INVESTIGATING THE FUNCTION OF DNA METHYLATION IN MYCOBACTERIUM SMEGMATIS

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Abstract

Mycobacterium tuberculosis is the second deadliest single pathogen on the planet after HIV. This project was designed to study the ability of the bacteria to survive hypoxia by using *Mycobacterium smegmatis* as a model organism. Studying how these bacteria are able to survive hypoxic environments is important because during tuberculosis infections, the bacteria form granulomas that become hypoxic. Understanding how the bacteria are able to survive and proliferate in these granulomas is essential to effectively combat tuberculosis infections. This project focused on the *msmeg_3213* gene in *M. smegmatis*, which is predicted to code for the DNA adenine methyltransferase MamA. In *M. tuberculosis,* MamA increases the expression of certain genes and is important for the bacteria's ability to survive hypoxic environments. However, the exact role MamA and DNA methylation play in helping the bacteria survive hypoxia is not known. The goal of this project was to determine the function of DNA methylation in *M. smegmatis*. A knockout construct was designed, and PCR and Gibson Assembly were used to synthesize and assemble it. The knockout plasmid was transformed into *E. coli* in an attempt to clone the plasmid; however, no correct clones were obtained from the *E. coli* transformations. If the cloning of the plasmid in *E. coli* had been successful, the plasmid would have been purified from the cells, and then transformed into *M. smegmatis* to try to remove the gene. If I had been able to successfully delete the *msmeg_3213* gene from *M. smemgatis*, the viability of the transformed bacteria would have been tested in both hypoxic and normal environments.

Introduction

Mycobacterium tuberculosis (MT) is a bacteria that causes the disease known as tuberculosis (TB). The WHO lists tuberculosis as the second largest cause of death by a single pathogen after HIV (WHO, 2015). The bacteria is extremely infectious because it is very easily spread through the air in mucous particles expelled when an infected person coughs (Frieden *et al.*, 2003). The WHO reported around 9 million new cases of TB in 2013, and that number is expected to rise in coming years. The immune system naturally fights the infection by forming granulomas around the bacteria within the body. A strong immune system can keep the bacteria in a latent state within the granulomas; however, if the immune system becomes compromised the bacteria may become active and pose a larger threat to the host (Frieden *et al.*, 2003). Tuberculosis infections are often difficult to combat because the bacteria are able to survive in a latent state while inside the oxygen deprived microenvironment of the granulomas (Rex, Kurthkoti e Varshney, 2013).

The mechanism by which *Mycobacterium tuberculosis* and other *Mycobacteria* species are able to survive hypoxia appears to be controlled through the expression of various genes when the bacteria are under stress. Bacteria in conditions with limited oxygen sources showed an upregulation in certain genes used in the energy metabolism cycle that were not being expressed in bacteria that were provided sufficient nutrient and oxygen sources. The expression of these new genes causes the bacteria to begin utilizing an NAD^+/NADH-independent enzyme, induce the production of cytochrome *bd* oxidase (used to scavenge oxygen in hypoxic conditions), and upregulate dehydrogenases required to make oxygen so that the cell is able to continue the Krebs cycle in hypoxia and nutrient starved environments

(Berney and Cook, 2010). Cytochrome *bd* oxidase has a high affinity, making it ideal to bind to any oxygen near in the environment. *Mycobacteria* utilizes cytochrome *bd* oxidase, menaquinone oxidoreductases (*ndh2*), and cytochrome bc_1-aa_3 to create a small electrical potential while under hypoxia (Berney and Cook, 2010).

Berney et al. showed that the regulation of proline in *Mycobacteria* is important in the bacteria's ability to survive hypoxia. Proline is an amino acid that is able to be broken down and used as a source of carbon and nitrogen within the cell. The metabolism of proline is mediated by the enzymes Δ1-pyrroline-5-carboxylate dehydrogenase (PruA) and proline dehydrogenase (PruB) and the protein PruC. The addition of proline to bacteria in hypoxia has shown increases in the overall biomass when compared to bacteria being grown in hypoxia without additional proline (Berney *et al.*, 2012).

The proteins DosRS have also been shown to be involved in *M. tuberculosis*'s ability to survive hypoxia. The DosRS/DosT two component system is comprised of DosRS and DosT. DosR is a transcriptional activator, meaning that it is a protein responsible for increasing the transcription of a set of genes. Rustad et al. found that during hypoxia about 100 new genes are expressed within the tuberculosis genome, and that nearly all of the induced genes became induced by the DosR/DosS system (Rustad et al., 2009). Unfortunately, as later tests would show, bacteria with mutant DosR genes were shown to be able to survive hypoxia as well as the wild type strains they were being compared to. The data gathered by Rustad et al. showed that the DosR gene plays a role in the bacteria's response to hypoxia, but is not required for the bacteria to enter a dormant state. The DosR gene can be activated by multiple stressors including nitrous oxide, carbon monoxide, SDS, and low pH. Bacteria that were actively

reproducing have been shown to express the DosR gene, which would indicate that it is not responsible for inducing a dormant state in the bacteria because during a dormant state the bacteria would not be able to reproduce (Rustad et al., 2009).

Srivastava et al proposed that methyltransferases may be an important part of determining the virulence of *Mycobacteria tuberculosis* as 5-methylcytosine was found to present in the virulent strain *Mycobacteria tuberculosis* $H_{37}R_v$, but absent in the avirulent strain *Mycobacteria tuberculosis* $H_{37}R_a$; however, later results gathered by Srivastava et al showed that methyltransferases were not the cause of the bacteria's virulence as 5-methylcytosine was found to be in strains of *Mycobacterium smegmatis* (a nonvirulent bacteria), and N^6-methyladenine was found in all strains (Srivastava, Gopinathan e Ramakrishnan, 1981). The presence of methyltransferases in other *Mycobacteria* strains is important, as Scarlet Shell et al proposed that DNA methylation may play an important role in the ability of *Mycobacteria* strains to survive hypoxic environments. Shell et al found that gene regulation and expression under normal conditions in *M. tuberculosis* is partly regulated by a methyltransferase designated MamA. MamA is an enzyme coded for by the gene *Rv3263* and produces N_6-methyladenine within the bacterial DNA. When the gene was removed from the bacterial DNA, a 15-39% decrease in viable cells was observed when growing in hypoxic environments (Shell *et al.*, 2013).

This project aims to continue the research of Shell et al by continuing to investigate the function of DNA methylation within *Mycobacteria,* and how the loss of MamA affects the cell's ability to survive hypoxia. Restrictions within the workspace have prevented the use of *Mycobacterium tuberculosis* in lab, and so this project has utilized *Mycobacterium smegmatis*

as a model organism in its place. PCR and Gibson assembly were used to create a knockout plasmid targeting the *M. smegmatis* gene *msmeg_3213*, and a hygromycin resistance gene was built into the plasmid to ensure selectivity when growing bacteria on/in 7H9 and 7H10 media.

Methods

This project has utilized PCR and Gibson Assembly in an effort to construct a plasmid knockout for the msmeg_3213 gene in *Mycobacterium smegmatis*. The knockout was composed of the plasmid *PJM1* and an upstream and downstream flank section of DNA from the *M. smegmatis MC^{2}155* strain. The upstream and downstream flanks were constructed using PCR and then joined to the plasmid using Gibson Assembly.

PCR1

The upstream flank of the knockout was constructed using PCR before being added to the plasmid. The forward primer used was SSS740 and the reverse primer used was SSS741. The full list of components used can be seen in Table 1 below.

Order Mixed	Component	Per RXN (µL)	2.5x Master Mix (µL)
1	Q5 Buffer	5	12.5
3	F Primer (SSS740)	1.25	3.1
4	R Primer (SSS741)	1.25	3.1
5	Dntp	0.5	1.25
2	5x Q5 High GC Enhancer	5	12.5
7	Q5 Hot Start Polymerase	0.25	0.63
6	Water	10.75	27
8	Template (MC^{2}155)	1	-
		25µL total volume	60µL total (24µL per RXN)

Table 1- PCR1 Components

The master mix was split into two PCR tubes and the template was added to each tube. The tubes were then placed in a thermocycler to allow the reaction to run. The temperatures and times used in the thermocycler can be seen in Table 2 below.

Steps	°C	Time (s)
1	98	30
2	98	10
3	71	20
4	72	25
5	72	120
6	10	∞

Table 2- PCR1 Thermocycler Conditions

When step 4 was reached, the machine automatically cycled back to step 2. This repeating process occurred 25 times before the machine was allowed to reach step 5. When the final cycle was reached, the tubes were removed from the machine and run in an agarose gel.

PCR2

The downstream flank of the knockout was made using another PCR. The Forward Primer used was SSS742 and the Reverse Primer was SSS743. The Full list of components can be seen below in Table 3.

Order Mixed	Component	Per RXN (µL)	2.5x Master Mix (µL)
1	Q5 Buffer	5	12.5
3	F Primer (SSS742)	1.25	3.1
4	R Primer (SSS743)	1.25	3.1
5	Dntp	0.5	1.25
2	5x Q5 High GC Enhancer	5	12.5
7	Q5 Hot Start Polymerase	0.25	0.63
6	Water	10.75	27
8	Template (MC^2155)	1	-
		25µL total volume	60µL total (24µL per RXN)

Table 3- PCR2 Components

Template was added to two PCR tubes along with the master mix, and the tubes were placed into the thermocycler. The full list of temperatures and times can be seen in Table 4 below.

Steps	°C	Time (s)
1	98	30
2	98	10
3	68	20
4	72	25
5	72	120
6	10	∞

Table 4- PCR2 Thermocycler Conditions

Steps 2 through 4 were cycled 25 times before step 5 was allowed to be reached. When the final step was reached the PCR product was run through an agarose gel.

Agarose Gels

The gels were made by combining 0.6g Agarose with 60mL TAE Buffer and microwaving until completely mixed. After removed from the microwave 0.5µL EtBr was added to the solution. The mixture was then poured into a mold and allowed to harden. Before a PCR product was run through the gel, it was first mixed with 5µL loading dye. The PCR product was then pipetted into the corresponding wells and run using electrophoresis for 35-45 minutes.

Gel Extraction

The PCR product was purified from the gel so that it was able to be used in the Gibson Assembly. The gel band was sliced from the gel using a razorblade to cut and a UV light to identify the DNA in the gel. The gel slices were then placed in microcentrifuge tubes and weighed. After weighing the gel slices QG buffer was added to the tubes. 3 parts buffer were

added for every 1 part of gel. The tubes were then incubated in a heat block at 50°C for 10 minutes until the gel had completely melted.

After the gel slices had incubated and melted the liquid was applied to a spin column and spun at 1400rpm for 1min. The flow through was discarded, 0.75mL Buffer PE was applied to the spin column, and the column was spun for 1 min. The flow though was discarded and the column was spun again for a minute to remove any residual flow through remaining in the column. The column was then moved to a microcentrifuge tube and 30µL elution buffer was applied. The tube was left to sit for 1 minute, and then spun for 1 minute. The concentration of PCR product was found using Qubit 2.0. The information for the purification of PCRs 1 and 2 can be seen in Tables 5 and 6 respectively.

Tube #	Gel Weight (g)	µL QG Buffer added	Concentration (ng/µL)	ng DNA
1	0.348	1044	58	1682
2	0.409	1227	70	2030

Table 5- PCR1 Gel Extraction

Tube #	Gel Weight (g)	µL QG Buffer added	Concentration (ng/µL)	ng DNA
1	0.216	648	13	377
2	0.222	666	6.74	195

Table 6- PCR2 Gel Extraction

Cutting Plasmid

Before the plasmid could be used in the Gibson Assembly it first needed to be cut. The plasmid was cut with Not1 and Spe1. The components used to cut the plasmid were mixed in a PCR tube and incubated at 37°C for 1-2 hours. A 3.5x master mix was made so that a control could be run for each enzyme cutting point. The full list of components can be seen in Table 7 below.

Component	Per RXN (µL)	3.5x Master Mix (µL)
10x NEB buffer	2	7
Plasmid	4.386	15.3
Water	12.614	44
Not1	0.5	-
Spe1	0.5	-

Table 7- Plasmid Cutting Components

After incubating, the cut plasmid was run through an agarose gel to confirm that the cut plasmid was the correct size.

Gibson Assembly1

The Gibson Assembly was used to create the knockout by combining the upstream and downstream flanks with the plasmid. The Gibson Assembly protocol from Synthetic Genomics was followed. The DNA fragments were mixed and diluted with water. The DNA fragments were then added to the Gibson Assembly master mix and mixed thoroughly. After being mixed the solution was incubated at 50°C for 1 hour. The amount of DNA fragments used can be found below in Table 8.

Component	ng DNA used	Starting Concentration	µL used
Vector	68.172	32.6 ng/µL	2.1
Upstream Flank	28.52	58.0 ng/µL	0.5
Downstream Flank	27.82	13.0 ng/µL	2.1

Table 8- Gibson Assembly1 DNA Fragments

The full set of Gibson Assembly conditions can be found below in Table 9 below.

Component	Volume (µL)
Fragments	4.7
Master Mix	5
Water	0.3
Total Volume	10

Table 9-Gibson Assembly 1 Conditions

After allowing the mixture to incubate for an hour, the Gibson Assembly is ready to perform transformations.

Gibson Assembly 2

The first Gibson Assembly protocol did not yield positive results after attempting transformations. Because of this, the protocol for the assemblies was slightly edited. The final volume for the assembly was dropped from 10µL to 5µL. This meant that the Gibson Assembly was comprised of 2.5µL DNA fragments and 2.5µL master mix. The volume of each fragment added had to be recalculated to fit the new volume. The new values for the DNA fragments can be seen below in Table 10.

Component	ng DNA used	Starting Concentration	µL used
Vector	16.3	32.6 ng/µL	0.5
Upstream Flank	29	58 ng/µL	0.5
Downstream Flank	19.5	13 ng/µL	1.5

Table 10- Gibson Assembly 2 DNA Fragments

The new volume of DNA fragments was added to the new volume of master mix. The full list of components for the new volumes can be seen below in Table 11.

Component	Volume (µL)
Fragments	2.5
Master Mix	2.5
Total Volume	5

Table 11- Gibson Assembly 2 Conditions

After adding and mixing all components the mixture was incubated at 50°C for an hour.

E. coli Transformation

The Gibson Assembly product was used to transform E. coli in an attempt to clone the plasmid. A chemical transformation protocol was followed. To start, an aliquot of NEB 5-alpha competent E. coli cells were thawed for 10 minutes on ice. 2µL of Gibson Assembly product was then added to the thawed cells, and they were placed on ice for 30 min. The cells were then heat shocked in a water bath at 42°C for exactly 30s. After experiencing the heat shock, the cells were placed back on ice for 5 minutes. 200µL of SOC was added to the tube, and the tube was placed in a shaking incubator at 37°C for 1 hour. After the bacteria had incubated, they were pipetted onto two LB agar plates containing hygromycin with a concentration of 150ng/ml. 200µL was pipetted onto plate 1 and 20µL was pipetted onto plate 2. The plates were then wrapped in tin foil and placed in the warm room to incubate at 37°C.

Resuspending Colonies

Colonies that had grown on the plates after a 24 hour period were picked and grown in an LB media containing hygromycin to increase selectivity of cell growth. The cells were grown in a 50mL conical tube. 5mL of LB broth was pipetted into the tube, and 15µL of hygromycin was mixed into the broth. Two colonies were selected from each plate and each colony was placed in its respective tube. The concentration of hygromycin in the tube was 150ng/mL taken from a stock concentration of 50mg/mL.

Results

PCR, Gibson Assembly, and chemical transformation protocols were followed in an attempt to create a knockout construct that was able to effectively remove the *msmeg_3213*

gene from *Mycobacrerium smegmatis*. This project was unable to produce any verifiable, successful results. Throughout the project issues such as low DNA concentration measured after PCR, low colony formation, and colony formation on control plates was observed. If the Gibson Assembly was performed correctly, the upstream and downstream flanks should have been inserted into the plasmid as seen below in Figure 1.

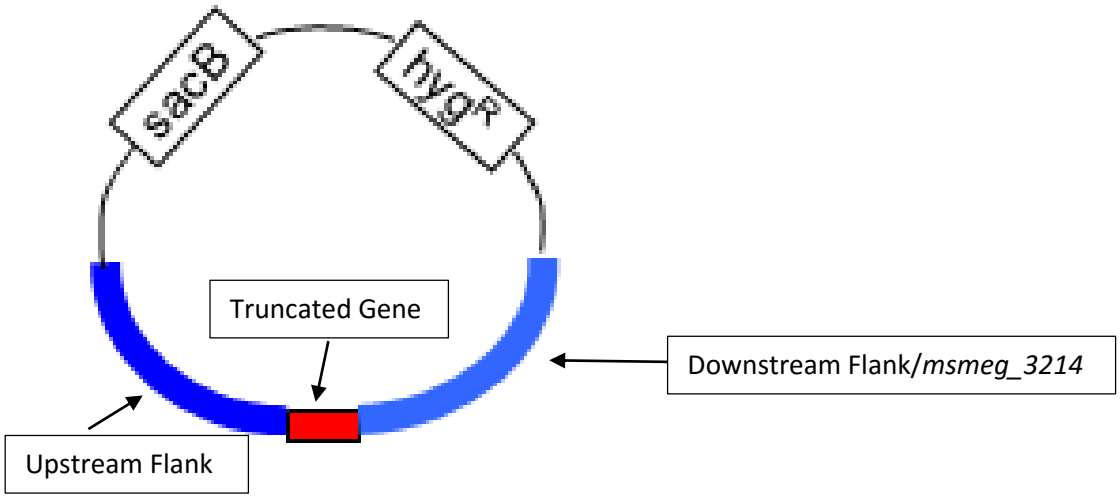

Figure 1: Plasmid with knockout

The fully constructed plasmid inserted itself into the bacterial genome through homologous recombination between the upstream flank and the matching part of the gene as depicted below in Figure 2.

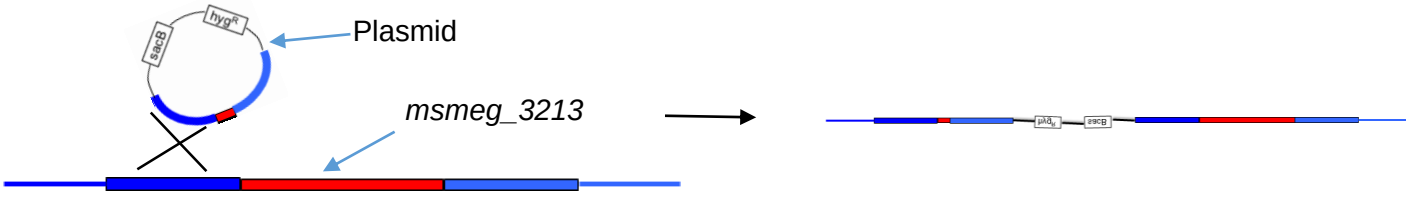

Figure 2: First Recombination

After the initial recombination, the transformed cells are able to survive in selective media due to the *hygR* gene found in the PJM1 plasmid. A second recombination removes the excess DNA

found in the plasmid, leaving behind the truncated version of the *msmeg_3213* gene as well as the full *msmeg_3214* gene in the downstream flank as seen below in Figure 3.

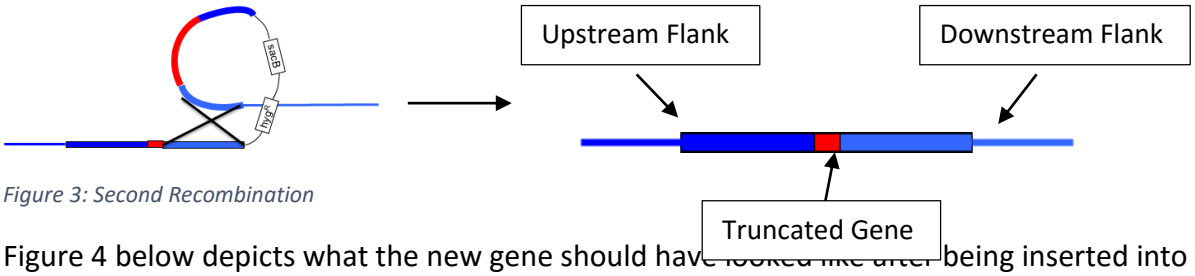

Figure 3: Second Recombination

Figure 4 below depicts what the new gene should have looked like after being inserted into the genome.

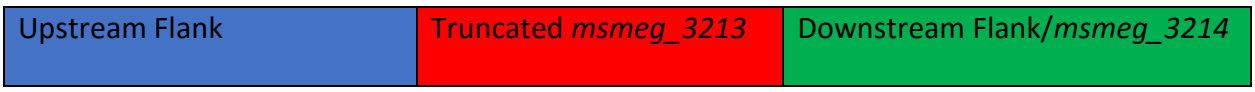

Figure 4: Knockout Insert

It was important to keep the full *msmeg_3214* gene intact so that any effects seen in transformed bacteria were caused only by the loss of *msmeg_3213*. The truncated gene left in the insert contained the 11bp overlap found between *msmeg_3213* and *msmeg_3214*, and the downstream flanks contains the remainder of the *msmeg_3214* gene.

After conducting PCR and purifying the product from an agarose gel, fluctuations were seen in the concentration of DNA purified from the gel. A picture of an agarose gel after running DNA can be seen below in Figure 5.

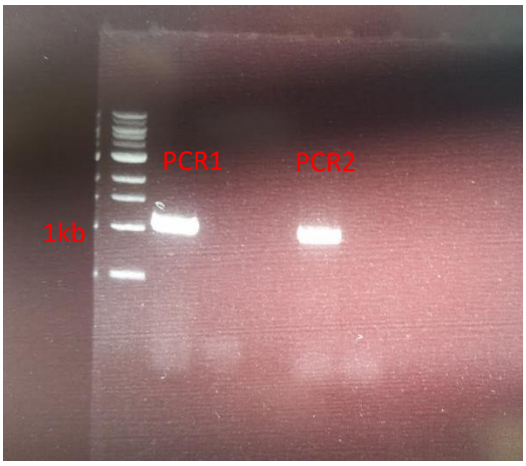

Figure 5: PCR 1&2 Gel

Seen in the picture above, the PCR results appeared to be around the 1kb line, indicating that they are about the correct size. As stated above, fluctuations in DNA concentration were seen after purifying the lines of DNA from the gel. The concentration of PCR1 product was 58ng/µL and the concentration of PCR2 product was 13ng/µL. The large difference in concentration was a cause for concern; however the PCR products were still used for Gibson Assembly.

Gibson Assembly was used to insert the PCR products into the PJM1 plasmid so that it could then be cloned in *E. coli*. The picture below shows an example of the gel used to ensure that the plasmid was the correct size before using Gibson Assembly to insert the DNA fragments produced in the previous PCRs.

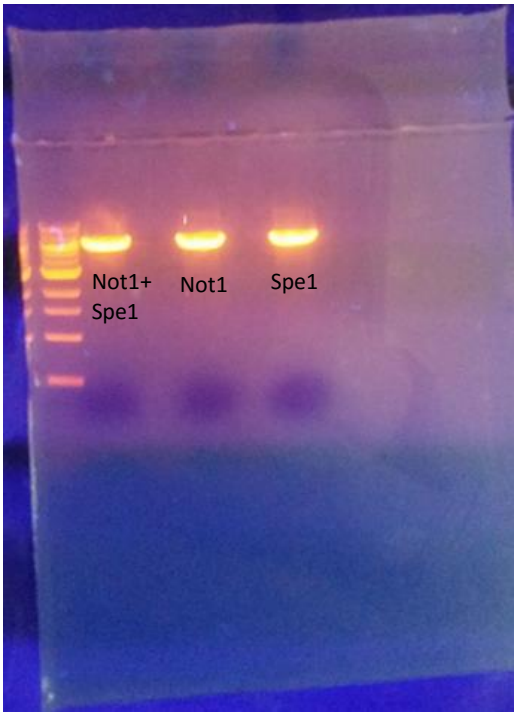

Figure 6: Post-Plasmid Cutting Gel

The band in the second well (containing the plasmid cut by both Not1 and Spe1) appears to be at the correct height, just barely above the bands for the plasmid cut with only Not1 or Spe1. After confirming that the plasmid was cut and the PCR products were the desired size, Gibson Assembly was performed to insert both the upstream and downstream flanks into the plasmid.

The product of the Gibson Assembly was cloned into *E. coli* cells using chemical transformation methods. More problems were encountered during the cell culture phase of the transformation. The colonies formed on the positive transformation plate appeared very small. A picture of the transformed plate can be seen below in Figure 7.

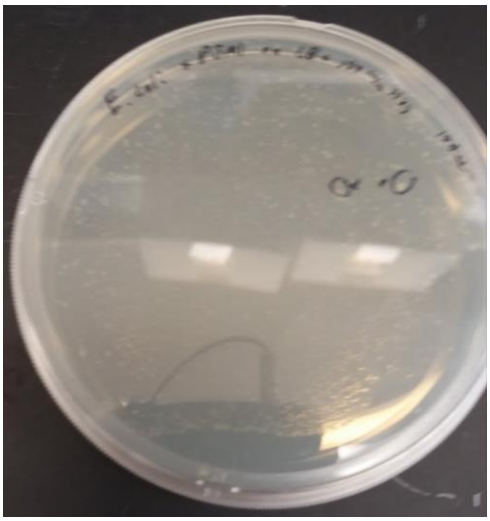

Figure 7: Transformed E. coli

There were many small colonies on the plate above; however none of them grew to be much larger than the tip of a pipette. The small colonies forming on the transformed plate did not seem to be of importance because the same type of small colonies were seen to form on the negative control plate as seen below in Figure 8.

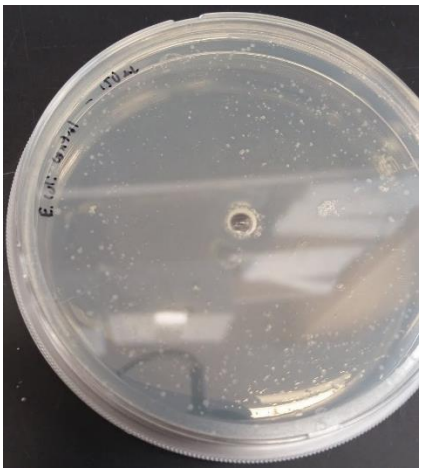

Figure 8: Transformation Negative Control

The formation of small colonies on the control plate is an indicator that there may have been something wrong with the transformation. Colonies from both the transformed plate and the

control plate were picked and transferred to a conical tube containing liquid media. There was no growth observed in liquid media containing 150µL hygromycin.

Discussion

This project was designed to remove the *msmeg_3213* gene from *Mycobacterium smegmatis*, which is believed to code for the methyltransferase MamA. PCR and Gibson Assembly were utilized to build a knockout construct which could be transformed into the bacteria. The results gathered from the experiments performed during the project indicate that the attempts to build a knockout construct, clone the construct using *E. coli*, and then to remove the gene from *M. smegmatis* were failures. No colonies formed on the selective plates that were also able to proliferate within liquid media. This result indicates that the colonies observed on the plates may have not been truly resistant to hygromycin due to the transformation, but may have undergone a mutation which allowed them to form small colonies on the plates.

The reason for the failed transformations is not entirely clear. There are a few possibilities for why the results were not positive. The first possibility is that there was an error while building the knockout construct. If the PCRs and Gibson Assembly did not work properly, it would cause the transformation to fail and the plasmid would not target the correct spot in the bacterial genome. Another possibility for the failure of the transformations may have been the conditions used during the transformation protocol. If the water bath was not at the correct temperature, that may have caused issues transforming the plasmid into *E. coli*. A final reason that the transformation may have failed is that the *msmeg_3214* gene may be toxic to *E. coli*.

The *msmeg_3214* gene overlaps with the *msmeg_3213* gene with 11bp. The entire *msmeg_3214* gene is also found on the downstream flank of the knockout construct as seen in Figure 6. If the gene is toxic to *E. coli* then it could also be the cause for the slowed growth seen and inability of the transformed *E. coli* to proliferate in liquid media.

The function of MamA, and its role in hypoxia survival, is still important for future researchers to study. I would recommend that any continuations of this project test that the *msmeg_3214* gene is toxic to *E. coli*. Another way to test the function of the *msmeg_3213* gene might be to create a point mutation that inactivates the gene, rather than fully removing it from the bacterial genome. Another knockout strategy might be to remove the *msmeg_3213* gene from the genomic location where it is found, and replace it in a different part of the genome with an inducible promoter. Throughout the project, the Shell lab was in communication with the lab of Eric Rubin, who found that the *msmeg_3213* gene is essential in *Mycobacterium smegmatis*. Removing the gene will cause the cells to die if the gene is essential; however, the inducible promoter would allow a researcher to decide whether or not the promoter is activated and the newly inserted gene will be expressed.

Appendix

Primer Name	Primer Sequence	Primer Description
SSS740	CGAATTGGAGCTCCACCGCG GTGGCACGAGCTGACGTTCC TCGAC	Upstream Flank for making msmeg_3213 knockout construct Forward Primer
SSS741	CGTTCGCCGCGTCACCCCGT CATTGTTGACGACA	Upstream Flank for making msmeg_3213 knockout construct Reverse Primer
SSS742	CAATGACGGGGTGACGCGG CGAACGGTAAGGT	Downstream Flank for making msmeg_3213 knockout construct Forward Primer

SSS743	TACATTATACGAAGTTATGG ATCCAGCTCTGAGACAAGCT CACATCT	Downstream Flank for making msmeg_3213 knockout construct Reverse Primer

Table 12: Primer Sequences

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