Identification of the Genetic Basis of Persistence in *Mycobacterium smegmatis*

Josephous Miculob

Supervisor: Sharon Kendall

The causative agent of tuberculosis (TB), *Mycobacterium tuberculosis* (Mtb), infects a third of the world's population. After entering the lungs, Mtb is phagocytosed by alveolar macrophages. Mtb can persist inside the phagosomes of these macrophages despite being a hostile environment due to the presence of reactive oxygen and nitrogen species (ROS and RNS) produced by NADPH oxidase and inducible nitric oxide synthase, respectively. ROS and RNS can react with bacterial lipids, carbohydrates, proteins and nucleic acids causing free radical stress. Mtb may also be exposed to antibiotic stress such as rifampicin, a first-line drug used for TB treatment. Despite these various stressors, Mtb can persist for long-periods of time within the patient. The aim of this summer project was to determine the genetic basis of how Mtb can persist for long periods of time inside the patient. This was done by measuring the survivability of a transposon mutant library (Table 1) of *Mycobacterium smegmatis* (Msm), a common model for Mtb, following treatment with hydrogen peroxide (H_2O_2), DETA/NO, and rifampicin.

Table 1: Transposon Library Mutated Genes

Mutant	Tn Insertion Point	Gene Description
1	MSMEG_2434	Metallopeptidase
2	MSMEG_6284	Cyclopropane-fatty-acyl-phospholipid synthase
3	MSMEG_1300	HP
4	MSMEG_6024	Acetoacetyl-CoA synthase
5	MSMEG_6540	Virulence factor Mce family protein
6	MSMEG_1730 and MSMEG_2818	ISMsm1, transporfase orfB
7	-	-
8	-	-
9	MSMEG_3711	HP
10	MSMEG_0017	ABC transporter, permease/ATP-binding protein
11	MSMEG_1137	Amino acid permease-associated region
12	MSMEG_1229	DNA gyrase, B subunit
13	MSMEG_4244	3-deoxy-7-phosphoheptulonate synthase
14	MSMEG_0345	Conserved HP: Permease MlaE
15	MSMEG_5166	Na^+/solute symporter

Blanks (-) = sequencing did not work so the Tn insertion point was not determined.
HP (hypothetical protein) analysed using PFAM (https://pfam.xfam.org/) to find domains, if possible.

The transposon (Tn) mutant library consisted of 15 mutants (Tn1-Tn15). Before treating the mutants with the various stressors, growth of the mutants over time was compared with wild-type (WT) Msm. This was done by measuring the optical densities of liquid cultures of the mutants in a 24-well plate over a 60hrs. The mutant cultures were grown in 7H9 broth supplemented with ADC (albumin dextrose catalase), glycerol and kanamycin. WT Msm was grown in a similar broth but without kanamycin. The transposon mutants all showed similar growth curves with each other and with the WT Msm, with all cultures reaching stationary phase by 40 hours. The WT growth curve was also compared with another WT growth curve from a 6-well plate instead. No major differences were seen between the growth curves, suggesting more samples may be screened in future experiments as a 24-well plate could be used instead of a 6-well plate.

Preliminary experiments were conducted before subjecting the transposon mutants to the various stressors to determine the concentrations that should be used for treatment. This involved exposing WT Msm to various concentrations of stressors. These concentrations were based of similar research using these stressors in mycobacteria. The ideal concentration would be one that resulted in killing without being fully bactericidal to allow for differentiation of susceptibilities between the transposon mutants. Survivability was measured by calculating the number of colony-forming units (CFUs) per ml of culture after exposure.

The concentrations of H_2O_2 tested were 0.2, 2, and 20mM whereas DETA/NO concentrations tested were 0.05, 0.5, and 5mM. Aliquots of WT liquid culture at OD 0.3 grown in the same liquid medium described above were exposed to the various concentrations of hydrogen peroxide and DETA/NO listed above for 3h at 37C. To count any CFUs, Miles and Misra spotting was performed which involved performing 10-fold serial dilutions of the aliquots after the 3h exposure and pipetting 20µl of each in triplicate on LEMCO agar. CFUs were counted, allowing the concentration of bacteria (CFU/ml) to be calculated. The highest concentrations of both H_2O_2 and DETA/NO resulted in the biggest decrease in CFU/ml compared with the unexposed controls however only 5mM DETA/NO showed significant decrease. This was unexpected as these experiments were based on the significant killing observed at lower concentrations in previous experiments.

A reason why no significant decreases in CFU/ml was seen with most of the concentrations used may be the presence of catalase in the 7H9 broth. Catalase is an enzyme that breaks down H_2O_2. This was tested by comparing growth over time of a Msm Tn mutant in the presence of higher concentrations of H_2O_2 (40, 80, and 176mM) with or without ADC. It was found that growth was still viable without ADC but to be optimal, ADC is required. It also showed that concentrations of hydrogen peroxide above 40mM are completely bactericidal. There are several factors that may have contributed to this. Firstly, Tn3 was the only viable starter culture as the others failed to grow overnight so this mutant may had been more susceptible to hydrogen peroxide compared with the others and the WT. Any concentrations above 20mM may also be completely bactericidal as higher concentrations were used in this experiment. More of the hydrogen peroxide would have also been present as it wasn't being broken down by catalase. Despite these factors, this experiment was informative, and the protocol was modified accordingly to add a wash step to remove ADC. Killing by DETA/NO was also tested with or without ADC in the broth which showed significant decrease in CFU/ml in the experiment lacking ADC. Higher concentrations of DETA/NO (20 and 50mM) were also tested and showed concentration-dependent decreases in CFU/ml compared with the control. However, 50mM DETA/NO requires 500µl of 0.1M NaOH to dissolve it. This was tested as a vehicle control and was found to have a substantial killing effect compared with using 200µl NaOH (used to dissolve 20mM DETA/NO). It was then decided that the concentration of DETA/NO to expose to the Tn mutants would be 20mM.

A washing step to remove ADC and a test for 40mM of H_2O_2 was added in a repeat of the preliminary experiment. It was found that higher concentrations (20mM and 40mM) resulted in significant decreases in CFU/ml but were not completely bactericidal. The higher concentrations showed the largest decreases in CFU/ml, but a strange growth pattern was observed. 'Spots' earlier along the dilution series tend to show confluent growth as the bacteria are more concentrated. Individual CFUs would then be distinguishable further along the dilution series as the bacteria become more diluted. No colonies earlier in the dilution series and colonies appearing further along was observed after 20mM (Figure 1) and 40mM exposure. Hydrogen peroxide may be diluted along the dilution series. At earlier dilutions, the concentration of hydrogen peroxide is enough to inhibit growth but as it becomes more diluted along the dilution series, it eventually reaches a

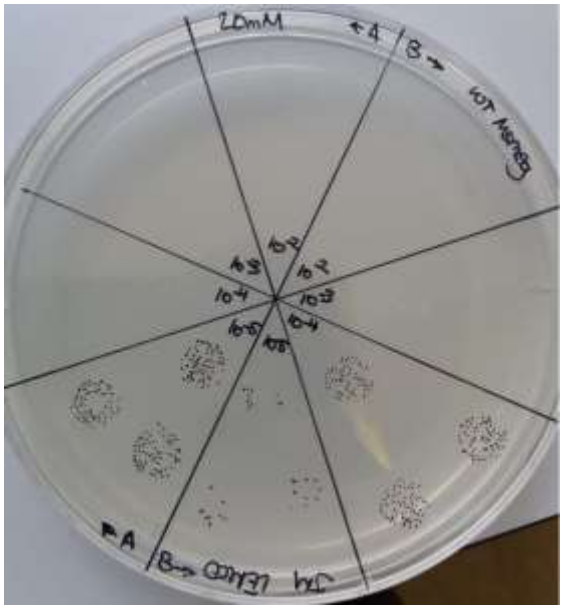

Figure 1: Miles and Misra Spotting Following 20mM H_2O_2 exposure of Msm after 3h. No colonies are seen earlier in the dilution series (10^{-2}-10^{-3}). Colonies appeared further along the dilution series (10^{-4}-10^{-5}).

concentration that allows for growth. The protocol was then modified further to include a wash step after the 3h exposure of the stressors. Despite this, it was decided to use 20mM of H_2O_2 as significant decreases in CFU/ml was still observed.

The same preliminary experiment was conducted with rifampicin, testing concentrations of 3, 15, and 150µg/ml which are 5, 10, and 15-fold the MIC of rifampicin for Mtb. Surprisingly, no significant decreases in CFU/ml were observed so higher concentrations (500µg/ml and 5mg/ml) were tested. A concentration-dependent decrease in CFU/ml was observed but Msm was still able to grow. A reason Msm is less susceptible to rifampicin is that Msm expresses ADP-ribosyltransferase which Mtb lacks. This enzyme is involved in making rifampicin ineffective. Future experiments should consider knocking out the Arr gene, which encodes ADP-ribosyltransferase, to create better Msm models for Mtb. Exposing the Tn mutants to rifampicin was not conducted due to lack of time but future experiments should also do this.

In the interest of time, only 5 of the 15 Tn mutants were screened (Tn2, Tn4, Tn9, Tn12, Tn14) using the modified protocol from the preliminary experiments and the decided concentrations of H_2O_2 and DETA/NO (20mM, respectively). After H_2O_2 exposure, all the Tn mutants showed similar decreases in CFU/ml as with the WT except for Tn4, which was completely killed off. Tn4 has a defective acetoacetyl-CoA synthase. This enzyme makes acetoacetyl-CoA which is a precursor in the mevalonate pathway which itself is involved in cell wall biosynthesis in bacteria. The cell wall is known to be one of the targets for hydrogen peroxide's mechanism of action so a defect in cell wall biosynthesis may make Tn4 more susceptible to it compared with the other mutants. DETA/NO exposure resulted in similar decreases in CFU/ml as the WT for all mutants except for Tn4. Surprisingly, Tn4 showed an increase in CFU/ml following DETA/NO exposure. This experiment should be repeated in future to validate these results.