CHARACTERIZATION OF DRUG RESISTANCE IN *MYCOBACTERIUM TUBERCULOSIS* VIA SATURATING MUTAGENESIS OF DRUG TARGETS

A Master's Thesis Presented

By

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Basic and Biomedical Sciences

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Basic and Biomedical Sciences

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Thank you to Dr. Kenan Murphy for his expertise in recombineering and for providing the pKM402 Che9 RecT *M. smegmatis* strain.

Abstract

Mycobacterium tuberculosis isolates from multiple drug resistant or extensively drug resistant patients show a particular set of mutations in drug targets conferring resistance. However, the selection of drug-resistant strains *in vitro* yields an alternative set of mutations, thought to result from the cost-benefit associated with drug resistance. Mutations allowing for survival under antibiotic may not be beneficial when presented with the host environment or with a drug-free environment. These fitness effects drive the natural evolution of this bacterium. Using recombineering a large cohort of mutations was generated within two drug targets, *inhA* and *gyrA,* to study *in vitro* the variability of mutations allowable under either isoniazid or ofloxacin, respectively. As a proof of concept this process was carried out in *Mycobacterium smegmatis*. Analysis of survivors allowed for identification of novel mutations and substitutions, as well as showing mutations previously found only in clinical isolates can be present in laboratory isolates.

Table of Contents

Acknowledgements iii

Abstract iv

List of Tables vi

List of Figures vii

Chapter I Introduction 1

Overview 1

Recombineering 2

Chapter II Recombineering *inhA* and *gyrA* 6

Introduction 6

Isoniazid 6

Fluoroquinolone 8

Materials and Methods 10

Results 14

Discussion 18

Appendix 21

Bibliography 31

List of Tables

Table II.1 Commonly found mutations in *inhA* conferring resistance to INH from clinical and laboratory isolates. 6

Table II.2 Commonly found amino acid mutations in *gyrA* conferring resistance to FQ from clinical and laboratory isolates. 8

Table II.3 Mutations in *inhA* and *gyrA* determined following sequencing of survivors... 18

List of Figures

Figure I.1 Accepted model for the molecular action of recombineering. 4

Figure II.1 *M. tuberculosis* InhA modeled with bound NADH. 7

Figure II.2 Modeled mycobacterium GyrA. 9

Figure II.3 Transformation with the mutated *inhA* oligo increases the frequency of resistance to 20µg/mL INH and transformation with the mutated *gyrA* oligo increases the frequency of resistance to 0.5µg/mL OFX. 15

Figure II.4 PCR check for pKM402 inhA and pKM402 gyrA survivors. 16

Figure II.5 Number of mutations per gene for *inhA* and *gyrA* isolates, excluding silent mutations. 17

Figure II.6 *M. tuberculosis* InhA modeled with bound NADH and mutated residues found in survivors. 19

Chapter I Introduction

Overview

Tuberculosis (TB) is an infectious disease concentrated primarily within the respiratory tract and is caused by the bacterium *Mycobacterium tuberculosis* (*M. tuberculosis*). Once exposed, approximately 10% of individuals develop active disease characterized by cough, fever, lethargy, and weight loss (Raviglione, 2006). Despite such a small percentage of individuals developing active disease, there are an estimated 1.5 million TB related deaths every year worldwide making it the second leading cause of death from an infectious disease behind HIV (WHO, 2011). Unfortunately, this plight has become an even greater problem with the development of drug resistant *M. tuberculosis* strains. Some individuals are shown to have disease resistant to not just one antibiotic, but multiple or even all known treatments. Multiple drug resistant tuberculosis (MDR-TB) patients demonstrate resistance to the first line drugs rifampicin and isoniazid (INH), while extensively drug resistant tuberculosis (XDR-TB) patients have MDR-TB with added resistance to the class of second line drugs, fluoroquinolones (FQ), and at least one of the second line injectable agents amikacin, kanamycin, and/or capreomycin (WHO, 2010).

Bacterial isolates from these MDR-TB or XDR-TB patients are consistently shown to have a particular set of mutations in drug targets conferring resistance. However, spontaneous drug resistant mutants generated in the laboratory for drug resistance studies often yield an alternative set of mutations. This differing spectrum of mutations hinders

the study of drug resistance evolution. Without a reliable model for the evolution of drug resistance, more efficient anti-tubercular drugs will continue to be elusive. Therefore, the cause of these variations between clinical and laboratory isolates, as well as a method to predict which mutations will arise needs to be explored.

The cost-benefit associated with drug resistance is one explanation for this difference between clinical and laboratory isolates. This cost-benefit is linked to the possibility of decreased function of a protein after mutation. This is especially the case for drug targets, as the drug often blocks an active or binding site of an essential bacterial protein thereby killing the bacteria. Therefore, mutations conferring drug resistance often alter the activity of the targeted protein.

The environment encountered by a population of bacteria plays a major role in whether or not the decreased function of a vital protein would be detrimental. For bacteria undergoing drug selection, the conditions *in vivo* and *in vitro* are exceedingly different. For instance, a protein necessary for survival when challenged by the host immune response would not be as necessary for a bacterium grown in culture. It is these fitness effects associated with differing environmental pressure that drives the development of the global population of bacteria.

Recombineering

Studying the evolution of drug resistance in an *in vitro* environment has proven

problematic without satisfactory experimental alternatives accounting for these environment differences. Therefore, a unique approach to the study of drug resistance evolution was taken, the opposite of selecting for spontaneous mutants under antibiotic pressure. A large cohort of possible mutations within an antibiotic target was generated to study the variability of mutations allowable under antibiotic selection *in vitro*. This mutational library was created in *Mycobacterium smegmatis* (*M. smegmatis*) through the use of a technique known as recombineering, to be explained in detail below. Analysis of the drug susceptibility allowed for the identification of novel mutations, as well as showing mutations previously observed only in clinical isolates can be present in laboratory isolates.

Genetic techniques such as homologous recombination have greatly increased the abilities of researchers to manipulate many model organisms, from bacteria to mice. However, many of these methods contain pitfalls resulting in decreased efficiency. As is the case with conventional cloning, unique restriction enzymes cannot always be found, which is especially difficult when working with large pieces of DNA. Additionally, large pieces of DNA can be fragile thereby adding another complication to working with large DNA molecules. On the other hand, large DNA molecules can be accurately manipulated using homologous recombination. Also, no restriction enzyme sequences are required; homologous recombination requires only a discrete region of homologous sequence. Through the added convenience of phage mediated homologous recombination, termed recombineering, shorter regions of homology can be used (Court

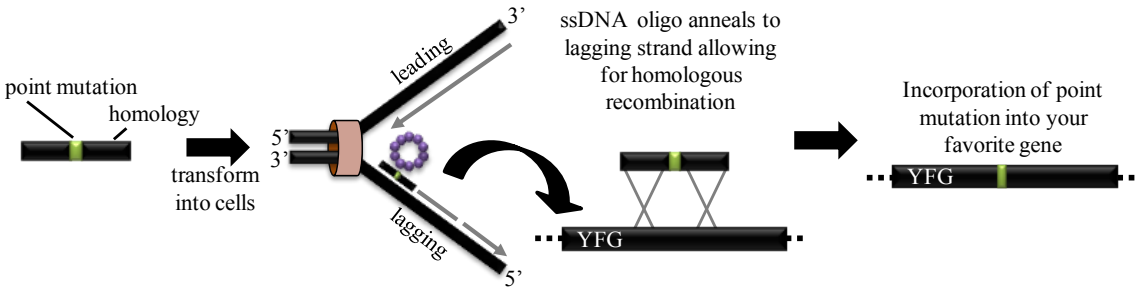


Figure I.1 Accepted model for the molecular action of recombineering.
The ring structured single stranded DNA annealing protein (SSAP) aids in annealing the mutated single stranded DNA oligo to the lagging strand of DNA at the replication fork (adapted from van Kessel et al., 2008 and Murphy and Marinus, 2010).

et al., 2002). For instance in *Escherichia coli* (*E. coli*), only 50 nucleotides flanking the region to be changed need to be of homologous sequence (van Kessel et al., 2008). Recombineering takes advantage of the bacteriophage proteins RecE and RecT for facile generation of single point mutations, deletions, and insertions (van Kessel and Hatfull, 2008). RecE, a DNA exonuclease, creates single stranded DNA which is annealed to replicating DNA by RecT, a single stranded DNA annealing protein (SSAP), allowing for homologous recombination (Figure I.1). This bacteriophage based system does not require RecA, which is advantageous due to the possibility of unwanted recombination events with RecA (Court et al., 2002). With the discovery of homologous proteins in mycobacteriophages it became possible to perform similar experiments in mycobacteria. The mycobacteriophage Che9c encodes the homologous proteins gp60 and gp61, which were demonstrated to have biochemical similarity to RecE and RecT, respectively (van Kessel and Hatfull, 2007). Fortunately, when working with single stranded DNA (ssDNA) only the RecT function is necessary; also recombineering is more efficient when using ssDNA (Court et al., 2002 and Ellis et al., 2001). This allowed for the convenience of using a synthetically made oligo as the substrate for recombination. It

has been demonstrated that recombineering is efficient in mycobacteria with as little as 70bp of homology; however, to mutagenize large regions of genes, a series of 100bp oligos were used.

Chapter II Recombineering *inhA* and *gyrA*

Introduction

Isoniazid

Isonicotinic acid hydrazide, also known as isoniazid (INH), was introduced in 1952 as the first effective treatment for tuberculosis, and it continues to be the primary first line drug against tuberculosis (Vilchèze and Jacobs, 2007). The principal action of INH is against the mycobacterial cell wall by inhibiting the biosynthesis of mycolic acids, which results in a loss of acid-fastness and eventual cell lysis. This inhibition is accomplished through interaction with the NADH dependent enoyl-acyl carrier protein reductase (InhA) of the fatty acid synthase II system. Before interaction with InhA, INH needs to be activated by KatG (catalase-peroxidase-peroxynitritase). KatG activation results in reaction with NADH thereby creating a NAD-INH adduct which binds to the InhA activation site (Wahab et al., 2009).

Unfortunately, within the first year of its use, resistance to INH was identified in clinical isolates (Steenken et al., 1952). INH resistance arises through several routes including mutations in: the *inhA* active site, the *inhA* upstream region/promoter, and within *katG* (Wang et al., 2011). Approximately 22% of isolates, however, have no known resistance mechanism. Conversely, the mechanism of action for INH previously discussed was determined through study

Table II.1 Commonly found mutations in *inhA* conferring resistance to INH from clinical and laboratory isolates.

I16T[=]	V78A[$]
I21T[^]	S94A[~]
I21V[^]	I95P[$]
I47T[$]	I194T[^]

Clinical isolates indicated in black text and laboratory isolates in gray text
[~]Banerjee et al., 1994
[=]Dessen etal., 1995
[$]Basso et al., 1998
[^]Ramaswamy et al., 2003

of mutations found in a relatively small number of clinical isolates. Mutations within *inhA* decrease the affinity of the protein for the NAD-INH adduct via either structural changes that alter the architecture of active site or mutations within the active site itself (Oliveira et al, 2006). Despite the active site spanning 42 amino acids and the NADH binding site spanning 185 amino acids there are only 8 known resistance-associated mutations within *inhA* (Table II.1). This surprisingly low number in the mutations allowable to alter the InhA binding site led to the question: Are the reported clinical and laboratory mutations the only viable options for INH resistance arising from mutation in *inhA* or can novel mutations be isolated?

Using the known amino acid sites for mutation and the amino acids within the InhA binding site, a 400bp region was selected for randomization via recombineering (Figure II.1 and Supplementary Table 1). As an initial study, these experiments were performed in *Mycobacterium smegmatis* (*M. smegmatis*), a non-pathogenic soil dwelling relative of *M. tuberculosis* commonly used in the laboratory due to its ease of use. Therefore, it was necessary to determine the similarly between the sequences for *inhA* so the mutations could

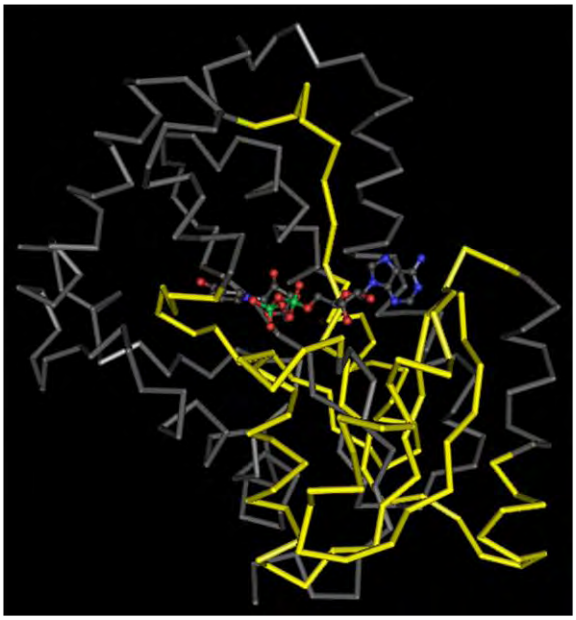


Figure II.1 *M. tuberculosis* InhA modeled with bound NADH.
Region mutagenized for recombineering highlighted in yellow (PDB ID: 2AQ8).

be properly tracked. The *M. smegmatis inhA* amino acid sequence is 94% identical to the *M. tuberculosis* sequence, with all the known mutations aligning properly (Supplementary Figure 2). Therefore, it was determined *M. smegmatis* would be a suitable model.

Fluoroquinolone

The second line class of drugs known as fluoroquinolones is characterized by structural similarity to nalidixic acid and by the antimicrobial mode of action (Gorzynski et al., 1989). Fluoroquinolones target the type II topoisomerases, DNA gyrase and topoisomerase IV, of bacteria (Piton et al., 2010). *M. tuberculosis* is unique in that it only encodes one of the two type II topoisomerases, the DNA gyrase. Gyrase is an A2B2 complex, and fluoroquinolones specifically target the subunit gyrase A (GyrA). Fluoroquinolones bind the DNA-gyrase complex and stabilize the normally transient enzymatic reaction, thereby stalling DNA replication.

Table II.2 Commonly found amino acid mutations in *gyrA* conferring resistance to FQ from clinical and laboratory isolates.

T80A[+]	A90V[~]
A83V[=]	S91P[~]
D87N[=]	D94N[~]
D87A[=]	D94A[~]
D87N[=]	D94G[~]
G88C[~]	D94H[~]
G88C[+]	D94N[~]
G88A[$]	D94Y[~]
D89N[^]	S95T[*]
D89G[+]	L96P[*]
A90V[~]	D111N[*]

Clinical isolates indicated in black text and laboratory isolates in gray text
[~]Takiff et al., 1994
[=]Alangaden et al., 1995
[$]Matrat et al., 2006
[^]VonGroll et al., 2009
[*]Ali et al., 2011
[+]Campbell et al., 2011

The history of the fluoroquinolones is much more recent than that of INH with the introduction of the class as a treatment for TB in the early 1980s, and new compounds continue to be introduced into the drug market. The continuing development of new

versions of fluoroquinolone as well as the rise in MDR and XDR-TB made including a fluoroquinolone in this study highly applicable. The MIC_{90} values for the fluoroquinolones against *M. tuberculosis* range from 0.05mg/L to greater than 8mg/L (Jacobs, 1999). Ofloxacin (OFX), a commonly used antibiotic with a MIC_{90} value of 0.2mg/L was the fluoroquinolone chosen for this work.

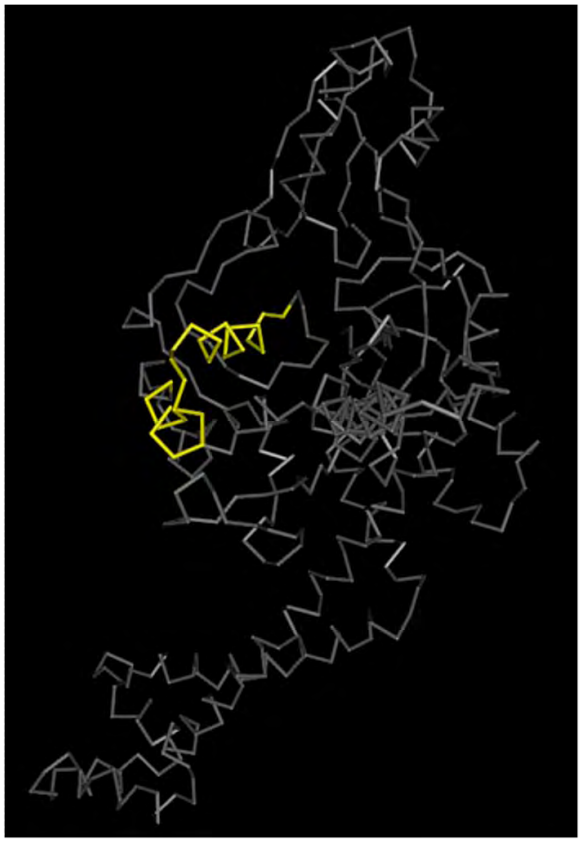


Figure II.2 Modeled mycobacterium GyrA. Region mutagenized for recombineering highlighted in yellow (PDB ID: 3IFZ).

Over 20 different mutations in *gyrA*, causing varying degrees of resistance to any of the fluoroquinolones, have been identified in either clinical or laboratory isolates (Table II.2). All of these mutations fall in the region of *gyrA* that has been designated as the fluoroquinolone resistance determining region (QRDR), which extends from residue 74 to 113 in *M. tuberculosis*. The QRDR falls within the DNA-gate of GyrA and includes some of the most important residues for interaction with DNA. Mutations within the QRDR decrease the affinity of the drug for the DNA-gyrase complex via disruption of the quinolone binding pocket (QBP) (Piton et al., 2010). The QBP is the binding site for FQ made from residues within the QRDR as well as DNA. Of the 30 amino acids that make up the QRDR only

11 mutations have been identified from either clinical or laboratory isolates. Similar to *inhA*, this low number of mutations allowable for drug resistance led to the question: Are the reported clinical and laboratory mutations the only viable options for OFX resistance arising from mutation in *gyrA* or can novel mutations be isolated?

Using the known amino acid sites for mutation and the amino acids within the QRDR a 100bp region was chosen for randomization via recombineering (Figure II.2 and Supplementary Table 1). Again, it was necessary to determine the similarly between the *M. smegmatis* and *M. tuberculosis* sequences for *gyrA* so the mutations could be properly tracked. The *M. smegmatis gyrA* amino acid sequence is 95% identical to the *M. tuberculosis* sequence, with all the known mutations aligning properly (Supplementary Figure 3). Therefore, it was determined *M. smegmatis* would be a suitable model.

Materials and Methods

Bacterial strains. Wild type *M. smegmatis*, mc^2-155, competent cells were generated by growing in 7H9 media supplemented with 10% OADC and 1% $Tween_{80}$ at 37°C while shaking to an OD of 1.0, spinning down the cultures at 4°C for 10min at 4000rpm, washing twice with 10% glycerol, and resuspending the culture in 10% glycerol at 1/10 of the original culture volume. Competent cells were frozen and stored at -80°C in 400µL aliquots.

Che9 RecT *M. smegmatis* strain pKM402 was designed and provided by Kenan Murphy. The Che9 RecT expression plasmid, pKM402, was made as follows: RecT gene

generated by PCR from pJV53 with primers 655-GTAGTACATATGGCTGAAAATGCTGTCACCAAG and 656-TTTCCCCCCCCCCCGACGTCAGGT cut with NdeI and NheI and ligated into pKM287 backbone (pKM287 is a derivative of pUV15tet-O). Plasmid maps for pKM402 expression plasmid and pUV15tet-O can be seen in Supplemental Figure 1.

pKM402 competent cells were generated by growing in 7H9 media supplemented with 10% OADC, 1% $Tween_{80}$, and kanamycin (20μg/mL) at 37°C while shaking to an OD of 0.5 before inducing the expression of RecT with the addition of ATc50 and growing covered at 37°C while shaking to an OD of 1.0. The cultures were then similarly spun down at 4°C for 10min at 4000rpm, washing twice with 10% glycerol, and resuspending the culture in 10% glycerol at 1/10 of the original culture volume. Due to the decreased recombineering efficiency of frozen competent cells, pKM402 competent cells were always made fresh.

Determination of MBC. The antibiotic concentration at which the bacteria are killed (MBC) was determined via plating. Approximately 10^8 cells of mc^2-155 were plated on individual plates containing either isoniazid (INH) or ofloxacin (OFX) at concentrations ranging from 0.1μg/mL – 100μg/mL for INH and from 0.01μg/mL – 100μg/mL for OFX. Plates were incubated for 3 days at 37°C and then checked for bacterial colonies. The concentration at which no additional colonies above the background rate of spontaneous mutation were observed was used as the antibiotic concentration for low level resistant mutants (20μg/mL for INH and 0.5μg/mL for OFX). High level resistant mutants were isolated using 30μg/mL for INH and 5.0μg/mL for OFX.

Custom oligos. Oligos were designed so that translated proteins would contain a single mutated amino acid within the region of interest. This was accomplished by producing oligos that have mutations at every position within the region of interest at a probability of 1%. For instance, at position 46 of *inhA* the wild type nucleotide is an adenine, and since the oligos are 100 bases in length, the oligo would be produced using a mixture of 99% A, 0.33% C, 0.33% G, and 0.33% T. However, since the company used (Integrated DNA Technologies) is unable to produce mixtures containing less than 1% of any given nucleotide the percentage was changed to 97% A, 1% C, 1% G, and 1% T.

Transformation. 0.5µg or 1.0µg of oligo DNA was added directly to competent cells before transferring the cell solution to a cuvette for electroporation. Using the Bio-Rad Gene Pulser Xcell, the following settings were used for electroporation: 2500V, 25µF, and 1000Ω. The electroporated cells were then transferred to 3mL 7H9 media supplemented with 10% OADC, and 1% $Tween_{80}$ in round bottom tubes. For outgrowth the tubes were placed at 37°C on a rotating wheel. Per Kenan Murphy's suggestion the usual outgrowth time for *M. smegmatis* after electroporation of 1 generation (3hrs) was extended to 4-6 generations (12-18 hrs) to allow for propagation of the mutation across all chromosomes. The cultures were then spun down at 4°C for 10min at 4000rpm before resuspension in 7H9 media supplemented with 10% OADC, and 1% $Tween_{80}$ for plating. Approximately 10^8 cells were plated on individual plates containing 20µg/mL INH, 30µg/mL INH, 0.5µg/mL OFX, or 5.0µg/mL OFX. Plates were incubated for 3 days at 37°C and then checked for bacterial colonies.

Primers. Primers were generated to flank the mutated regions of interest with at least 15

bases between the end of the primer and the adjoining region of interest for the purpose of sequencing. The position of the primer in relation to the region of interest can be seen in Supplementary Table 1. For sequencing and PCR of *gyrA*: gyrA_Fs-ACGCGATGTACGACTCGGCT; gyrA_Rs-AAGTTGCCCTGGCCGTCCACCA. For sequencing and PCR of *inhA*: inhA_F-ATGACAGGCCTACTCGAAGG; inhA_R-GCGTCGGGTCCTTCATGTTC. Also used were a set of positive control primers, S1109 and S1116, given by Kadamba Papavinasasundaram that is known to amplify the region of *M. smegmatis* DNA going across the pknA-pknB junction. This gene organization exists in other mycobacteria, but in other organisms the primers do not bind with high specificity under the conditions used.

PCR. Following incubation of the transformed cells on selection media colonies were picked. The picked colonies were transferred to 3mL 7H9 media supplemented with 10% OADC, and 1% $Tween_{80}$ in round bottom tubes and placed at 37°C on a rotating wheel for 3 days. Before sequencing, the cultures were checked for the presence of *inhA* and *gyrA* using PCR. The PCR conditions used were those established to be specific for the positive control primers, S1109 and S1116: initial denaturation for 3 minutes at 96°C followed by for 30 cycles, denaturation for 30 seconds at 96°C, annealing for 30 seconds at 60°C, and extension for 1 minute 30 seconds at 72°C.

Sequencing. Purified PCR products were submitted to GENEWIZ, Inc. for sequencing using the primers indicated above. The isolate sequences were aligned to the wild type mc^2-155 sequence for either *inhA* or *gyrA* to identify mutations.

Results

Increase in survivors following transformation with oligo. Following transformation of pKM402 *M. smegmatis* cells and subsequent incubation, the frequency of drug resistant mutants was determined. Cells transformed with the mutated *inhA* oligo gave rise to approximately twice as many survivor colonies when plated on 20µg/mL INH than mock transformed cells (Figure II.3A). Similarly, cells transformed with the mutated *gyrA* oligo show a two fold increase in survivor colonies when plated on 0.5µg/mL OFX (Figure II.3C). To approximate the frequency of mutation for transformed cells the amount of colonies present on plates was divided by the amount of cells plated (Figure II.3B and Figure II.3D).

A size restriction was applied when the colonies on INH were counted because of the great difference in the size of the survivor colonies. The control colonies on INH showed two distinctive sizes, either pinprick sized colonies or normal sized colonies. The transformed colonies, on the other hand, showed a range of colony sizes from pinprick to large. The pinprick sized colonies were considered as non-resistant and were not included in the adjusted values shown.

PCR of isolates shows presence of *inhA* and *gyrA*. Following growth of picked survivor colonies in broth, the cultures were used for PCR to test for the presence of either *inhA* or *gyrA*. PCR products run on an agarose gel confirmed the presence of the indicated genes (Figure II.4).

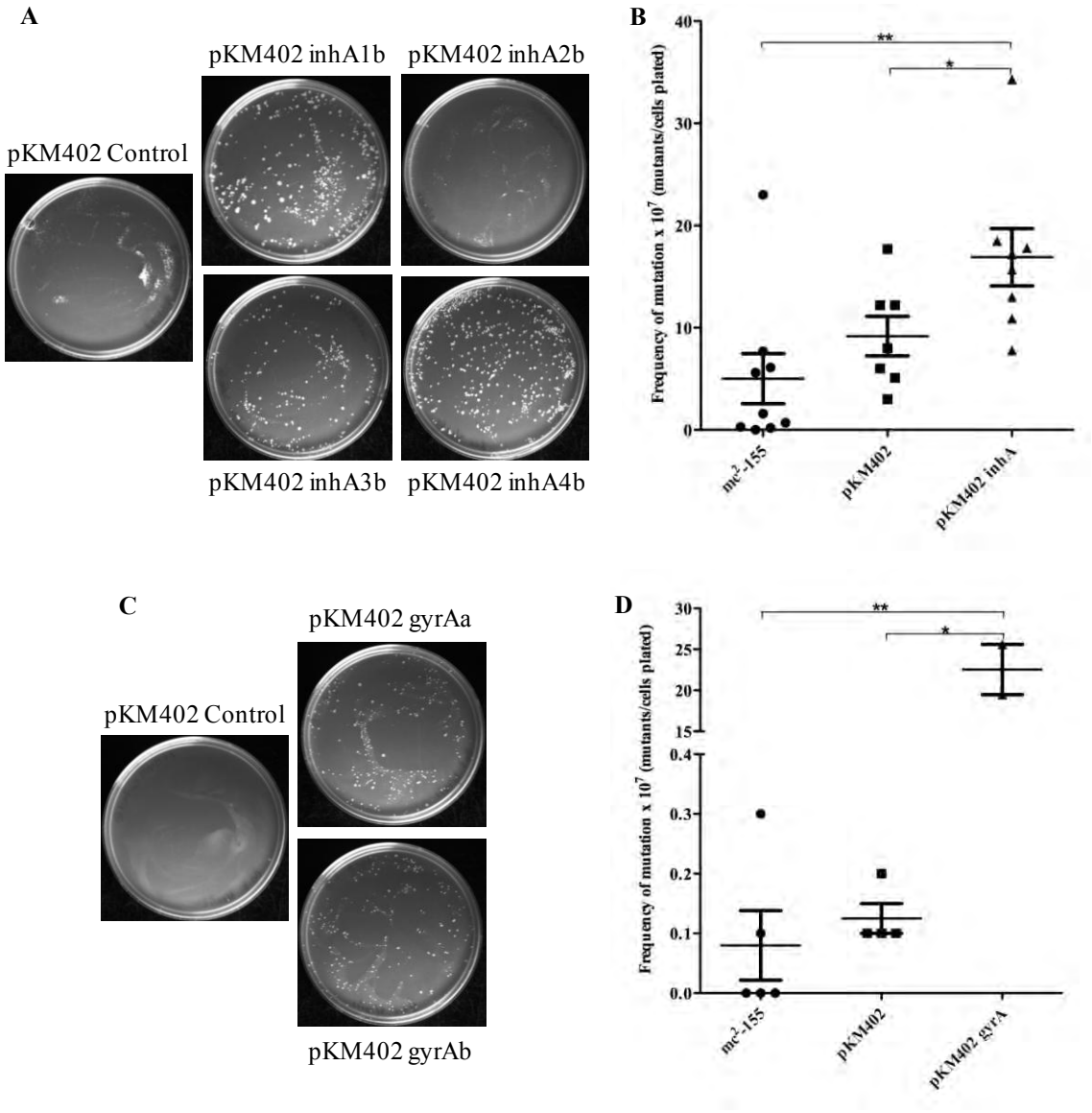


Figure II.3 Transformation with the mutated *inhA* oligo increases the frequency of resistance to 20µg/mL INH and transformation with the mutated *gyrA* oligo increases the frequency of resistance to 0.5µg/mL OFX.

A Example of colony counts. **B** Frequency of spontaneous mutation for mc^2-155 and pKM402 on 20µg/mL OFX compared to the frequency of mutation of pKM402 with *inhA* oligo. For mc^2-155 n=9, pKM402 n=7 and pKM402 inhA n=8. Using unpaired t test, *p value<0.05, **p value<0.01 **C** Example of colony counts. **D** Frequency of spontaneous mutation for mc^2-155 and pKM402 on 0.5µg/mL OFX compared to the frequency of mutation of pKM402 with *gyrA* oligo. For mc^2-155 n=5, pKM402 n=4 and pKM402 gyrA n=2. Using unpaired t test, *p value<0.0005, **p value<0.0001

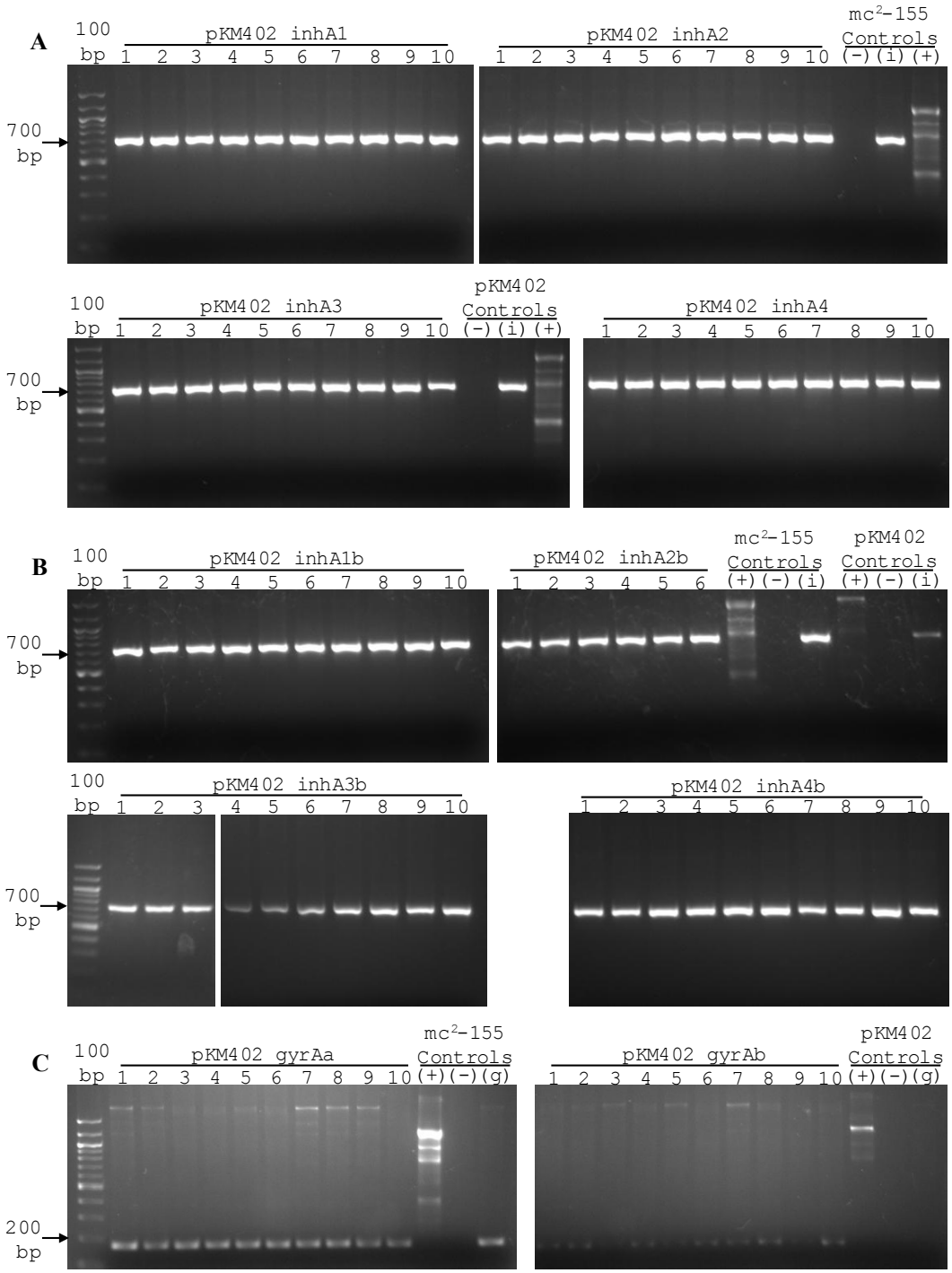


Figure II.4 PCR check for pKM402 inhA and pKM402 gyrA survivors.
A Cells transformed with 0.5µg *inhA* oligo DNA. **B** Cells transformed with 1.0µg *inhA* oligo DNA. **C** Cells transformed with either 1.0µg or 0.5µg *gyrA* oligo DNA. Controls were performed using wild type mc^2-155 and the RecT pKM402 strains; (+) primers targeting a 1.26kb segment present only in the mycobacterial genome, (-) no primers added, (i) primers targeting *inhA,* and (g) primers targeting *gyrA*.

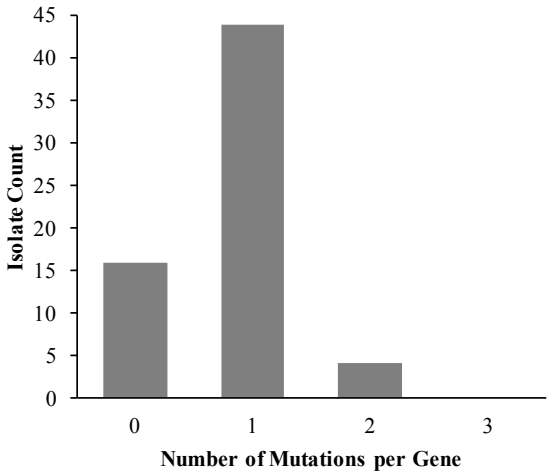


Figure II.5 Number of mutations per gene for *inhA* and *gyrA* isolates, excluding silent mutations.
In total, 54 inhA and 10 gyrA isolates were sequenced.

Sequencing of isolates shows no novel mutations. PCR products were sequenced to determine if any mutations in the region mutagenized were present. The percentage of isolates containing mutations in the region sequenced was high; 48/64 (75%) isolates contained mutations. Of the isolates with mutations, 8/48 contained two mutations per gene, and of those, 4 had a silent mutation as the second mutation (Figure II.5).

Four novel mutations were determined within the region of *inhA* mutated and no novel mutations were determined within the region of *gyrA* mutated (Table II.3). This is evident by comparing the survivor mutations with the established laboratory derived and clinical isolates discussed earlier (Table II.1 and Table II.2). However, the novel mutations in *inhA* were paired with currently established isolate mutations. Only one mutation was determined to be outside of the regions mutated, and was present in an isolate with *inhA* mutated (Supplementary Table 5). This isolate, though, contained a

currently established isolate mutation, and is thus considered as one of the naturally occurring spontaneous mutants.

Discussion

The aim of this work was to explore the ability of recombineering based mutagenesis to determine drug resistance. The first major conclusion was that novel mutations in INH resistant isolates were only identified as second site mutations. These "novel" mutations were paired with mutations previously established to confer resistance in either clinical or laboratory isolates, therefore the role these second site mutations play in the overall resistance has yet to be determined. Of the four "novel" mutations N86T is thought to be the only insignificant change because the amino acid change is a conservative substitution. The other three mutations (E31K, G83D, and Y182L) are thought to cause

Table II.3 Mutations in *inhA* and *gyrA* determined following sequencing of survivors.

Survivor Mutation		Established Isolate		Incidences
		Laboratory	Clinical	
inhA	I21N	-	I21T, V	7
	I21N, E31K	-	I21T, V	1
	I21T	-	I21T, V	2
	I47A*	-	I47T	1
	S94A	S94A	-	10
	S94A, G83D	S94A	-	1
	S94A, N86T	S94A	-	1
	I194N	-	I194T	6
	I194S	-	I194T	7
	I194S, Y182L*	-	I194T	1
gyrA	D95A	D94N+	D94A, G, H, N, Y	2
	D95N	D94N	D94A, G, H, N, Y	4
	D95Y	D94N	D94A, G, H, N, Y	4

*Mutation reported is the amino acid present in *M. tuberculosis.*

+There is an accepted inconsistency in the numbering system for *gyrA* (Maruri et al., 2012).

possible structural changes in the InhA active site (Figure II.6). The E31K mutation is at the C-terminal end of the α1 helix, which at the N-terminal end is a part of the active site. Therefore, it is thought the positioning of the α1 helix could be altered, disrupting the active site interactions. The G83D mutation is just downstream of the clinical mutation V78A, in the α3 helix. Unfortunately, the role the V78A mutation plays in resistance has not been explored, but the N-terminal end of the α3 helix is a part of the active site. Therefore, again it is thought the positioning of the α3 helix could be altered, disrupting the active site interactions. The Y182L mutation is in an unusual position within the protein, the α5β6 linker, providing the potential for alteration of either the α5 helix and/or the β6 sheet, which both have residues in the active site. As a future experiment, the importance of these mutations could be determined by creating single mutants, and testing for INH resistance.

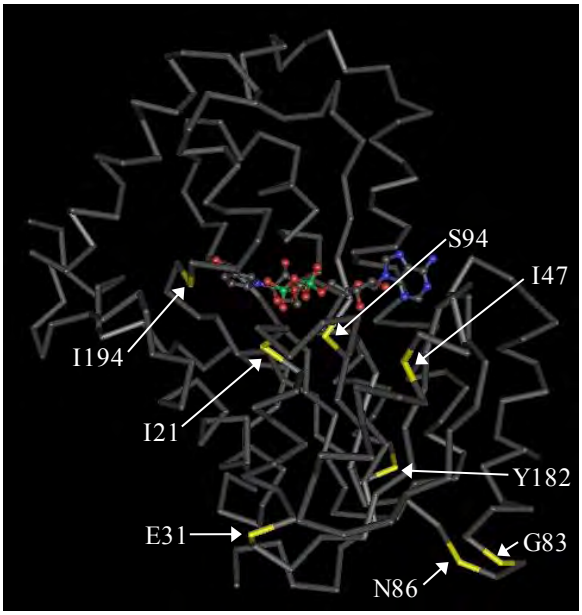


Figure II.6 *M. tuberculosis* InhA modeled with bound NADH and mutated residues found in survivors.
All eight mutated residues (I21, E31, I47, G83, N86, S94, Y182 and I194) are shown in yellow.

Another major development from this work is that mutations previously determined only in clinical isolates are viable laboratory isolates. In *inhA* only one laboratory isolate mutation was previously determined, S94A, so the viability of other mutations *in vitro*

was in question. This work did find that mutations in I21, I47, and I194 are all viable *in vitro*. Why no mutations other than S94A have been found in laboratory isolates could be the result of no other experiments having been reported since the 1994 Banerjee et al study.

Finally, it was determined that amino acid residues previously determined in clinical isolates have more variability, in other words, novel substitutions were determined. For instance, residue 47 is commonly found mutated from Ile to Thr in clinical isolates, but in this work the mutation found was I47A. Similar mutational differences were found for I21 and I194. These mutations were found to generate viable bacteria *in vitro*; however, these mutants may not be viable *in vivo* which could explain their absence in clinical isolates. The viability of these mutants *in vivo* could be tested in mice.

The experiment performed with *gyrA* demonstrated the inflexibility of FQ conferring mutations. Of the 10 isolates sequenced all 10 were at the same residue, and all 10 were previously established amino acid changes from either clinical or laboratory isolates. However, only 10 isolates were sequenced, and it has been determined up to 90% of all FQ resistant isolates have mutations in either D94 or A90 (Zhang and Telenti 2000). With continued work it is possible other mutations within the QRDR could be found.

Appendix

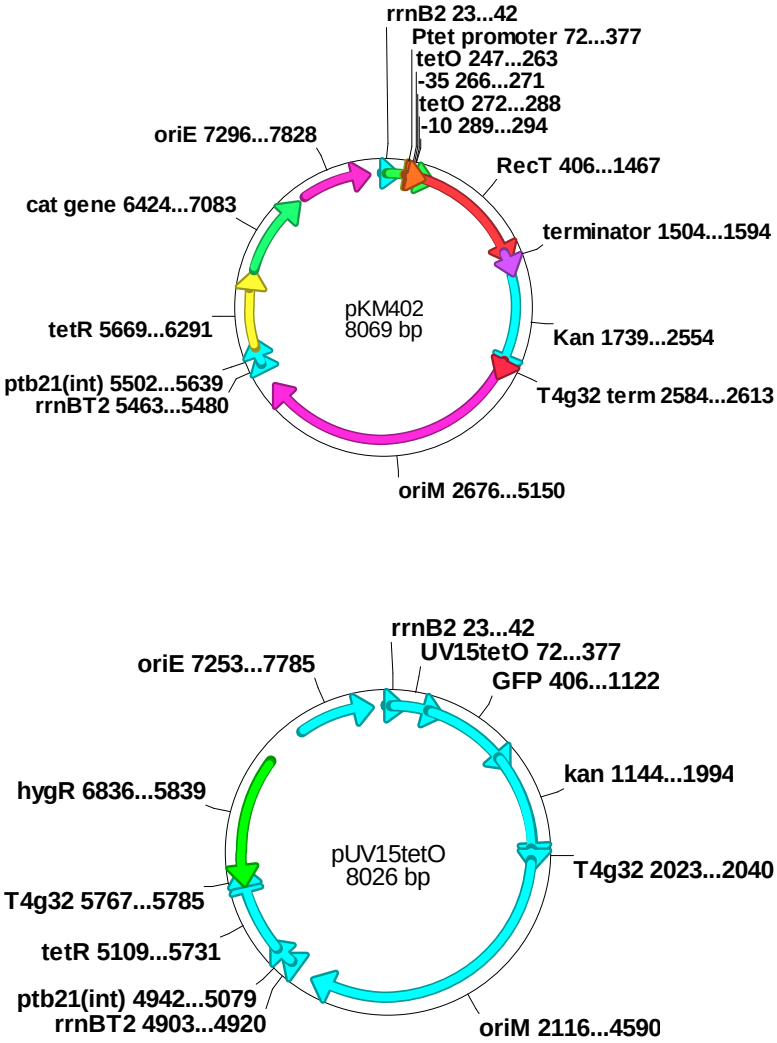


Figure A1. Plasmid design for pKM402 (Che9 RecT expression plasmid).
The pKM402 plasmid was made as follows: Generated RecT gene by PCR from pJV53 with primers 655 and 656. Cut with NdeI and NheI and ligated into pKM287 backbone (pKM287 is a derivative of pUV15tet-O).
Primer 655 RecT-NdeI GTAGTACATATGGCTGAAAATGCTGTCACCAAG
Primer 656 RecT-NheI TTTCCCCCCCCCCCGACGTCAGGT (NheI site is in the vector)

```
Score = 491 bits (1265), Expect = 6e-176, Identities = 235/269 (87%), Positives = 254/269
(94%), Gaps = 0/269 (0%)
M sm.  1   MTGLLEGKRILVTGIITDSSIAFHIAKVAQEAGAELVLTGFDRLKLVKRIADRLPKPAPLLELDVQNEEH 70
           MTGLL+GKRILV+GIITDSSIAFHIA+VAQE GA+LVLTGFDRL+L++RI DRLP  APLLELDVQNEEH
M tb.  1   MTGLLDGKRILVSGIITDSSIAFHIARVAQEQGAQLVLTGFDRLRLIQRITDRLPAKAPLLELDVQNEEH 70

M sm.  71  LSTLADRITAEIGEGNKIDGVVHSIGFMPQSGMGINPFFDAPYEDVSKGIHISAYSYASLAKAVLPIMNP 140
           L++LA R+T  IG GNK+DGVVHSIGFMPQ+GMGINPFFDAPY DVSKGIHISAYSYAS+AKA+LPIMNP
M tb.  71  LASLAGRVTEAIGAGNKLDGVVHSIGFMPQTGMGINPFFDAPYADVSKGIHISAYSYASMAKALLPIMNP 140

M sm.  141 GGGIVGMDFDPTRAMPAYNWMTVAKSALESVNRFVAREAGKVGVRSNLVAAGPIRTLAMSAIVGGALGDE 210
           GG IVGMDFDP+RAMPAYNWMTVAKSALESVNRFVAREAGK GVRSNLVAAGPIRTLAMSAIVGGALG+E
M tb.  141 GGSIVGMDFDPSRAMPAYNWMTVAKSALESVNRFVAREAGKYGVRSNLVAAGPIRTLAMSAIVGGALGEE 210

M sm.  211 AGQQMQLLEEGWDQRAPLGWNMKDPTPVAKTVCALLSDWLPATTGTVIYADGGASTQLL 269
           AG Q+QLLEEGWDQRAP+GWNMKD TPVAKTVCALLSDWLPATTG +IYADGGA TQLL
M tb.  211 AGAQIQLLEEGWDQRAPIGWNMKDATPVAKTVCALLSDWLPATTGDIIYADGGAHTQLL 269
```

Figure A2. Sequence alignment (protein BLAST) between *Mycobacterium smegmatis inhA* and *Mycobacterium tuberculosis inhA*.

```
Score = 1535 bits (3975), Expect = 0.0, Identities = 753/835 (90%), Positives = 797/835
(95%), Gaps = 1/835 (0%)
M sm.  1   MTDTTLPPEGEAHDRIEPVDIQQEMQRSYIDYAMSVIVGRALPEVRDGLKPVHRRVLYAMYDSGFRPDRS 70
           MTDTTLPP+ ++ DRIEPVDI+QEMQRSYIDYAMSVIVGRALPEVRDGLKPVHRRVLYAM+DSGFRPDRS
M tb.  1   MTDTTLPPD-DSLDRIEPVDIEQEMQRSYIDYAMSVIVGRALPEVRDGLKPVHRRVLYAMFDSGFRPDRS 69

M sm.  71  HAKSARSVAETMGNYHPHGDASIYDTLVRMAQPWSLRYPLVDGQGNFGSPGNDPPAAMRYTEARLTPLAM 140
           HAKSARSVAETMGNYHPHGDASIYD+LVRMAQPWSLRYPLVDGQGNFGSPGNDPPAAMRYTEARLTPLAM
M tb.  70  HAKSARSVAETMGNYHPHGDASIYDSLVRMAQPWSLRYPLVDGQGNFGSPGNDPPAAMRYTEARLTPLAM 139

M sm.  141 EMLREIDEETVDFIPNYDGRVQEPTVLPSRFPNLLANGSGGIAVGMATNIPPHNLGELAEAVYWCLENYE 210
           EMLREIDEETVDFIPNYDGRVQEPTVLPSRFPNLLANGSGGIAVGMATNIPPHNL ELA+AV+W LEN++
M tb.  140 EMLREIDEETVDFIPNYDGRVQEPTVLPSRFPNLLANGSGGIAVGMATNIPPHNLRELADAVFWALENHD 209

M sm.  211 ADEEATCEAVMERVKGPDFPTSGLIVGTQGIEDTYKTGRGSIKMRGVVEIEEDSRGRTSIVITELPYQVN 280
           ADEE T  AVM RVKGPDFPT+GLIVG+QG  D YKTGRGSI+MRGVVE+EEDSRGRTS+VITELPYQVN
M tb.  210 ADEEETLAAVMGRVKGPDFPTAGLIVGSQGTADAYKTGRGSIRMRGVVEVEEDSRGRTSLVITELPYQVN 279

M sm.  241 HDNFITSIAEQVRDGKLAGISNIEDQSSDRVGLRIVVELKRDAVAKVVLNNLYKHTQLQTSFGANMLSIV 350
           HDNFITSIAEQVRDGKLAGISNIEDQSSDRVGLRIV+E+KRDAVAKVV+NNLYKHTQLQTSFGANML+IV
M tb.  240 HDNFITSIAEQVRDGKLAGISNIEDQSSDRVGLRIVIEIKRDAVAKVVINNLYKHTQLQTSFGANMLAIV 349

M sm.  351 DGVPRTLRLDQLIRLYVDHQLDVIVRRTRYRLRKANERAHILRGLVKALDALDEVIALIRASQTVDIARA 420
           DGVPRTLRLDQLIR YVDHQLDVIVRRT YRLRKANERAHILRGLVKALDALDEVIALIRAS+TVDIARA
M tb.  350 DGVPRTLRLDQLIRYYVDHQLDVIVRRTTYRLRKANERAHILRGLVKALDALDEVIALIRASETVDIARA 419

M sm.  421 GLIELLDIDDIQAQAILDMQLRRLAALERQKIVDDLAKIEAEIADLEDILAKPERQRGIVRDELKEIVDK 490
           GLIELLDID+IQAQAILDMQLRRLAALERQ+I+DDLAKIEAEIADLEDILAKPERQRGIVRDEL EIVD+
M tb.  420 GLIELLDIDEIQAQAILDMQLRRLAALERQRIIDDLAKIEAEIADLEDILAKPERQRGIVRDELAEIVDR 489

M sm.  491 HGDARRTRIVPADGEVSDEDLIAREDVVVTITETGYAKRTKTDLYRSQKRGGKGVQGAGLKQDDMVNHFF 560
           HGD RRTRI+ ADG+VSDEDLIAREDVVVTITETGYAKRTKTDLYRSQKRGGKGVQGAGLKQDD+V HFF
M tb.  490 HGDDRRTRIIAADGDVSDEDLIAREDVVVTITETGYAKRTKTDLYRSQKRGGKGVQGAGLKQDDIVAHFF 559

M sm.  561 VCSTHDWILFFTTQGRVYRAKAYELPEASRTARGQHVANLLAFQPEERIAQVIQIKSYEDAPYLVLATRN 630
           VCSTHD ILFFTTQGRVYRAKAY+LPEASRTARGQHVANLLAFQPEERIAQVIQI+ Y DAPYLVLATRN
M tb.  560 VCSTHDLILFFTTQGRVYRAKAYDLPEASRTARGQHVANLLAFQPEERIAQVIQIRGYTDAPYLVLATRN 529

M sm.  601 GLVKKSKLSDFDSNRSGGIVAINLREGDELVGAVLCSAEDDLLLVSANGQSIRFSATDEALRPMGRATSG 700
           GLVKKSKL+DFDSNRSGGIVA+NLR+ DELVGAVLCSA DDLLLVSANGQSIRFSATDEALRPMGRATSG
M tb.  600 GLVKKSKLTDFDSNRSGGIVAVNLRDNDELVGAVLCSAGDDLLLVSANGQSIRFSATDEALRPMGRATSG 699

M sm.  701 VQGMRFNEDDRLLSLNVVRPDTYLLVATSGGYAKRTSIDEYSVQGRGGKGILTIQYDRKRGSLVGALIVD 770
           VQGMRFN DDRLLSLNVVR  TYLLVATSGGYAKRT+I+EY VQGRGGKG+LT+ YDR+RG LVGALIVD
M tb.  700 VQGMRFNIDDRLLSLNVVREGTYLLVATSGGYAKRTAIEEYPVQGRGGKGVLTVMYDRRRGRLVGALIVD 769

M sm.  721 DDTELYAITSTGGVIRTAARQVRKAGRQTKGVRLMNLAEGDTLIAIARNADEDEAAESISESDAD 835
           DD+ELYA+TS GGVIRTAARQVRKAGRQTKGVRLMNL EGDTL+AIARNA+E     ++  + AD
M tb.  720 DDSELYAVTSGGGVIRTAARQVRKAGRQTKGVRLMNLGEGDTLLAIARNAEESGDDNAVDANGAD 834
```

Figure A3. Sequence alignment (protein BLAST) between *Mycobacterium smegmatis gyrA* and *Mycobacterium tuberculosis gyrA*.

Table A1. *Mycobacterium smegmatis* wild type sequences for *inhA* and *gyrA*.

Gene	Sequence (5'→3')
inhA	**ATGACAGGCC TACTCGAAGG** caagcgcatc ctcgtcacgg ggatcATCAC CGATTCGTCG ATCGCGTTCC ACATCGCCAA GGTCGCCCAG GAGGCCGGCG CCGAACTGGT GCTGACCGGT TTCGACCGCC TGAAGTTGGT CAAGCGCATC GCCGACCGCC TGCCCAAGCC GGCCCCGCTG CTGGAACTCG ACGTGCAGAA Cgaggagcac ctgtcgactc tggccgaccg gATCACCGCC GAGATCGGTG AGGGCAACAA GATCGACGGT GTGGTGCACT CGATCGGGTT CATGCCGCAG AGCGGTATGg gcatcaaccc gttcttcgac gcgccgtacg aggatgtgtc caagggcatc cacatctcgg cgtactcgta cgcctcgctc gccaaagccg ttctgccgat catgaatccg ggcggcggca tcgtcggcat ggacttcgac cccacgcgcg cgatgccggc ctacaactgg atgaccgtcg ccaagagcgc gctcgaatcg GTCAACCGGT TCGTCGCGCG TGAGGCGGGC AAGGTGGGCG TGCGCTCGAA TCTCGTTGCG GCAGGACCGA TCCGCACGct ggcgatgagc gcaatcgtgg gcggtgcgct gggcgacgag gccggccagc agatgcagct gctcgaagag ggctgggatc agcgcgcgcc gctgggctg**G AACATGAAGG** **ACCCGACGC**c cgtcgccaag accgtgtgcg cactgctgtc ggactggctg ccggccacca ccggcaccgt gatctacgcc gacggcggcg ccagcacgca gctgttgtga t
gyrA	atgactgata cgacgctgcc gccggaaggc gaggcgcatg accggatcga accggtcgac atccagcagg agatgcagcg cagctacatc gactacgcca tgagcgtgat cgtgggccgc gcgctgcccg aggtgcgcga cggtctcaag cccgtgcacc gccgcgtgct gt**ACGCGATG TACGACTCGG CT**tcgtcgga tcgcagccac gccaaatccg cgcgctccGT TGCCGAGACG ATGGGTAACT ACCATCCGCA CGGCGACGCC TCGATCTACG ACACCCTGGT CCGCATGGCC CAGCCGtggt cgttgcgcta cccgc**TGGTG GACGGCCAGG GCAACTT**cgg ctcgccgggt aacgatccgc cagcggccat gcgttacacc gaagcgcgac tcactccgtt ggcgatggag atgttgcgtg aaatcgacga ggagacagtc gatttcatcc cgaactacga cggacgggtg caggagccca cggttctgcc gagccggttt cccaacctgt tggccaacgg ttcgcgcggt atcgccgtgg gcatggccac caacaacccg ccgcacaacc tcggcgagct cgcggaggcc gtgtactggt gcctggagaa ttacgaggcc gacgaggaag ccacctgcga ggccgtgatg gagcgggtca agggacccga cttccccacg tccggcctga tcgtgggcac ccagggtatc gaggacacgt acaagaccgg ccgcgggtcg atcaagatgc gtggcgtcgt cgagatcgag gaggacagcc ggggacggac cagcatcgtc atcaccgagc tgccctacca ggtcaaccac gacaacttca tcacctcgat cgccgagcag gtgcgcgacg gcaagctcgc gggcatctca aacatcgagg accagtccag tgaccgtgtg ggcctgcgaa ttgtcgtgga gctcaagcgc gatgcggtcg ccaaggtggt gctgaacaac ctctacaagc acacccagct gcagaccagc ttcggcgcca acatgctgtc gatcgtcgac ggtgtgccgc gcacgctgcg cctggaccag ctgatccgcc tgtacgtcga ccaccaactc gatgtcatcg tgcggcgcac ccggtaccgg ctgcgcaagg ccaacgaacg ggcccacatc ctgcgtggtc tggtcaaggc actcgatgcc ctcgacgagg tcatcgcgtt gatccgggcg tcgcagaccg tcgacatcgc gcgtgccggc ctgatcgagc tgctcgacat cgacgacatc caggcccagg cgatcctcga catgcagctg cgcaggctcg cagccctcga gcggcagaag atcgtcgacg acctcgccaa gatcgaggcc gagatcgccg acctcgagga catcctcgcc aagcccgagc ggcagcgcgg gatcgtgcgt gacgagctca aggagatcgt cgacaagcac ggcgacgcgc gtcggacccg catcgtgccc gccgacgggc aggtcagcga cgaggatctc atcgcccgcg aagacgtggt ggtcaccatc accgagaccg gctacgccaa acgcaccaag accgacctgt accacagcca gaaacgcggc ggcaaaggtg tgcagggtgc cggcctcaag caggacgaca tggtcaacca cttcttcgtc tgctcgacgc acgactggat cctgttcttc accacacagg gtcgcgtgta ccgggcgaag gcgtacgagt tgcccgaggc gtcccgcacc gcgcgtggcc agcacgtcgc gaacctcctg gccttccagc ccgaggagcg catcgcgcag gtcatccaga tcaagagcta cgaggatgcg ccctacctgg tgctcgcgac ccgcaatggt ctggtgaaga agtccaagct gtccgacttc gactccaacc gctccggcgg catcgtcgcg atcaacctgc gggaaggcga cgaactggtc ggtgcggtgc tgtgctcggc agaggacgac ctgctcctgg tgagtgccaa ccgccagtcg atccgcttct cggcgaccga cgaggcgctg cggcccatgg gtcgcgccac ctccggtgtg cagggcatgc ggttcaacga ggacgaccgc ctgctgtcgc tcaacgtcgt ccggcccgat acgtatctgc tggtcgcgac atcgggtggc tacgccaagc gcacctcgat cgacgagtac tcggtgcagg gccgcggcgg caagggcatc ctgacgatcc agtacgaccg caaacgtggc agtctggtcg gagcgttgat cgtcgacgac gacaccgagc tgtacgcgat cacgtccaca ggtggtgtca tccgcactgc cgcacgtcag gtccgcaagg ctggtcgcca gacaaagggc gttcgcttga tgaacctggc cgagggcgac acactgattg ccatcgcccg caacggacga ggacgaggcg gccgagtcga tcagcgaatc cgacgcggac accgccgagt cacccgaggc gtgatggaaa cgctgaggtc ccgaaaggtc ctcggcccga gctaaggagc tgtaggtgag ttcacccaac gagccgggat acccgcgcgc gggggaccgg ccgggcgcca ccaacggcac gggtccgggc ggcgacagcg gagccctcag cagcaactcg acgcgggcca cggggcacat caccgattcc ggtgacgtgc ccccgtggca gcgcggggtc tcccgcaccg ctcagcaacc tggtcagccg ctcggcgaca ccgagcagca gccccgtccc gcgccgaggc ccgagccccg cgagacccgc caggagccgc ggcctgaaca tcgcaccgag gcctacgcga gcgagttgcc ggacctctcg ggtccggtcc cccgctcacc gcagcgcaag accggggccg acgcgccccg gg

PRIMER FOR SEQUENCING

area of interest to mutate

INHA1 OLIGO

INHA2 OLIGO

INHA3 OLIGO

INHA4 OLIGO

GYRA OLIGO

unmutated regions

Table A2. Oligos designed for this work.
Wild type oligos manufactured by Sigma-Aldrich. Mutant oligos manufactured by IDT and indicated by a 2 digit percentage. For instance, at a particular position a mixture of 97% A, 1% C, 1% G, and 1% T is denoted by 97010101. 1=97010101, 2=01970101, 3=01019701, 4=01010197

Name	Sequence (5'→3')
GyrA_Mt	CCGCGCGCTCC34T32C31312G14333T11241221422G21233C31232C42G14241231212C24G34C23C14332C21322GTGGTCGTTGCG
GyrA_WT	CCGCGCGCTCCGTTGCCGAGACGATGGGTAACTACCATCCGCACGGCGACGCCTCGATCTACGACACCCTGGTCCGCATGGCCCAGCCGTGGTCGTTGCG
InhA1_Mt	TCACGGGGATC14212C31442342314232G44221214232C11334C32C21331332C33C32C31124G34G24G12C33T442GACCGCCTGAA
InhA1_WT	TCACGGGGATCATCACCGATTCGTCGATCGCGTTCCACATCGCCAAGGTCGCCCAGGAGGCCGGCGCCGAACTGGTGCTGACCGGTTTCGACCGCCTGAA
InhA2_Mt	TGACCGGTTTC31223C24G11344334C11323C14232C31223C24G22C11322G32C22G24G24G31124C31234G213112GAGGAGCACCT
InhA2_WT	TGACCGGTTTCGACCGCCTGAAGTTGGTCAAGCGCATCGCCGACCGCCTGCCCAAGCCGGCCCCGCTGCTGGAACTCGACGTGCAGAACGAGGAGCACCT
InhA3_Mt	TGGCCGACCGG14212C32C31314233T31333C11211314231233T34G34G21242G142333T4214322G21313233T143GGCATCAACCC
InhA3_WT	TGGCCGACCGGATCACCGCCGAGATCGGTGAGGGCAACAAGATCGACGGTGTGGTGCACTCGATCGGGTTCATGCCGCAGAGCGGTATGGGCATCAACCC
InhA4_Mt	CGCTCGAATCG34C11223344234C32G23T31332G33C11334G33C34G23C42G11424C34T32G32A33A22G14223C12GCTGGCGATGAG
InhA4_WT	CGCTCGAATCGGTCAACCGGTTCGTCGCGCGTGAGGCGGGCAAGGTGGGCGTGCGCTCGAATCTCGTTGCGGCAGGACCGATCCGCACGCTGGCGATGAG

Table A3. Primers designed for this work.

Purpose	Name	Sequence (5'→3')
For sequencing and PCR of *gyrA*	gyrA-Fs	173-ACGCGATGTACGACTCGGCT-193
	gyrA-Rs	326-AAGTTGCCCTGGCCGTCCACCA-348
For sequencing and PCR of *inhA*	inhA-F	001-ATGACAGGCCTACTCGAAGG-021
	inhA-R	690-GCGTCGGGTCCTTCATGTTC-710

Table A4. Results of sequencing of pKM402 inhA survivor isolates

Survivor Isolate	Survivor Mutation		Established Isolate	
	Nucleotide	Amino Acid	Laboratory	Clinical
inhA1-1	61-atc 61-acc	I21T	-	I21T, V
inhA1-2	61-atc 61-aac	I21N	-	I21T, V
inhA1-3	-	NM	-	-
inhA1-4	-	NM	-	-
inhA1-5	55-tcg 55-tca	S19S	-	-
	61-atc 61-acc	I21T	-	I21T, V
inhA1-6	-	NM	-	-
inhA1-7	61-atc 61-aac	I21N	-	I21T, V
inhA1-8	-	NS	-	-
inhA1-9	-	NS	-	-
inhA1-10	-	NS	-	-
inhA2-1	139-att 139-gcc	I47A*	-	I47T
inhA2-2	-	NM	-	-
inhA2-3	-	NM	-	-
inhA2-4	-	NM	-	-
inhA2-5	-	NM	-	-
inhA2-6	-	NM	-	-
inhA2-7	-	NM	-	-
inhA2-8	-	NS	-	-
inhA2-9	-	NS	-	-
inhA2-10	-	NS	-	-

Table A4 continued. Results of sequencing of pKM402 inhA survivor isolates

Survivor Isolate	Survivor Mutation		Established Isolate	
	Nucleotide	Amino Acid	Laboratory	Clinical
InhA3-1	-	NM	-	-
InhA3-2	280-tcg 280-gcg	S94A	S94A	-
InhA3-3	280-tcg 280-gcg	S94A	S94A	-
InhA3-4	-	NM	-	-
InhA3-5	280-tcg 280-gcg	S94A	S94A	-
InhA3-6	280-tcg 280-gcg	S94A	S94A	-
InhA3-7	280-tcg 280-gcg	S94A	S94A	-
	580-atc 580-agc	I95I	-	-
InhA3-8	-	NS	-	-
InhA3-9	-	NS	-	-
InhA3-10	-	NS	-	-
InhA4-1	580-atc 580-agc	I194S	-	I194T
InhA4-2	544-tac 544-ttg	Y182L*	-	-
	580-atc 580-agc	I194S	-	I194T
InhA4-3	580-atc 580-aac	I194N	-	I194T
InhA4-4	580-atc 580-aac	I194N	-	I194T
InhA4-5	580-atc 580-aac	I194N	-	I194T
InhA4-6	580-atc 580-agc	I194S	-	I194T
InhA4-7	580-atc 580-aac	I194N	-	I194T
InhA4-8	-	NS	-	-
InhA4-9	-	NS	-	-
InhA4-10	-	NS	-	-

*Mutation reported is the amino acid present in *M. tuberculosis*

Silent mutations indicated in gray text; NS, not sequenced; NM, no mutation

Table A5. Results of sequencing of pKM402 inhAb survivor isolates.

Survivor Isolate	Survivor Mutation		Established Isolate	
	Nucleotide	Amino Acid	Laboratory	Clinical
inhA1-1b	61-atc 61-aac	I21N	-	I21T, V
inhA1-2b	61-atc 61-aac	I21N	-	I21T, V
	91-gag 91-aag	E31K	-	-
inhA1-3b	61-atc 61-aac	I21N	-	I21T, V
inhA1-4b	61-atc 61-aac	I21N	-	I21T, V
inhA1-5b	61-atc 61-aac	I21N	-	I21T, V
inhA1-6b	61-atc 61-aac	I21N	-	I21T, V
inhA1-7b	-	NS	-	-
inhA1-8b	-	NS	-	-
inhA1-9b	-	NS	-	-
inhA1-10b	-	NS	-	-
inhA2-1b	580-atc 580-aac	I194N	-	I194T
inhA2-2b	-	NM	-	-
inhA2-3b	-	NM	-	-
inhA2-4b	-	NM	-	-
inhA2-5b	-	NM	-	-
inhA2-6b	-	NM	-	-
inhA3-1b	-	NS	-	-
inhA3-2b	-	NS	-	-
inhA3-3b	-	NS	-	-

Table A5 continued. Results of sequencing of pKM402 inhAb survivor isolates.

Survivor Isolate	Survivor Mutation		Established Isolate	
	Nucleotide	Amino Acid	Laboratory	Clinical
inhA3-4b	280-tcg 280-gcg	S94A	S94A	-
inhA3-5b	257-aac 257-acc	N86T	-	-
	280-tcg 280-gcg	S94A	S94A	-
inhA3-6b	280-tcg 280-gcg	S94A	S94A	-
inhA3-7b	280-tcg 280-gcg	S94A	S94A	-
inhA3-8b	247-ggg 247-gat	G83D*	-	-
	280-tcg 280-gcg	S94A	S94A	-
inhA3-9b	280-tcg 280-gcg	S94A	S94A	-
	285-ggg 285-ggt	G96G	-	-
inhA3-10b	280-tcg 280-gcg	S94A	S94A	-
inhA4-1b	580-atc 580-agc	I194S	-	I194T
inhA4-2b	580-atc 580-aac	I194N	-	I194T
inhA4-3b	580-atc 580-agc	I194S	-	I194T
inhA4-4b	532-gag 532-gaa	E178E	-	-
	580-atc 580-agc	I194S	-	I194T
inhA4-5b	580-atc 580-agc	I194S	-	I194T
inhA4-6b	580-atc 580-agc	I194S	-	I194T
inhA4-7b	580-atc 580-aac	I194N	-	I194T
inhA4-8b	-	NS	-	-
inhA4-9b	-	NS	-	-
inhA4-10b	-	NS	-	-

*Mutation reported is the amino acid present in *M. tuberculosis*

Silent mutations indicated in gray text; NS, not sequenced; NM, no mutation

Table A6. Results of sequencing of pKM402 gyrAa survivor isolates

Survivor Isolate	Survivor Mutation		Established Isolate	
	Nucleotide	Amino Acid	Laboratory	Clinical
gyrAa-1	280-gac 280-ggc	D95A	D94N*	D94A, G, H, N, Y
gyrAa-2	280-gac 280-tac	D95Y	D94N	D94A, G, H, N, Y
gyrAa-3	280-gac 280-aac	D95N	D94N	D94A, G, H, N, Y
gyrAa-4	280-gac 280-tac	D95Y	D94N	D94A, G, H, N, Y
gyrAa-5	280-gac 280-aac	D95N	D94N	D94A, G, H, N, Y
gyrAa-6	280-gac 280-tac	D95Y	D94N	D94A, G, H, N, Y
gyrAa-7	280-gac 280-aac	D95N	D94N	D94A, G, H, N, Y
gyrAa-8	280-gac 280-tac	D95Y	D94N	D94A, G, H, N, Y
gyrAa-9	280-gac 280-gcc	D95A	D94N	D94A, G, H, N, Y
gyrAa-10	280-gac 280-aac	D95N	D94N	D94A, G, H, N, Y

*There is an accepted inconsistency in the numbering system for *gyrA* (Maruri et al., 2012)

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