Using Gene Overexpression as a Potential Method to Elucidate Antibiotic Resistance Mechanisms in Mycobacteria

by

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DISSERTATION

Presented to the Faculty of the Medical School

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF MEDICINE WITH DISTINCTION IN RESEARCH

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MD with Distinction in Research Thesis

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Abstract

Mycobacterium Tuberculosis remains a major public health threat, with 9 million new cases and 1.5 million deaths in 2013. Also concerning is the rise of antbiotic resistance, leading to the development of Multiple Drug Resistant (MDR) and Extensively Drug Resistance (XDR) *Mycobacterium tuberculosis* strains. The development of these strains is multifactorial involving both extrinsic factors and intrinsic factors. Extrinsic factors include treatment noncompliance and a delay in diagnosis with existing drug sensitivity methods. Intrinsic factors include heterogeneity in the bacterial population and localization in cavitations and other areas where antibiotic penetration is difficult. Our study analyzed the potential role of a potential mechanism of antibiotic resistance, overexpression of existing wild type M. tuberculosis genes involved in mycobacterial antibiotic defense.

We cloned seven M. tuberculosis wild type genes using PCR and the subcloned those constructs into E. Coli. Further subcloning steps placed these genes into shuttle vectors that allowed for transformation into *M tuberculosis and M. smegmatis.* The M. Smegmatis mutant overexpressing the GyrA gene was selected for dose-response studies to establish whether GyrA overexpression led to increased fluoroquinolone resistance. Two *M. smegmatis* GyrA mutant and one wild type strain were incubated with increasing concentration of Moxifloxacin. Initial dose-response studies did not show yield an adequate dose-response curves under the study conditions. However, the MIC was higher for the GyrA mutant strains than for wild type *M. smegmatis,* showing that these GyrA mutants were likely more resistant than wild type and that the role of gene overexpression in *M. tuberculosis* antibiotic resistance merits further study.

Introduction

Tuberculosis remains a major public health issue. According to the WHO, there were 9 million new cases in 2013 along with 1.5 million deaths (WHO, 2014). 360,000 of these deaths were among HIV positive individuals. 2.13 billion people worldwide are infected with *Mycobacterium tuberculosis*, but the majority have latent infection without active disease (WHO 2013). A patient with latent tuberculosis has a positive TB Quantiferon or Tuberculin skin test but no clinical, radiographic or microbiologic evidence of tuberculosis infection (CDC, ATS 2000)(CDC 2014). These patients have no evidence of TB on chest x-ray and have a negative sputum acid-fast-bacilli (AFB) smear. Patients with active TB typically have

symptoms consistent with tuberculosis and may have evidence of TB on chest x-ray or a positive AFB smear.

Antibiotic resistance in Mycobacterium tuberculosis is a growing problem as current strains of multiple-drug resistant (MDR) TB propagate and new ones arise through evolutionary selection. According to the CDC, MDR TB is defined as bacteria that are resistant to Isoniazid and Rifampin. Per the WHO, MDR- TB accounts for 3.5% of new cases and 20.5% of previously treated cases in 2013, with overall 480,000 cases of MDR-TB (WHO 2014). Recently, XDR-TB has been a growing problem as well. XDR-TB are bacterial strains resistant to Isoniazid, Rifampin, any fluoroquinolone, and at least one injectable second-line drug, such as Kanamycin (Muller 2013). Approximately 9% of patients with MDR-TB had XDR-TB (WHO 2014).

Cases of MDR and XDR TB arise either during the course of treatment or by transmission of existing resistant strains, otherwise known as primary resistance (Gandhi NR 2010). Factors contributing to acquired drug resistance include the initiation of an inadequate treatment regimen, poor drug quality, and patient nonadherence to treatment. Factors contributing to primary resistance include a delay in diagnosis using conventional drug susceptibility methods and a prolonged time to sterilization of sputum cultures due to the decreased efficiency of second-line drugs (Gandhi NR 2010). Although the contribution of primary versus acquired resistance to cases of MDR and XDR-TB cases is difficult to determine, studies from Chinese MDR TB strains determined over half of MDR/XDR TB cases are due to primary resistance. Zhao et al analyzed 4379 culture-positive TB cases from Shanghai, and determined that 60% of MDR-TB and 55% of XDR-TB cases occurred in patients with no history of TB treatment, suggesting but not conclusively establishing primary resistance as the method of acquisition(Zhao 2009). More recent research involving genotyping of XDR strains and MDR strains that later became XDR showed that 40% of XDR-TB strains were highly clustered in one of the highly endemic areas of TB in China, suggesting primary resistance is highly important. They also analyzed 9 MDR TB strains that became XDR, 4 of which were transmitted via primary resistance, 4 via acquired resistance, and 1 via exogenous reinfection (Wang 2015). In a South African study of patients with MDR-TB/XDR TB priorly treated for less resistant strains, the spoligotypes for the follow-up isolate differed from the original isolate, suggesting exogenous reinfection even in these priorly treated patients (Andrews 2008).

Mycobacteria evolve resistance to isoniazid and rifampicin at 10^{-8} and 10^{-9} mutations/mycobacterium/cell division(David 1970), independent of antibiotic exposure. This means that every time an individual mycobacterium divides, there is a 10^{-8} to 10^{-9} chance it will develop a mutation conferring resistance to these antibiotics. Considering bacterial burden during active *M. tuberculosis* infection is 10^{8} bacilli in an individual lesion, *M. tuberculosis* must be treated with multiple drugs, as resistance will inevitably arise with monotherapy (Gillespie 2002). Mutations conferring antibiotic resistance may arise in the gene encoding for the drug target or its regulatory region, or in the gene products involved in the activation of pro drugs (Muller 2013).

Clinically, Tuberculosis is commonly a latent infection, with evidence of TB infection on PPD or Quantiferon test but no clinical, microbiologic, or radiographic evidence of disease. In these cases, Mycobacterium are present but not actively dividing. Even in clinically active cases, M. tuberculosis has a very slow generation time, and its slow metabolic activity makes it a difficult antibiotic target (Gillespie

2002). Furthermore, macrophages, which constitute the first line of host defense against TB infection, place further selective pressure on the bacteria by forcing it to adapt to the harsh intracellular environment. However, despite the phenotypic shifting of mycobacteria occurring in macrophages, recent data shows that isoniazid, pyrazinamide and ethambutol can effectively kill intracellular mycobacteria independently of their intracellular replication rate (Raffetseder 2014). Many antibiotics, such as Rifampin and Isoniazid, are more effective at killing growth-phase cultures vs. stationary cultures (Wayne 1994). Further adding to the therapeutic challenges are the different microenvironments that the organism occupies in the host. It can form cavitations, areas of caseous necrosis and empyemas, making antibiotic penetration difficult and creating an acidic environment that may denature many drugs. These differing microenvironments lead to various different populations of bacteria present in the host (Gillespie 2002). This heterogeneity increases the likelihood of developing resistant strains.

Mycobacteria do not readily take up plasmids, so it is generally thought that horizontal gene transfer does not play a role in the development of resistant *M. tuberculosis* strains(Gagneux S 2007). However, recent data suggests that recombination, and therefore horizontal gene transfer, may have played a role in *M. tuberculosis* evolution. This was evident in studies that found excess genetic diversity in regions of the *M. tuberculosis* genome encoding for key adaptive function and few signs of genome degradation, therefore showing recombination events played a role in M tb evolution and that it is not as genetically isolated as previously thought (Namouchi A 2012). However, a putative role for recombination in the evolution of antibiotic resistance has yet to be established.

Also important to the understanding of antibiotic resistance are the rise of "persister" cells, bacterium that have differentiated into phenotypes with reduced sensitivity to antibiotics(Balaban 2011). This phenomena was first described following antibiotic exposure, but can also be caused by intracellular signals, quorum sensing, and starvation(Balaban 2011). This is not due to mutations, but to phenotypic changes undergone by the bacterium to adapt to a new environment, but could lead to a resistant mutation arising as it increases the time the bacteria is exposed to the drug (Muller 2013).

Understanding the mechanisms of antibiotic resistance in M. tuberculosis is an active research field, and several genes have been identified that may code for antibiotic resistance. However, it is unclear for whether elevated expression of these genes in M. tuberculosis leads to increased resistance. My research focused primarily on six different known or putative antibiotic resistance genes, as shown in the table below.

Table 1: Potential Genes involved in Mycobacterium tuberculosis antibiotic resistance

Gene	Antibiotic Resistance[1]	Gene product	Mechanism of resistance
PncA	Pyrazinamide	Pyrazinamidase	Elimination of pro-drug conversion[2]
KasA	Isoniazid	β-ketoacyl-[acyl-carrier protein] synthase 1	Alteration of drug target[3]
KasB	Isoniazid	β-ketoacyl-[acyl-carrier protein] synthase 2	unknown
GyrA	Fluoroquinolone	DNA Gyrase	Alteration of drug target[2]

RpoB	Rifampicin	βsubunit RNA polymerase	Alteration of drug target[2]
EmbB	Ethambutol	Arabinosyl transferase	Alteration of drug target[2]

1. TB Mutation Database https://tbdreamdb.ki.se/Info/Default.aspx
2. Muller, B. B., Sonia Rose, Graham, Gagneux, Sebastien (2013). "The Heterogeneous Evolution of Multidrug-resistant Mycobacterium Tuberculosis." Trends Genet **29**(3): 160-169.
3. Slayden, R., Barry, CE (2002). "The Role of KasA and KasB in the biosynthesys of meromycolic acids and isoniazid resistance in *Mycobacterium tuberculosis* " Tuberculosis **82**(4/5): 149-160.

Materials and Methods

We used PCR to clone 7 genes (the genes listed in table 1 plus the RpoB gene with an ATG start site). The constructs were subcloned into E. Coli using the TOPO TA Cloning Kit (Life Sciences). Cloning was performed by first transforming One Shot Topo 10 chemically competent E. Coli cells, with the pCR 2.1 TOPO vector (both from Life Sciences) used to directly insert the PCR product. The pCR 2.1 vector contains an Ampicillin resistance cassette to select transformants. Transformants were grown in LB-agar plates containing ampicillin. Colonies were subsequently grown in LB broth with Ampicillin. The plasmid was subsequently extracted using a Miniprep kit (Quiagen) and the insert was isolated by digests with the EcoR1 restriction enzyme. The digested product was run on an agarose gel to verify the correct gene size. Plasmids containing the correct insert were used for Sanger sequencing. Sequencing was done by using the primers for using T7 as the forward primer and M13Rev as the reverse primer (available through the Sequencing Core). The DNA sequences were analyzed using Vector NTI to evaluate for the orientation of the gene insert. Plasmids with the gene insert in the reverse direction were digested nondirectionally with HindIII. The 5 gene products (PCNA, KasA, KasB GyrA, RpoB) were excised by directed restriction digests (EcoRV and BamH1 or with Hind III restriction enzymes). The inserts were isolated from agarose gels using a gel extraction kit (Quiagen).

The inserts were subcloned into a M. tuberculosis shuttle vector, either pSum-MCS (multiple cloning site)-2 or MCS-3, as per the chart below. The pSum vectors have a kanamycin resistance gene allowing for selection of transformants. The subcloning step was performed in a similar fashion to the original cloning step. Transformation was done by cutting the vector, purifying the vector and ligating the vector with the insert using T4 ligase. This was used to transform E. Coli. The transformed DH5α cells were subsequently grown in LB agar plates containing 10 mg/l kanamycin. Transformants were grown in liquid LB media with kanamycin and the plasmid extracted using the miniprep kit. Plasmids were then digested using either EcoRV/BamH1 or HindIII and then ran on agarose gels.

Plasmids that had taken up the right insert were sent for Sanger sequencing to verify the gene insert was in the right direction. Lastly, the transformants who had taken up the insert in the correct direction were then regrown in a larger volume of LB (200 ml) and the plasmid extracted using a Maxiprep kit (Quiagen). The plasmids extracted using the maxiprep kit were sequenced using primers to provide sequencing for the entire gene.

Mutational analysis was performed using oligonucleotides (Sigma Aldrich) to sequence the internal portion of the two longer genes, RpoB and GyrA. This was to verify no mutations had been inserted

during the PCR reaction. Oligonucleotide primers were designed as shown in table two. Since Sanger sequencing can only accurately sequence 600-700 bp of DNA at a time, primers were designed to analyze the internal parts of these longer genes that were not adequately sequenced by earlier reactions.

Of these, Pcna, KasB, and GyrA had no mutations and were tested in *Mycobacterium tuberculosis* H37Ra, an avirulent strain of tuberculosis. Transformation was performed via electroporation. Following electroporation, the transformants were grown on 7H9 liquid media with 10% OADC initially. These were then plated onto 7H10 Middlebrook Agar plates containing kanamycin and 10% OADC. Transformants that grew in these plates after three weeks were then grown in 7H9 Middlebrook media with Kanamycin and 10% OADC. After the liquid cultures had grown, these were tested for uptake of the plasmid by bead beating a portion of the culture, extracting the plasmid via a miniprep kit, and then retransforming into E. coli.

Table 2: Oligonucleotides for Internal Sequencing

Name	Oligonucleotide	Location
RpoB-1	GGACATCTACCGCAAGCTGCG	BP 807-827
RpoB-2	CAACATCGGTCTGATCGGC	BP 1456-1476
RpoB-3	CTGGGCAAGAACCTGCTGGTG	bp 2125-2145
GyrA-1	GTGATCACCGAGTTGCCGTATCAG	bp 807-831
GyrA-2	GGATCGTGCGCGACGAACTCG	bp 1430-1450

As M. tuberculosis has such lengthy generation time, we also transformed M. smegmatis, a fast-growing mycobacterium and M tb model organism, in order to more quickly complete the dose-response studies. M smegmatis was grown for 4 days in 7H9 Middlebrook broth media and then electroporated using the PcnA, GyrA and KasB plasmids. These were grown in fresh 7H9 overnight and plated on 7H10 Middlebrook agar plates containing 10 ug/ml Kanamycin.

Once M. smegmatis mutants were identified, dose-response studies using Moxifloxacin were conducted for 2 GyrA mutants and Wild Type M. Smegmatis.

For the GyrA studies, 2 GyrA mutants were subcultured, 2 ml stationary phase culture to 20 ml fresh 7H9 media containing 10 ug/ml Kanamycin. Likewise, 2 ml of stationary phase wild type M. Smegmatis was cultured in 20 ml plain 7H9 media. All three cultures were back-diluted the following day to an OD650 of approximately 0.6. 5 ml of each isolate were consequently incubated overnight in the following Moxifloxacin concentrations: 0 mg/l, 0.25 mg/l, 0.5 mg/l, 1 mg/l, 2 mg/l, 4 mg/l, 8 mg/l, and 16 mg/l. Cultures were incubated at each concentration in triplicate for a total of 24 cultures per isolate. The following day, 1 ml aliquots of each culture were washed twice to remove the Moxifloxacin. Washing was done by centrifuging each aliquot and 14000 rpm for 5 minutes and resuspending the bacteria in 1 ml of normal saline. Serial dilution was then performed by adding 200 ug of each 1 ml aliquot to 1.8 ml

of saline and adding 200 ul of that tube to the subsequent 1.8 ml tube. This was done a total of 5 times. These 10^0 to 10^{-5} dilutions were consequently plated on 7H10 agar plates, plain 7H10 plates for the wild type M. Smegmatis and 10 ug/ml kanamycin-containing plates for the 2 GyrA mutants.

For the next set of dose-response studies, we first determined the minimum inhibitory concentration (MIC) for each of the three isolates. These were done by first inoculating 7H10 agar plates with wild type M. Smegmatis and 7H10-Kanamycin plates with each of the GyrA mutants. These plates were then inoculated with Etest strips containing a gradient of moxifloxacin concentrations. Plates were read after 2 days of incubation. The dose response study was subsequently repeated with lower concentrations of moxifloxacin: 0 mg/l, 0.002 mg/l, 0.004 mg/l, 0.008 mg/l, 0.016 mg/l, 0.032 mg/l, 0.064 mg/l, 0.128 mg/l. The incubation period was shortened to 5 hours.

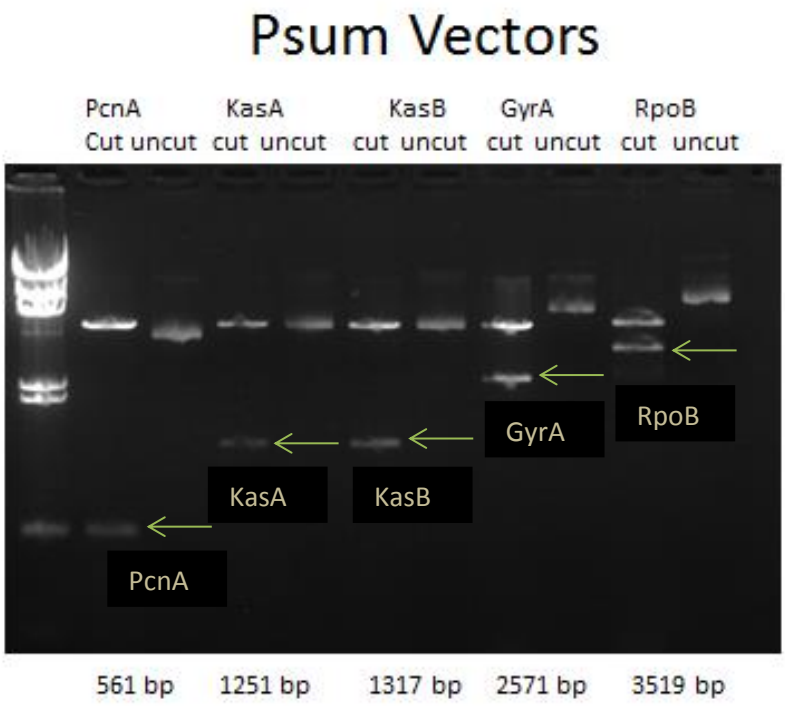
Results

The original cloning step yielded five of the seven of the original PCR products with the correct gene insert with the full length gene inserted into the pCR vector. These 5 genes were then cloned into the pSum vectors. See figure 1 for gel electrophoresis of both the undigested plasmid and digested plasmid of the pSum vectors.

Table 3: Genes cloned into pCR vectors:

Gene	Description	Antibiotic Resistance	Expected Size (Kb)	Cloned (Kb)	Cloning Strategy
PncA	Nicotinamidase	Pyrazinimide	561	561	HindIIi-MCS2
GyrA	DNA gyrase	Fluoroquinolones	2571	2571	HindIII-MCS2
RpoB	β Subunit RNA Polymerase	Rifampicin	3519	3519	EcoRV-BamH1- MCS3
KasA	β-ketoacyl-[acyl-carrier protein] synthase 1	Isoniazid?	1251	1251 KB	HindIII-MCS2
KasB	β-ketoacyl-[acyl-carrier protein] synthase 2	Isoniazid?	1317	1317 KB	HindIII-MCS2
EmbB	Arabinosyl transferase	Ethambutol	3297	~2.3	not yet cloned
RpoB+ATG	β Subunit RNA Polymerase	Rifampicin	3519	~1 KB	not yet cloned

Figure 1: Gel electrophoresis of Psum vectors. Location of gene insert is labelled with arrows



NO sequencing revealed a number of mutations in KasA and RpoB. PncA had a mutation that did not change the coding sequence of the amino acid.

Table 4: Results of Mutational analysis

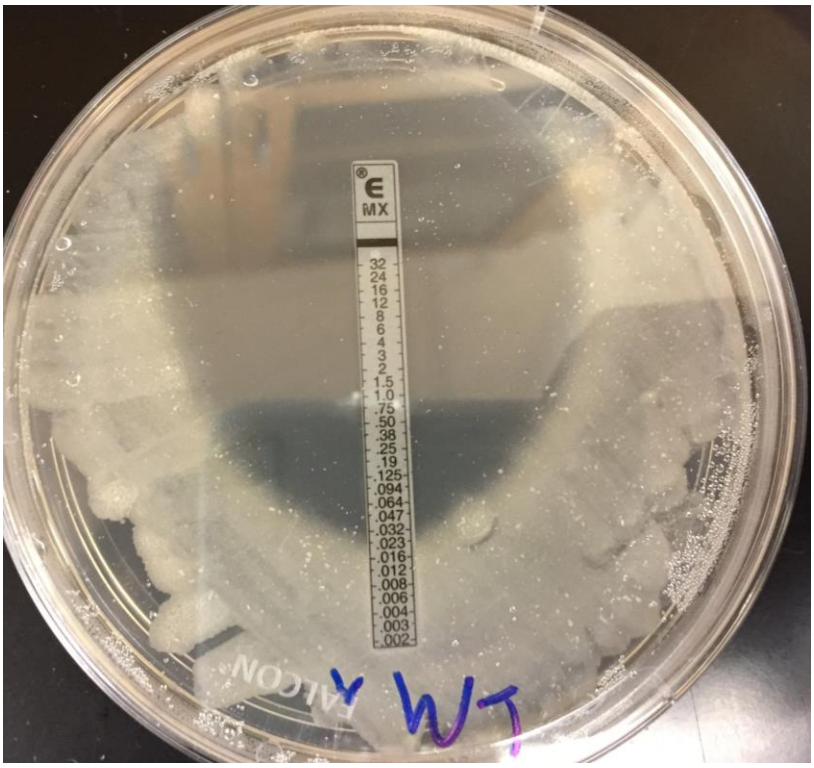
gene	Location	mutation	amino acid sub	
RpoB	1498	insertion G		near end of sequencing reaction
RpoB	1819	C->T	R->C	
RpoB	2146	G->A	A->T	
RpoB	2162	insertion G		near end of sequencing reaction
RpoB	2650	G->A		
RpoB	2876	A->G		
RpoB	3083	A->G		
GyrA	1666	G->A		not present in downstream sequencing reaction

GyrA	1678	G->A		not present in downstream sequencing reaction
GyrA	2096	insertion G		not present in downstream sequencing reaction
KasB	562	insertion G		not present in downstream sequencing reaction
KasA	51-52	CG->TA	A->T	
PcnA	512	C->T	A->V	

Result of Dose-Response Studies

Dose response studies were performed by incubating isolates of the two M. smegmatis GyrA and wild type M. smegmatis in increasing concentrations of moxifloxacin, to see if the percentage of bacteria killed at a given Moxifloxacin concentration varied between the three strains. The first dose-response curve yielded no bacterial growth for any colonies incubated even with the lowest concentration of ciprofloxacin. Only the isolates incubated with 0 mg/l of moxifloxacin grew. In preparation for the second round of dose-response studies, we determined the MIC using e test strips, as per the methods section. The MIC was slightly above 0.032 mg/l moxifloxacin for the GyrA mutants and slightly below 0.032 mg/l for the wild type M. Smegmatis. These results are portrayed in figure 5a-c. The repeated dose-response studies with lower concentrations of Moxifloxacin (0 mg/l, 0.002 mg/l, 0.004 mg/l, 0.008 mg/l, 0.016 mg/l, 0.032 mg/l, 0.064 mg/l, 0.128 mg/l) yielded the results seen in Figures 6. Under these conditions, we did not see significant M. Smegmatis kill by Moxifloxacin at any concentration, as the amount of bacteria seen did not significantly decrease with increasing antibiotic concentration

Figure 5: M. Smegmatis MIC Determination

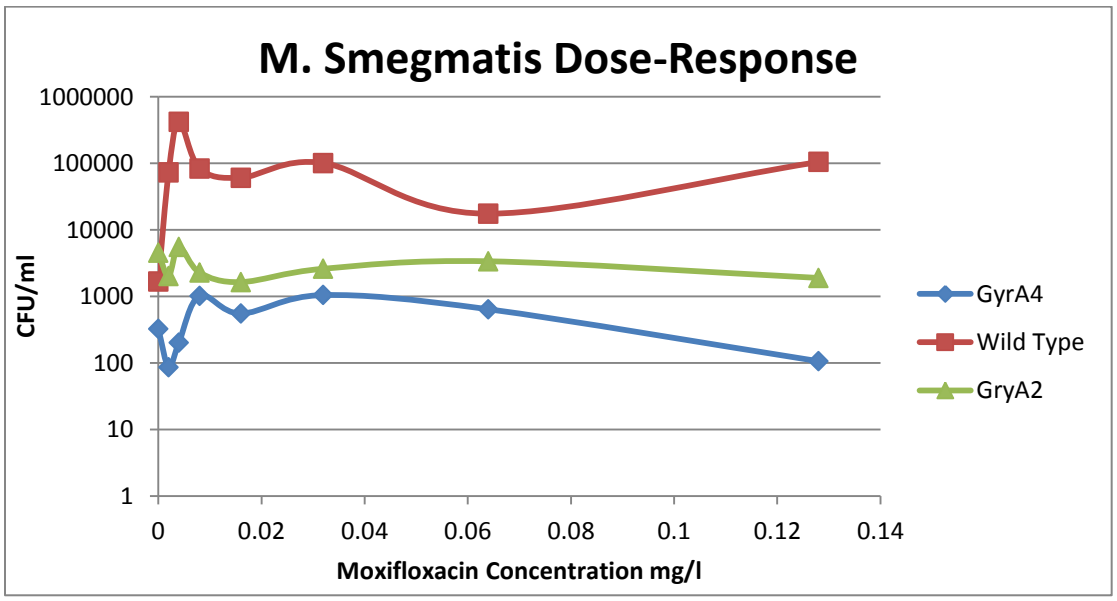


5a: Wild-Type M. Smegmatis MIC determination

5b: Gyra-2 Mutant M. Smegmatis MIC determination

5b: Gyra-2 Mutant M. Smegmatis MIC determination

Figure 6- M. smegmatis Dose-Response after 5 hours of Incubation: Wild Type and GyrA mutant strains #2 and #4



Discussion

The goal of the project is to perform accurate dose-response studies in order to determine if overexpression of wild type M. Tuberculosis genes can cause antibiotic resistance. The genes we studied are either known or thought to be involved in mycobacterial defense against antibiotics. Although mutations have been known to confer additional resistance, genes such as RpoB and GyrA that are involved in key mycobacterial replication functions are generally highly conserved (Gillespie 2002). Therefore, particularly for Moxifloxacin resistance, the role of GyrA overexpression is worth studying. The MIC determined via the e-test method did show that the GyrA M. smegmatis mutants did have a moderately higher MIC than the wild type mutant, indicating that overexpression of the GyrA gene is making the mutants more resistant to fluoroquinolones. More formal dose-response studies serve to further corroborate this observation.

Performing accurate dose-response studies in moderately fast-growing bacteria is difficult. The incubation period must be long enough to allow a response to be seen, but not so long that catch-up growth occurs. The concentrations used must also be close enough to the MIC for the isolate so that some, but not all, of the population is killed. Therefore, the next steps involve repeating the dose-response studies with the same low concentrations of Moxifloxacin used in the second dose-response study but this time plating the isolates after two different incubation periods: 12 hours and 24 hours. Varying the incubation period will help find the ideal incubation length that will allow for a dose-response curve to be seen without allowing for catch-up growth.

Another technical challenge that is impacting these studies is that the cost of fitness to the mutant bacteria of maintaining the plasmid. The mutant bacteria were grown in Kanamycin-containing media at all times in order to maintain the selective pressure necessary for plasmid retention. However, this comes at a cost to fitness. Although not formally quantified, the GyrA mutant isolates exhibited lower growth rates in comparison to wild type. This was evident in the overall lower colony counts seen in the GyrA mutants in comparison to the wild type over the course of the dose-response studies, even in those isolates incubated with no Moxifloxacin. A further step would be to create an M. Smegmatis mutant with a plasmid containing a Kanamycin resistance gene but no other genes to account for any effect the plasmid may have.

Another further step would be to repeat the study using the other mutants generated, namely the M. tuberculosis PncA, KasA, and GyrA mutants, along with the M. Smegmatis PncA mutants. This would more comprehensively answer our research question. This would also allow us to determine if there is a difference among the M. smegmatis mutants and M tuberculosis mutants as they compare to their respective wild type isolates; giving us more information as to whether the M smegmatis mutants are a valid model organism for these types of studies.

Another way to approach this problem is to avoid transforming via plasmids altogether. With the advent of Crispr-Cas technology, genes can be inserted directly into the genome. Cluster Repeated Interspace Short Palindromic Repeats (CRISP) RNA and their associated Cas proteins form part of prokaryotic defense against invading DNA from phages or plasmids. The system has two stages: the adaptation stage when the bacteria incorporate the invading DNA into their own genome, and the interference stage, in which the bacteria utilize their newly acquired nucleic acids to defend against that invader sequence(Deveau 2010). Crispr-Cas systems have been engineered to insert genes into a variety of prokaryotic and eukaryotic genomes. If such a system could be engineered for mycobacteria, then the genes we are studying could be directly inserted into their genome, abrogating the need for a plasmid and allowing for a more accurate analysis of the effect overexpression of these genes may have.

Works Cited

World Health Organization. Global Tuberculosis Report (2014)

Center for Disease Control, “Tuberculosis Fact Sheets: Latent TB infection vs Active TB disease” 2014.

Center for Disease Control, American Thoracic Society. (2000). "Diagnostic Standards and Classification of Tuberculosis in Adults and Children." American Journal of Respiratory and Critical Care Medicine **161**(4): 1376-1395

Andrews, J. R., Neel R. Gandhi, Prashini Moodley, N. Sarita Shah, Louise Bohlken, Anthony P. Moll, Manormoney Pillay, Gerald Friedland, A. Willem Sturm (2008). "Exogenous Reinfection as a Cause of Multidrug-Resistant and Extensively Drug-Resistant Tuberculosis in Rural South Africa." J Infect Dis. **198**(11): 1582-1589.

Balaban, N. (2011). "Persistence: mechanisms for triggering and enhancing phenotypic variability." Curr Opin Genet Dev. **21**: 768–775.

David, H. (1970). "Probability distribution of drug-resistant mutants in unselected populations of Mycobacterium tuberculosis." Appl Microbiol. **20**: 810–814.

Deveau, H., Garneau, Josiane E, Moineau, Sylvain (2010). "CRISPR/Cas System and Its Role in Phage-Bacteria Interactions." Annu. Rev. Microbiol. **64**: 475-493.

Gagneux S, S. P. (2007). "Global phylogeography of Mycobacterium tuberculosis and implications for tuberculosis product development." Lancet Infect Dis. **7**: 328-337.

Gandhi NR, N. P., Dheda K, Schaaf HS, Zignol M, van Soolingen D, Jensen P, Bayona J (2010). "Multidrug-resistant and extensively drug-resistant tuberculosis: a threat to global control of tuberculosis." Lancet **375**: 1830-1843.

Gillespie, S. (2002). "Evolution of drug resistance in Mycobacterium tuberculosis: clinical and molecular perspective." Antimicrob Agents Chemother. **46**: 267–274.

Muller, B. B., Sonia Rose, Graham, Gagneux, Sebastien (2013). "The Heterogeneous Evolution of Multidrug-resistant Mycobacterium Tuberculosis." Trends Genet **29**(3): 160-169.

Namouchi A, e. a. (2012). "After the bottleneck: Genome-wide diversification of the Mycobacterium tuberculosis complex by mutation, recombination, and natural selection." Genome Res. **22**: 721–734.

Raffetseder, J., Elsje Pienaar, Robert Blomgran, Daniel Eklund, Veronika Patcha Brodin, Henrik Andersson, Amanda Welin, Maria Lerm (2014). "Replication Rates of Mycobacterium tuberculosis in Human Macrophages Do Not Correlate with Mycobacterial Antibiotic Susceptibility." PLoS One **9**(11): e112426.

Wang, F., Lingyun Shaoa, Xiaoping Fana, Yaojie Shena, Ni Diaoa, Jialin Jina, Feng Suna, Jing Wua, Jiazhen Chena, Xinhua Wenga, Xunjia Cheng, Ying Zhanga, and Wenhong Zhanga (2015). "Evolution and Transmission Patterns of Extensively Drug-Resistant Tuberculosis in China." Antimicrob. Agents Chemother. **59**(2): 818-825.

Wayne, L. (1994). "Dormancy of Mycobacterium tuberculosis and Latency of Disease." Eur J Clin Microbiol Infect Dis. **13**(11): 908-914.

Zhao, M., Xia Li, Peng Xu, Xin Shen, Xiaohong Gui, Lili Wang, Kathryn DeRiemer, Jian Mei, Qian Gao (2009). "Transmission of MDR and XDR Tuberculosis in Shanghai, China." PLoS One **4**(2): e4370.