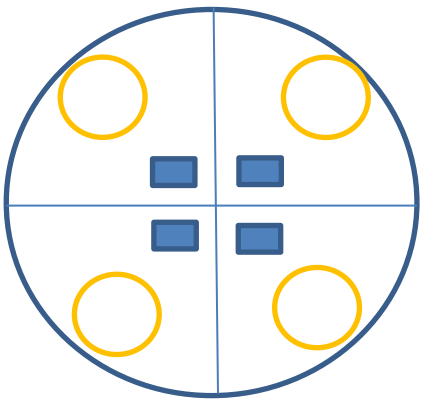
The above procedures were basic methods that were consistently used across most, if not all, of the experimental set-ups. Multiple experimental setups were created to optimize our results.

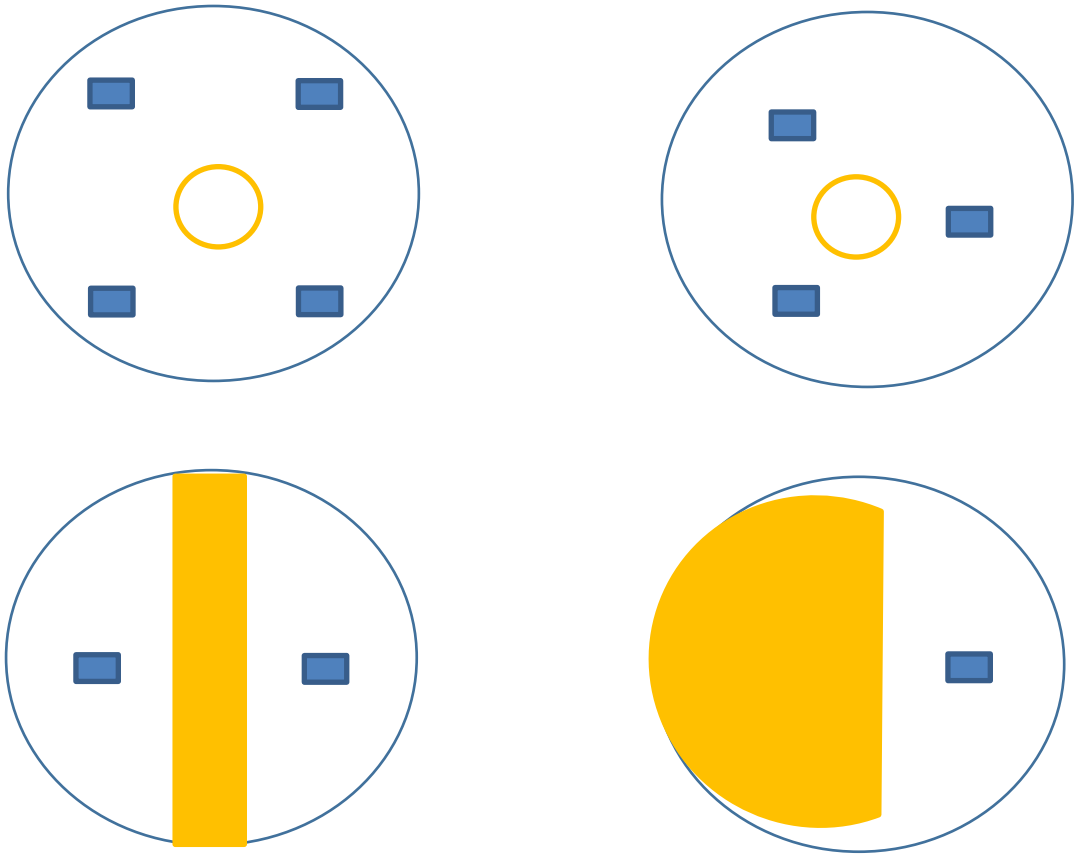
1st Setup: Numerous full-lawn *M. smegmatis (Msm)* plates were created from a saturated inoculum and *C. elegans* were transferred to these plates. As there has been little to no research in this area, we needed to understand the interaction between the two organisms. In addition to using 7h10 agar with OADC, kanamycin plates were made and kanamycin resistant *Msm* were plated onto these plates. There were two controls – a plate with only kanamycin resistant *Msm* and a plate containing *E. coli* with *C. elegans*. All plates were stored at 37 C for 3 days. One plate consisted of plain 7h10 kanamycin agar with no bacteria. *C. elegans* were placed here to see if they could eat the kanamycin medium. This plate was left at room temperature.

2nd setup: Along with the 1st setup, "area trials" were performed. These trials studied how the *C. elegans* and *Msm* interacted when placed at different areas on the plate (e.g. Do *C. elegans* move towards or away from *Msm*? Which direction does *Msm* grow towards?). The following set-ups were used for the area trials:

Legend: Orange circle/area- original seeding point of *Msm* colony. Blue rectangles- *C. elegans* transfer point.







<u>Note</u>: Each area trial set-up had a corresponding control group with no C. elegans. And a control group with E. coli and C. elegans.

All area trials were placed at 37 C for 3 days. As mentioned, these area trials were run at the same time as the initial full lawn trials. We found that most of the *C. elegans* died and failed to reproduce. We inferred the negative result was because 37 C was too warm to sustain *C. elegans* growth. However, the 37 C amplified bacterial trails formed by the *C. elegans*, thus making it easier to track *C. elegans* movements when they were still alive. This unexpected observation of trails of bacteria made by *C. elegans* helped us hypothesize that *Msm* was binding to *C. elegans*.

3rd Setup: A second set of area trials were run. However, in order to find the optimal temperature to grow both organisms together, some plates were grown at 37 C, some at 28 C, and some at room temperature. It was necessary to strike a balance between the *C. elegans* being able to reproduce and the *Msm* growing at a normal rate. Area trials were also set up with

kanamycin resistant *Msm*. It was found that room temperature was the ideal temperature– the *C. elegans* reproduced well and *Msm* still grew at a steady rate, although slower than at 37 C.

4th Setup: Now that the temperature had been optimized, it was necessary to more thoroughly study the interactions between *C. elegans* and *Msm* at different stages of growth. To do this, serial dilutions of *Msm* culture were performed (1:10 to $1:10^{5)}$ – this setup is the "density trials." Each dilution was spread on separate plates as full lawns and *C. elegans* were transferred onto each plate. Each dilution had a corresponding plate with the same dilution but without *C. elegans*. A control for the entire *Msm* density trial involved creating the exact same set-up with dilutions of *E. coli* (1:10 to $1:10^{5)}$ grown on 7h10 media (we observed that *E. coli* could grow on 7h10 media). All plates were kept at room temperature for seven days.

5th Setup: The density trials were repeated a second time. However, the second time, *E. coli* was diluted further. Instead of using *E. coli* dilutions from 1:10 to $1:10^5$, *E. coli* dilutions from $1:10^7$ to $1:10^{11}$ were used, as the *E. coli* colonies dilutions to 1:10 to $1:10^5$ were overgrown. The *Msm* dilutions remained were kept at 1:10 to $1:10^5$. All plates were kept at room temperature for seven days and results were recorded during the time period.

6th Setup: We ran a number of plain agar trials on both 7h10 and NGM media. *C. elegans* were transferred directly from older *Msm* plates onto plain agar plates – either 7h10 or NGM. We allowed a small portion of *Msm* to be transferred along with the *C. elegans* to see if *Msm* would bind to *C. elegans* and start growing far away from the transfer point. Each plate started out with an average of 2-10 *C. elegans*. Since *C. elegans* reproduce quickly, the initial populations were kept small. We pipetted using the worms onto the plates using 10-30 uL of autoclaved water. As a control, plain agar plates with no *C. elegans*, but with small amounts of *Msm* were also set up. All plates were kept at room temperature for 1.5 weeks.

7th Setup: Zones of inhibition were observed in the earlier trials. To determine if the inhibition was caused by secretion of chemical compounds from *C. elegans*, chemicals were extracted from the zones of inhibition via ultrafiltration (Reference: Mentor). Then, we placed a grid over a 7h10 plate and pipetted ~25ul of chemical extract onto different areas marked by the grid. Once the chemical extract dried, *Msm* was spread on top of the chemical extract locations. All plates were left at 37 C for five days.

Across all of the trials, we noticed *C. elegans* binding to *Msm*. To confirm this, the worms were gram stained (4) and Ziehl-Neelsen stained (1), a stain commonly used for mycobacteria (Reference: Mentor). Worms were fixed with formaldehyde onto chamber slides to prevent them from washing off when the staining was performed (Reference: Mentor). In addition, PCR tests were performed during the research to confirm if specific colonies were comprised of *Msm* (Reference: Mentor).

Results and Discussion:

Note: This section contains sets of images and graphs that show results. Each set of images and graphs are followed by analysis and discussion.

Results from Phase 1:

Figure 1.1: This is the control group. C. elegans are shown growing normally in E. coli plates. The clump in the middle shows *C. elegans* reproducing.

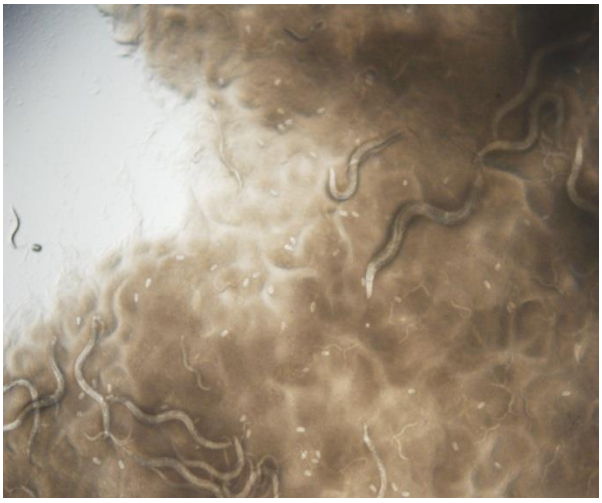


Figure 1.2: This shows *C. elegans* growth in *Msm*. The brown is *Msm* colony. *C. elegans* are traveling throughout *Msm*. *Msm* colony is fragmented- sign of eating.

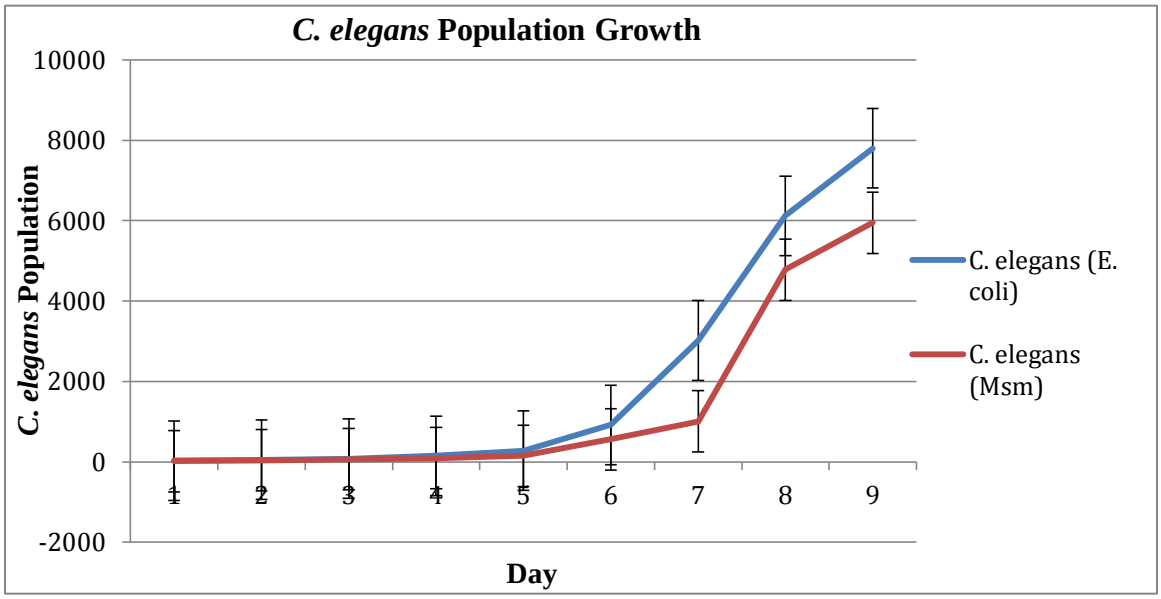


Figure 1.2: This graph measures C. elegans population growth when *E. coli* was the food source and when *Msm* was the food source. The graphs show exponential growth for both experimental settings and then logistical growth towards the end. Standard error bars are shown.

Phase 1 Analysis: The hypothesis for phase 1 was supported. It was determined that *C. elegans* thrive in the presence of *Msm* and exhibit normal development. To determine that *C. elegans* survived amidst *Msm* and were developing normally, *C. elegans* were grown in *Msm* and their development and growth was observed and compared to the control groups. Figure 1.1 shows that *C. elegans* grown in *E. coli* (the control food source) exhibit free movement and appear undeterred by *E. coli*. *C. elegans* appear to clump and develop normally, morphologically speaking. When *C. elegans* were grown in *Msm* plates, they grew similar to the control groups. As shown in figure 1.2, *C. elegans* move freely throughout the *Msm* colony and appear undeterred by the presence of new bacteria. There even appears to be fragmentation of the *Msm* colony to hint at *C. elegans* eating the bacteria. *C. elegans* in both groups exhibited normal neurological responses and their lifespans were also similar. In both groups, reproduction was not hindered as *C. elegans* in both groups laid eggs at regular time intervals.

The results also supported the hypothesis that *C. elegans* are able to convert *Msm* into a good food source. Figure 1.3 shows *C. elegans* population growth when exposed to *E. coli* and when exposed to *Msm*. It is evident that *C. elegans* population growth rate is similar when exposed to *E. coli* and when exposed to *Msm*. From days 1-5, both groups appear to experience slow growth. From days 6-8, there appears to be exponential growth in both groups. An exponential reproduction rate indicates that *C. elegans* are able to convert *Msm* into a food source. After day 8, there appears to be a leveling off in the growth rate, hinting at logistical growth. This is because the food source is running out and *C. elegans* are forced to slow their reproduction rate.

The results of phase 1 make this research particularly unique as no studies have determined *Msm* to be a food source for *C. elegans*. Additionally, very few studies have conclusively determined that *C. elegans* are able to thrive in the presence of *Msm*.

Results from Phase 2:

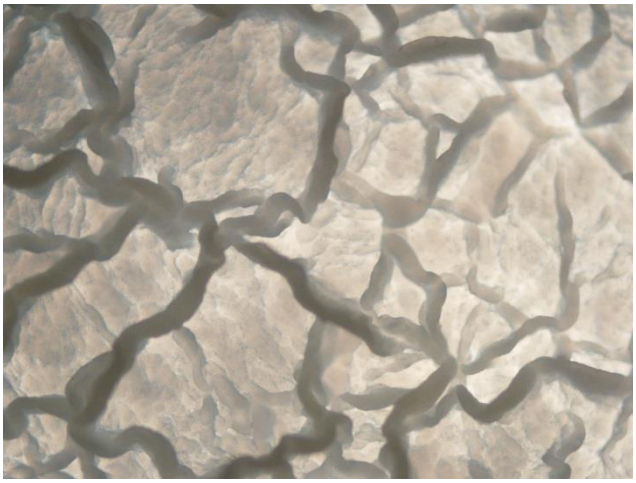


Figure 2.1: This is *Msm* control group. It shows that a normal *Msm* colony is thick and waxy- colony morphology. The black lines are cording of the colony.

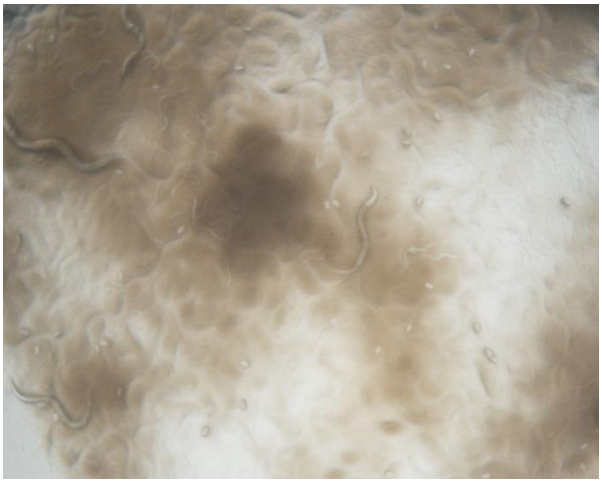


Figure 2.2: This shows *Msm* fragmentation because of *C. elegans*. It's important to note that the colony does not seem thick and there is a dark spot which hints at *C. elegans* consumption. It differs substantially from figure 2.1.

Figure 2.3: This is a control *Msm* colony seeded in the center of the plate. It's thick and waxy, also has an orange color.

Figure 2.4: This is an *Msm* colony like in figure 2.3, but it has been exposed to *C. elegans*. There is a clear zone of inhibition (highlighted by red) with significant fragmentation. Note: there is a difference in lighting between figure 2.3 and this figure.

Phase 2 Analysis: The results from phase 2 supported the hypothesis that *C. elegans* inhibited *Msm* growth and damaged the structure of the *Msm* colonies. There were two important pieces of evidence that substantiated the hypothesis. First, we observed that the morphology of the *Msm* colonies once exposed to *C. elegans* changed significantly. As figure 2.1 shows, the structure of the control *Msm* colony is as follows: thick, waxy, dry, with significant cording and lobate margins. Additionally, the control colonies appear to have an orange-yellow tint to them. All of these properties are a result of high lipid concentration within the cell wall (2). However, in the colonies exposed to *C. elegans*, the colonies appear fragmented (figure 2.2); this is most likely due to *C. elegans* eating *Msm*. The colonies also do not appear thick and have lost their waxy texture. This indicates cell wall degradation because the lipids in the cell wall of *Msm* give it a waxy texture and a lack of lipids would change the properties. The color appears darker and brown which indicates a significant decrease in lipid concentrations within the cell wall (8).

In addition, large zones of inhibition were observed in both kanamycin resistant strains of *Msm* and normal strains of *Msm* when exposed to *C. elegans*. The *Msm* colony in figure 2.4 has a zone of inhibition in the center of the colony. Although the zone is not as clear and distinct, it is still apparent enough to conclude that *C. elegans* are a significant inhibitor of *Msm* growth. The colony in figure 2.4 can be compared to the control colony in figure 2.3. In figure 2.3, the colony has no areas of lack of growth and the colony appears healthy and prolific.

The results of phase 2 are both significant and novel. No studies have observed an inhibition affect caused by *C. elegans* towards *Msm*. This is because few studies have looked at the interaction between the two organisms. These results give us confidence to speculate that *C. elegans* may be a source for anti-microbial compounds that target the cell wall of *Msm.*

Results from Phase 3:

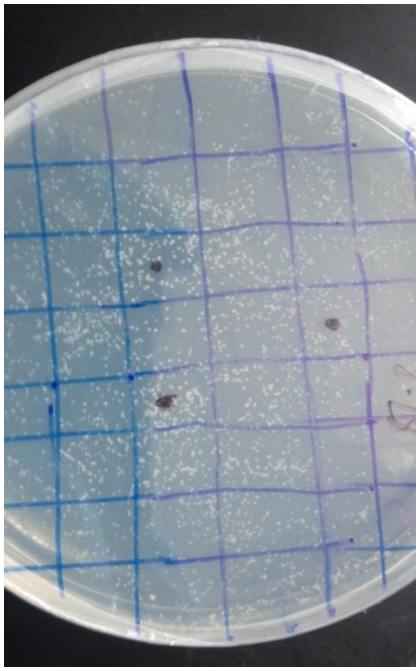


Figure 3.1: This shows a control diluted *Msm* colony. It's evident that there are no wet or gooey spots in the control groups- non-degraded cell wall.

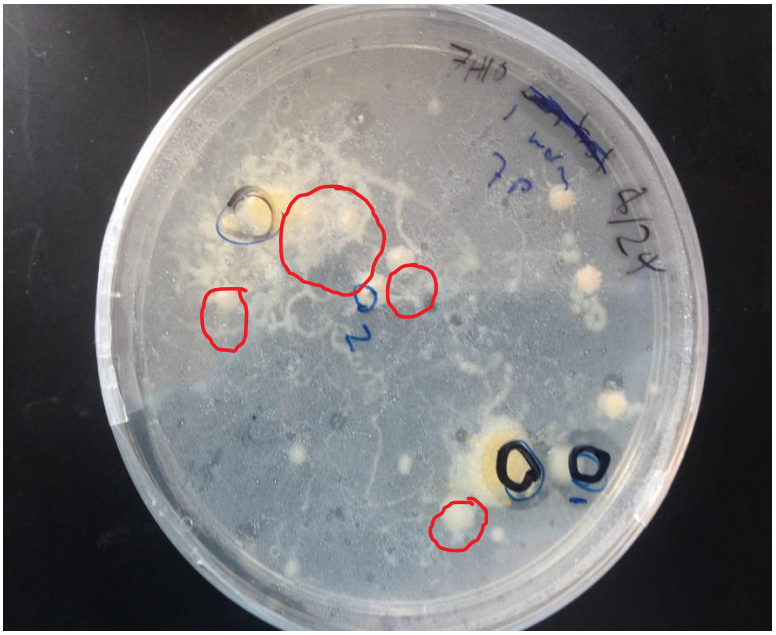


Figure 3.2: The wet spots in the *Msm* colony represent cell wall degradation due to *C. elegans*- the wet spots are a result of lipid breakdown in the cell wall.

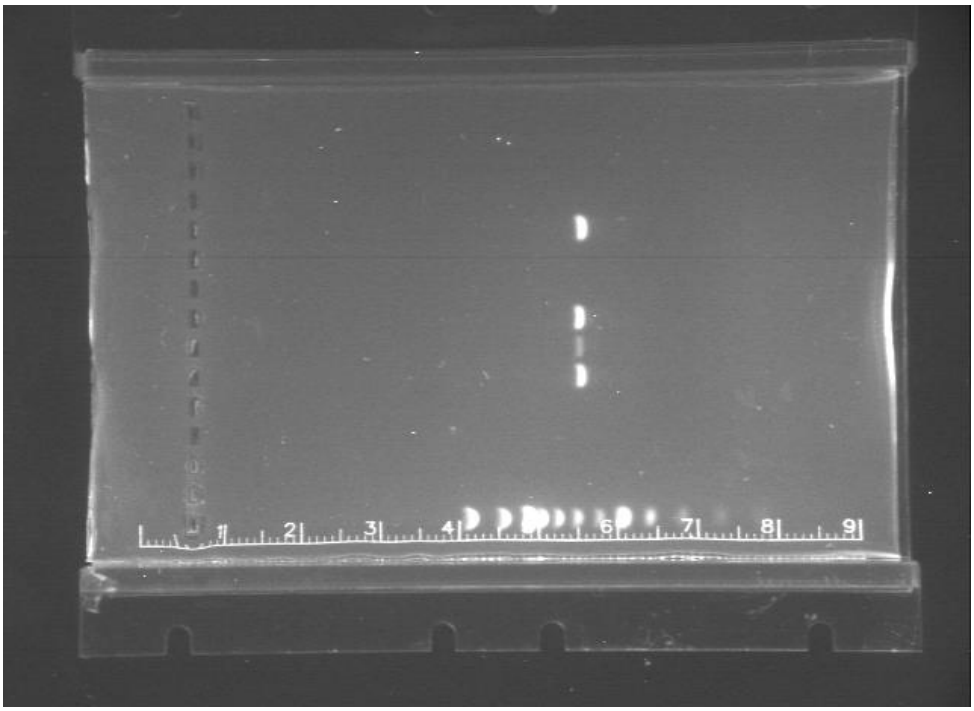


Figure 3.3: This is a PCR. The DNA fragments were taken from the wet spots in figure 3.2. The DNA bands that are glowing demonstrate that the wet spots are indeed degraded *Msm* colonies.

Phase 3 Analysis: The results thus far have established that *C. elegans* inhibit *Msm* growth and are able to convert *Msm* into a food source. The following results add a level of significance to the inhibition observation. Phase 3 results support the hypothesis that *C. elegans* are damaging the cell wall and perhaps inhibiting mycolic acid synthesis, although inhibition of mycolic acid synthesis cannot be asserted with confidence. Evidence of cell wall degradation comes from figure 3.2 where *Msm* colonies are spotted that display wet and gooey textures. This wet texture supports cell wall degradation because when the glycolipids and mycolic acids are broken down, the *Msm* colonies lose their thick and waxy textures (2). Figure 3.1 shows control *Msm* colonies that display the normal dry and waxy textures associated with normal *Msm* growth. There are clear differences between these colonies and the wet colonies. In order to confirm that the bacteria found in the wet colonies was *Msm;* we conducted a PCR of the DNA fragments of the

bacteria in that area. Figure 3.3 shows the PCR results and from the results, we can say with a degree of confidence that the wet colonies are of mycobacteria. In this PCR, bands that don't glow are from fragments we picked from *E. coli* plates to run as controls. This confirms that the PCR worked because only mycobacterial bands are glowing. Ultimately, the results give us confidence to conclude that *C. elegans* are responsible for cell wall degradation.

Unexpected Results:

Note: The results below are not part of the original phases, but are interesting observations that demonstrate a novel phenomenon discovered by the research. The images show that Msm is binding to C. elegans. The images are analyzed in the "discussion."

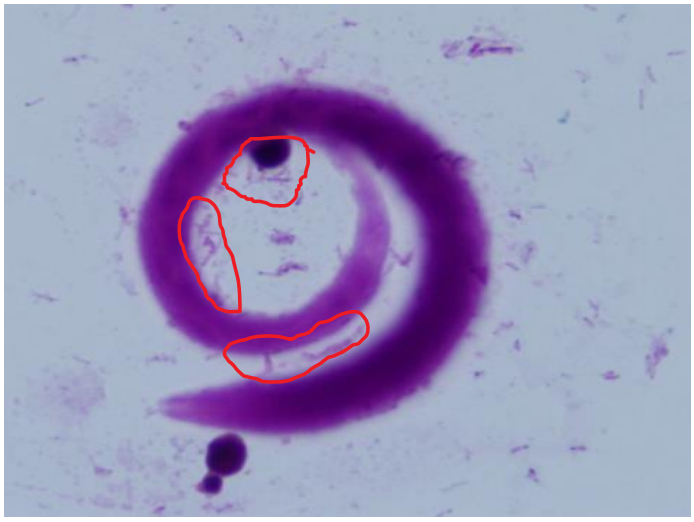


Figure 4.1: This is a Ziehl-Neelsen stained worm. The red circles show mycobacteria bacilli attached to the worm- evidence of binding. In the original image, the bacilli are pink. This image was zoomed to show the binding.

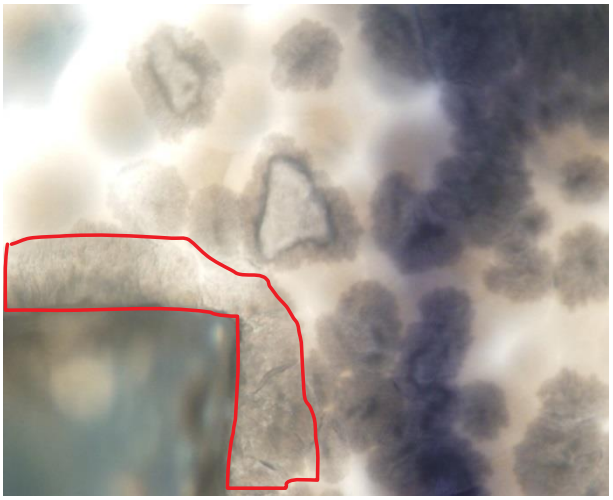


Figure 4.2: This shows *Msm* growth near an agar chunk. The agar chunk was placed far away from original *Msm* colony, but *Msm* was able to bind to *C. elegans* and began to grow far away from original seeding point.

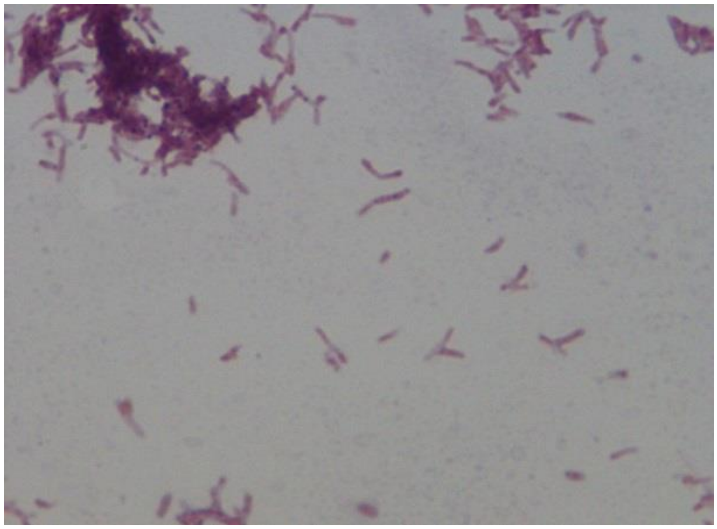


Figure 4.3: This is a control stain of *Msm* to compare to figure 41. This image is from source 12. Note: this image is more magnified than figure 4.1

Unexpected Results Analysis: Although these results were not part of the original phases of the experiment, they reveal a new phenomenon discovered by our research. The results show that *Msm* binds to the cuticle of *C. elegans.* This may give us insight into how *C. elegans* are able to break down the cell wall of *Msm*. There are two observations that hint at binding. First, we observed *Msm* growth far away from the original seeding point. As shown in figure 4.2, *Msm* appeared to grow in many plates near the agar chunks used to seed *C. elegans* onto the plates. To put this into perspective, the agar chunks were one to two inches away from the original seeding point of the *Msm* colony. In order to rationalize how *Msm* was growing far away from the original seeding point, it was inferred that *Msm* was binding to *C. elegans*. As the *C. elegans* travelled throughout the agar, we believe the *Msm* was deposited by the worms. This deposition of *Msm* resulted in new satellite *Msm* colonies in different areas of the agar.

The second observation was that when we stained the worms, we found *Msm* bacilli bound to the cuticle of the worm. As figure 4.1 shows, pink bacilli can be seen near the worm. This provides strong evidence to corroborate the assumption that *Msm* is binding to *C. elegans.*

Conclusion:

The methodology of the research was able to establish *C. elegans* as a source for antibacterial compounds capable of inhibiting the growth of Msm and degrading the cell wall. By successfully demonstrating in phase 1 that *C. elegans* are able to convert *Msm* into a new food source and survive in the presence of mycobacteria, we can conclude that *C. elegans* are not inhibited by *Msm*, but instead can thrive on *Msm*. On the other hand, we can conclusively say from phase 2 results that *Msm* is inhibited by *C. elegans* because of the presence of zones of inhibition and fragmentation of *Msm* colonies. The research added significance to the phase 2 results by demonstrating in phase 3 that *C. elegans* are able to degrade the cell wall of *Msm*, which is crucial to further development of *C. elegans* based antibiotics. In addition to supporting the hypotheses, the research observed a new phenomenon dubbed "binding" which may shed light onto how cell wall degradation is taking place.

We can conclude with confidence that these phenomena are occurring: 1) *Msm* is a novel food source for *C. elegans* 2) inhibition of *Msm* 3) binding of *Msm* to *C. elegans*. The experiments done to verify these phenomena are conclusive and rule out alternate explanations. In conjunction with our mentor's research on cell wall degradation, we can conclude that cell wall degradation is taking place on plates exposed to *C. elegans*. We believe that the binding of *Msm* to *C. elegans* may be an explanation for cell wall degradation. However, in order to verify the inhibition of mycolic acid synthesis, we would need to conduct mass spectroscopy on the mycobacteria found within the "wet" colonies in figure 2.2. Additionally, further analysis of *C. elegans* intestinal tract would reveal if there is digestion of mycolic acids, and confirming digestion of mycolic acids can lead to novel methods to inhibit mycolic acid synthesis.

In spite of the conclusive nature of the methodology, there are still some questions left unanswered. If we were to continue experimentation, we would hope to deal with these questions. First, how is the cell wall specifically being degraded? As mentioned before, further mass spectroscopic analysis of the cell wall would reveal the specific molecules that have been damaged or broken down. Additionally, we would perform analysis into the bonds formed between the lipids of the *Msm* cell wall and the lipids of the *C. elegans* cuticle to find a correlation between binding and degradation. Another question also remains unanswered: Does *C. elegans* secretion result in inhibition. Although we performed one experiment where the secretion was isolated, mixed with a phosphate buffer to form a solution, and then introduced to *Msm,* the experiment did not yield any conclusive results. Further experimentation would involve changing the concentration of the secretion and changing the concentration of the phosphate buffer to optimize the solution.

Despite the remaining unanswered question, the current results give crucial insight into the interactions between *C. elegans* and *Msm,* and these interactions can further be researched to potentially develop new antibiotics. The research has identified that there is some evidence that *C. elegans* are able to degrade the cell wall and inhibit *Msm* growth by eating, digesting, secreting antibacterial compounds, and perhaps binding to the lipid rich cell wall of *Msm*. This research can be applied to *Mtb* because it targets the cell wall of *Msm. Msm* and *Mtb* share approximately 2000 common homologs and share the same cell wall structure.

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