Proposal for a Thesis in the Field of Biotechnology

in Partial Fulfillment of Requirements for

the Master of Liberal Arts Degree

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I.

Tentative Title

"A Translational Recovery Mechanism May Provide a Novel Target for Drug Resistant Strains of *M. tuberculosis*"

II.

The Research Problem

Tuberculosis (TB) is second only to HIV/AIDS as the most deadly infectious disease in the world (World Health Organization [WHO] 2014). Though deadly, TB can often be effectively treated with a six month course of first-line antibiotics. Unfortunately, multiple drug resistant *Mycobacterium tuberculosis* (MDR-TB) populations are estimated to cause 5% of all TB cases (WHO, 2014) and they are not adversely affected by any of the first-line drugs. In these cases, second-line drugs can succeed but some strains are resistant to not only first and second line drugs, but to all currently available antibiotics (Velayati et al., 2009). To combat the problem of drug resistance there is an urgent need for the discovery of new medicines which treat these diseases by targeting novel bacterial systems.

Several TB antibiotics already target protein synthesis. Second-line drugs amikacin, kanamycin, and streptomycin interrupt the activity of the bacterial ribosome during translation, which can cause the ribosome machinery to stall. This can result in the production of toxic peptides and the loss of a viable ribosome complex, both of which can perturb *M. tuberculosis* growth and slow the spread of infection.

It has recently been shown that systems aiding in recovery and release of stalled ribosomes may be universally essential in prokaryotes (Feaga, Viollier, & Keiler, 2014). *E. coli* and other prokaryotes have multiple systems which aid in stalled ribosome recovery. One of the most characterized of these systems is *trans*-translation, which is mediated by tmRNA. The tmRNA/*trans*-translation system is not essential for growth in *Escherichia coli* because there are alternative ribosome stall recovery factors (Giudice & Gillet, 2014), but tmRNA is essential in

M. tuberculosis (Personne & Parish, 2014). This implies that *M. tuberculosis* might possess a ribosome recovery system that lacks the diversity of factors found in other species. For this reason, TB may be particularly vulnerable to drugs that affect ribosome recovery.

Drugs which target stalled ribosome recovery systems could augment the current drug regimen by increasing the efficacy of antibiotics already in use. The pro-drug pyrazinamide (PZA) works in a somewhat similar fashion. PZA has very little activity on its own but in the presence of other antibiotics PZA greatly enhances the effects of the drug combination as a whole. PZA is the most recent addition to the first line drugs used to treat TB and has drastically reduced the treatment time of the recommended TB treatment regimen. It was shown by Shi *et al.* (2011) that activated PZA does this by inhibiting tmRNA mediated *trans*-translation in *M. tuberculosis*.

Trans-translation is not the only ribosome recovery mechanism available to *M. tuberculosis*. One ribsosome recovery system uses an essential ribosome rescue factor called Pth. Pth is a peptidyl-tRNA hydrolase, (Giudice & Gillet 2014) which manages the build-up of toxic and inactive peptidyl-tRNA's that accumulate during protein biosynthesis and especially during ribosome stalling (Das & Varshney 2006). Pth is highly conserved and is essential in both *E. coli* and *M. tuberculosis* (Sharma et al., 2014), but studies investigating the crystal structures of *M. tuberculosis* Pth (MtPth) in comparison to the Pth structures of other bacterial species have shown that there are important structural differences in MtPth (Selvaraj et al., 2007). MtPth has several distinct conformational divergences in the substrate binding site. This may indicate a different substrate specificity (Sharma et al. 2014) and perhaps a unique function.

If MtPth plays an irreplaceable role in the cell it may be an excellent candidate for drug discovery research. MtPth is essential for growth in *M. tuberculosis*, but it would be helpful to

know whether disruption of MtPth production has a bacteriostatic or bactericidal effect. A bactericidal effect is most desirable in terms of drug development, but many drugs targeting translation in prokaryotes have a bacteriostatic effect. Regulated depletion mimics drug targeting of essential genes and can be used to determine whether a drug inactivating a given target is likely to have a static or cidal effect on the bacteria.

To learn more about the role of Pth in ribosome stall and recovery, we will investigate the effect of Pth regulation in concert with exposure to antibiotics which inhibit different aspects of protein synthesis. We will use Pth depletion via targeted proteolysis and over-expression strains propagated in the presence of translation-targeting drugs such as kanamycin, chloramphenicol, linezolid and other antibiotics listed in Table 1. The effect of Pth reduction or over-expression in combination with these drugs will inform us about the role of Pth and any relationship it may have to the activities of these antibiotics and their particular mechanisms of action.

This project will provide information about whether blocking Pth activity is a viable mechanism of action to target with antibiotic therapy. If it is a promising candidate for future target-directed drug discovery, this research will facilitate the process of drug development. Pth may be an especially drug-able target for mycobacteria, but it is also a highly conserved gene among prokaryotes. Although Pth is conserved among prokaryotes, eukaryotic organisms have multiple systems which perform activities similar to those carried out by Pth, so a drug that targets prokaryotic Pth would likely not be detrimental to the host. Therefore it may be possible to develop a broad-spectrum antibiotic targeting Pth, which could treat several diseases. Investigation of MtPth may also reveal novel aspects of ribosome stall recovery.

III.

Definition of Terms

Bactericidal: causes irreversible cell death

Bacteriostatic: may reduce cell number but instead of killing, causes growth cessation

BSL3: Biosafety Level 3, a category of the hazard level of a disease-causing agent

CCA site: a tri-nucleotide binding site

Crystal Structure: protein crystals used to study the molecular structure of that protein

Drug Target: an entity to which a drug is directed or bound and usually inactivates

Doubling time: the average generation time (time to double the population) of a particular microbial species

Essential: a gene or protein whose existence or function is essential for growth

MDR-TB: Multiple Drug Resistant Tuberculosis

Merodiploid: partially diploid, in this instance, containing two copies of the same gene

MIC: minimum inhibitory concentration (of a drug)

Mycobacteria: a genus of Actinobacteria which includes the pathogenic species *M. tuberculosis*

Proteolysis: lysis of proteins into smaller, nonfunctional units

Pth: peptidyl-tRNA hydrolase, removes tRNA from the peptide moiety

RT-PCR: reverse transcription polymerase chain reaction, a method of detecting mRNA transcripts as a means to estimate expression level

Recombineering: recombination-mediated genetic engineering, a technique based on homologous recombination used to manipulate genomes

Regulated depletion: a technique whereby the level of protein in a cell may be decreased by the addition of a substrate which controls proteolysis of a particular protein

Ribosome: the primary site of translation

tnSEQ: uses transposon mutagenesis and sequencing to identify relationships between networks of genes and associated phenotypes in a microorganism

Translation: the process of protein synthesis

tmRNA trans-translation: a system used to aid in the recovery of a stalled ribosome wherein the prime mediator is tmRNA

Western Blot: a technique using antibodies to detect the amount of a certain protein

XDR-TB: extensively drug resistant tuberculosis

IV.

Background

Tuberculosis

Roughly 9 million people contracted the respiratory disease tuberculosis (TB) in the year 2013 and of those infected 1.5 million died (WHO, 2014). At least some of these deaths were due to the lack of effective antibiotic treatment. TB is caused by the bacterium *Mycobacterium tuberculosis*, and the combined factors of HIV/AIDS prevalence and sub-lethal exposure to antibiotics are driving the evolution and spread of drug-resistant tuberculosis (Brites & Gagneux 2012). The growing problem of multiple drug resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) requires that new drugs and treatment plans be made available to the global population.

Currently available drug therapies have changed little in recent decades and there are major issues with the treatment. The regimen is long, complicated, and requires multiple drugs be taken simultaneously, risking harmful drug interactions (Mdluli, Kaneko, & Upton, 2014). All of these factors contribute to suboptimal patient adherence to treatment which leads to further propagation of infectious drug-resistant strains of TB (Falzon et al., 2011). New drugs must be effective against emerging MDR and XDR TB strains, shorten the treatment time, and use novel mechanisms of action to truly reduce the incidence of disease (Mdluli, Kaneko, & Upton, 2014).

Translation in *M. tuberculosis* Through the Lens of Antibiotics

Protein synthesis, also called translation, is an exceptionally important activity in any living cell. It is the process by which the structures making up the physical substance of an organism come into being as directed by the genome. The primary site of protein synthesis is the

ribosome. A bacterial ribosome consists of large (50S) and small (30S) subunits made up of both protein and RNA elements. The ribosome "reads" an mRNA transcript, recruits the appropriate aminoacylated tRNA according to the codon sequence, and catalyzes the formation of peptide bonds which create a growing chain that will form a functional protein upon completion.

The study of ribosome processes has been aided by the discovery of antibiotics which inhibit different aspects of translation. Some of these drugs also have the ability to target translation in *M. tuberculosis*. For example, streptomycin and linezolid are two antibiotics that can inhibit translation at its first step: initiation (Moazed & Noller, 1987, and Swaney et al., 1998). This step involves the placement of an initiator called fMet-tRNA^{fMet} at the start codon in the peptide (P) site (Schmeing & Ramakrishnan, 2009).

As the P-site holds the initiator tRNA, the acceptor site (A-site) admits an incoming aminoacyl-tRNA corresponding to the next codon in the mRNA transcript. This begins a stepwise process called elongation that will repeat until the end of the transcript has been reached. During elongation catalysis of a peptide bond attaches the peptide chain to the amino acid on the incoming aminoacyl-tRNA located in the A-site. Once that bond has been formed, the mRNA scrolls another codon step forward and the newly minted peptidyl-tRNA translocates from the A-site to the P-site. The uncharged tRNA that formerly occupied the P-site translocates to the exit site (E-site) and releases from the ribsosome.

There are several antibiotics which can throw a wrench into the elongation machinery. Hygromycin stabilizes the A-site, inhibiting translocation and inducing misreading of the mRNA transcript (Moazed & Noller, 1987). Both chloramphenicol and erythromycin bind to the 50S subunit of the ribosome and inhibit peptidyl transferase activity (Richter, Rüsch-Gerdes, & Hillemann 2007) such that the amino acid cannot be bound to the polypeptide chain. Finally,

elongation factor G (EF-G) which catalyzes translocation from A-site to P-site is vulnerable to fusidic acid (Collignon & Turnidge, 1999).

If the ribosome complex is not deterred by any of the aforementioned chemical factors, the mRNA transcript will end in a codon that signals the ribosome to terminate translation. There will be no tRNA binding to this codon but instead the stop codon will recruit release factors which cleave the polypeptide chain, now a completed protein, and help the ribosome dissociate. Once disassembled, the individual factors that made up the ribosome complex are free to prepare and regroup for a new translation cycle. This final step is just as important as the preceding two, and is also vulnerable to chemical attack.

Aminoglycosides like kanamycin and amikacin disrupt the fidelity of the translation process which can wreak havoc on termination by causing high rates of mistranslation (Palomino & Martin, 2014). Mistranslation is a faulty reading of the mRNA transcript. Incorrectly de-coding mRNA can lead to frame shifts which alters polypeptide formation such that it no longer corresponds to the mRNA sequence and can lead to the appearance of a stop codon long before the end of the transcript. This situation causes the formation of toxic peptides which can damage the cell and can even damage the ribosome complex in such a way that it will stall and remain locked, unable to dissociate. This removes that ribosome from the cell's essential pool of translation machinery.

Mechanisms of Ribosome Stall Recovery

Production of toxic peptides and the loss of viable ribosomes is highly detrimental to the cell. These events are frequent enough even without chemical intervention that all living cells

have evolved mechanisms designed to help recover from stalled ribosomes. These systems are themselves just as essential to cell viability as any of the components of translation.

There are two main types of ribosome stall according to Giudice and Gillet (2013). The first type of disruption is a ribosome pause, or no-go mRNA. This is often caused by the lack of sufficient charged tRNA or amino acids. It is called no-go because the A-site is not vacant, but occupied by an uncharged tRNA which has no amino acid to transfer to the polypeptide chain. This halts the ribosome's activity because it cannot perform translocation. Fortunately for the cell, there are several factors involved in the stringent response that can be activated by a no-go ribosome stall. The *relBE* toxin-antitoxin system was shown to allow for recovery of these stalled ribosomes in some bacterial species (Giudice & Gillet, 2013) and there may be other systems in place that have not yet been described.

Another type of ribosome stall, called non-stop, can be caused by the loss of a stop codon due to mRNA truncation or mistranslation. Without a stop codon the ribosome will stall at the end of the mRNA instead of proceeding with termination. In bacteria, *trans*-translation mediates the recovery from non-stop mRNA (Guidice & Gillet, 2013). *Trans*-translation is ubiquitous in bacteria (Feaga, Viollier, & Keiler, 2014) and has two main components, transfer-mRNA (tmRNA), and small basic protein (SmpB). Both tmRNA and SmpB work together with other factors to recognize and alleviate the non-stop stalled ribosome.

Unfortunately for bacteria relying on *trans*-translation, in addition to all of the previously mentioned drugs that target translation and cause ribosome stall, there is a drug that can target *trans*-translation. A drug called pyrazinamide (PZA) inhibits *trans*-translation in *M. tuberculosis* by binding to ribosomal protein S1 (RpsA), which is a part of the 30S subunit (Shi et al., 2011).

When the active form of PZA binds to RpsA, tmRNA can no longer bind to and rescue the stalled ribosome (Shi et al., 2011).

The Success of Pyrazinamide

PZA is one of the newest additions to the TB treatment regimen. While it has almost no discernable activity on its own *in vitro*, PZA is able to greatly enhance the effect of other drugs in the TB treatment regimen *in vivo*. PZA is now a first-line drug that has had the powerful effect of shortening the TB treatment course from 9-12 months to just six months. Since its discovery, drug combinations tested against TB infection that did not involve the use of PZA have been found to be significantly inferior to regimens including PZA (Nuermberger et al., 2008).

Trans-translation, the system inhibited by PZA, may play a role in recycling ribosomes in non-replicating bacteria, as well as rescuing stalled ribosomes. Bacteria in a latent, non-replicating state may experience a dearth of ribosomes as cell metabolism slows, increasing the importance of ribosome turnover. This latent state ribosome-turnover sensitivity may be part of the reason for PZA's success.

Peptidyl-tRNA Hydrolase (Pth)

The recovery process mediated by *trans*-translation requires that the mRNA be truncated (Ivanova et al., 2004) but sometimes this doesn't happen. If this is the case, the stalled ribosome must be processed in another way. An alternative recovery mechanism is premature drop-off and cleavage of peptidyl-tRNA by peptidyl tRNA hydrolase (Pth). Pth is an esterase which breaks peptidyl-tRNA apart into its tRNA and polypeptide components. It is a ubiquitous and essential gene in most prokaryotes, including *E. coli* and *M. tuberculosis*.

It was shown in *E. coli* that loss of Pth can lead to accumulation of peptidyl tRNA and cell death. This study by Menninger (1979) showed enhanced killing with erythromycin treatment while depleting Pth. The author postulated that the depletion of Pth and erythromycin treatment both increased the concentration of peptidyl tRNA which was toxic to *E. coli*. A different study found that over-expression of a key charged tRNA was able to rescue the cell from Pth depletion (Vivanco-Dominguez, Cruz-Vera & Guarneros, 2006) but it appears that the charged tRNA actually stimulated over-production of Pth during depletion.

More recent studies investigated the crystal structure of *M. tuberculosis* Pth (MtPth) and compared it to distant and closely related bacterial species. In 2007, Selvaraj *et al.* proposed a model for the structural changes that Pth must undergo during enzyme activity and compared *E. coli* Pth (EcPth) with MtPth. Although EcPth and MtPth are homologous (38% sequence identity) and MtPth has been shown to rescue EcPth depletion in *E. coli* (Das & Varshney, 2006) there are significant structural differences between the two species. These differences are elaborated in more detail by Sharma *et al*. (2014) who showed that MtPth possesses subtle differences in the active site, CCA site, and the acceptor site. The authors showed that MtPth is structurally divergent even from *Mycobacterium smegmatis* Pth especially in the CCA site. These structural studies indicate a different substrate specificity and perhaps a unique function is carried out by Pth in *M. tuberculosis*.

Further differences between *E. coli* and *M. tuberculosis* Pth were recently revealed by Personne and Parish (2014) who found that *M. tuberculosis* may have an unusual stalled ribosome recovery mechanism. The authors explain that while tmRNA (encoded by the gene *ssrA*) is not essential in *E. coli* and several other common bacterial species, it is essential in *M. tuberculosis*. Interestingly, smpB, the protein that works in tandem with tmRNA was dispensable

for growth in *M. tuberculosis*. Personne and Parish found that although tmRNA both marks nascent polypeptide for clearance and rescues stalled ribosomes in *M. tuberculosis*, the essential function was the latter. They propose that the essentiality of tmRNA and the lack of homologs of other known ribosome stall recovery factors indicate that *M. tuberculosis* ribosome numbers are limited or that ribosome stalling could be more detrimental in *M. tuberculosis* than in other bacterial species.

One ribosome stall recovery factor present in *M. tuberculosis* that was not mentioned in the previous report is Pth. Research by Feaga, Viollier and Keiler (2014) described the effect of deletion of the tmRNA encoding gene *ssrA* in a bacterial species that did not require it for growth. This group used tnSeq to investigate whether other ribosome recovery mechanisms that had been dispensable in the presence of *ssrA* suddenly became essential. One of the hits from this tnSeq screen was *pth*.

For all of these reasons it is plausible that MtPth could be a very good candidate for target-directed drug discovery. Investigation of MtPth may also reveal a novel approach to ribosome stall recovery and could lead to a better understanding of both Pth and ribosome stalling activity at large in *M. tuberculosis*. Drug development will be facilitated by the knowledge gained from these investigations.

V.

Research Methods

The purpose of this research is to investigate the role of Pth in *M. tuberculosis* using drugs that target prokaryotic translation mechanisms. Using genetic manipulation, Pth knock-down/depletion, over-expression, and the appropriate control strains have been created in *M. tuberculosis* H37Rv. The knock-down strain was recombineered (Murphy, 2012) at the native locus to insert a degradation tag at the C-terminus of Pth. With this tag Pth levels will be depleted by regulated proteolysis using the DAS-tag system described by Raju et al. (2014). This system uses enzyme protease called SspB which recognizes the DAS tag and cleaves it to reveal a sequence that targets the attached protein for degradation by the Clp protease system. SspB will be regulated by a Tetracycline-inducible promoter (Bertram & Hillen, 2008). The addition of anhydrous tetracycline (ATC) to the culture medium will stimulate expression of *sspB*, which will facilitate degradation of Pth. In addition to the DAS tag, this strain also carries a flag tag to aid in confirmation of proteolysis of Pth over time by Western blot analysis using an anti-flag antibody (Figure 1A).

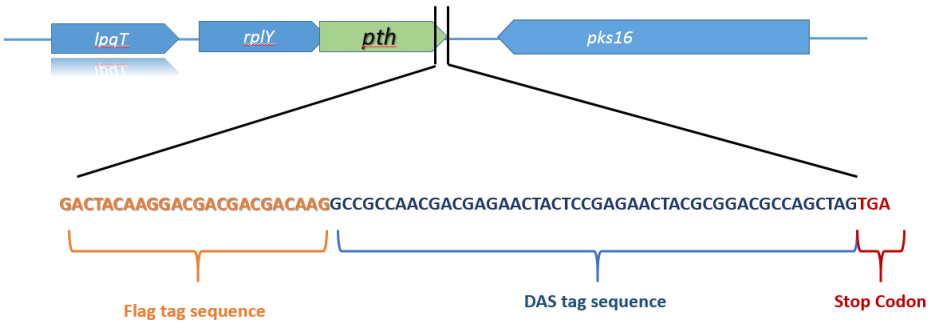
The first task will be to investigate whether depletion of Pth has a bactericidal or bacteriostatic effect (Figure 2). Most drugs targeting *M. tuberculosis* translational mechanisms have a bacteriostatic effect, but in terms of drug development, a bactericidal effect is generally more desirable. To determine the effect of a reduced concentration of Pth, the depletion strain will grow un-induced until mid-log phase. At mid-log phase the culture will be split into six smaller cultures. Three will continue to grow as normal, and three cultures will receive ATC to begin Pth proteolysis.

As a control, wild-type *M. tuberculosis* H37Rv will be grown to the same mid-log phase and split into nine smaller cultures. Three cultures for bactericidal control will be treated with isoniazid, a drug known to kill *M. tuberculosis*. The bacteriostatic control will be treated with ethambutol, a known bacteriostatic TB drug. The final three cultures will grow without treatment. Every 24 hours (one doubling time) the cultures will be plated for viable cells. After three days of antibiotic treatment, all of the cultures will be washed with ATC-free media and allowed to grow again for three more days. Figure 3 shows the expected outcomes of the bacteriostatic and bactericidal controls. Viable cell counts for the induced depletion strain will be compared to the antibiotic-treated controls.

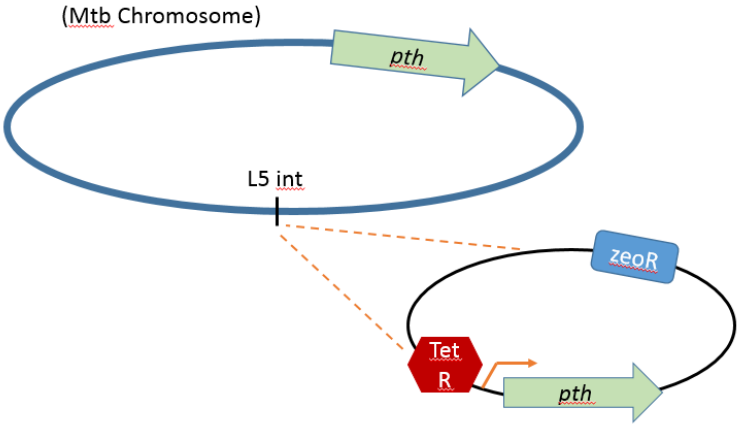
The *pth* over-expression strain was constructed by transforming *M. tuberculosis* H37Rv with a plasmid containing the *pth* gene under the control of a regulate-able promoter. The plasmid was integrated into the chromosome at the L5 mycobacteriophage integration site (Lewis & Hatfull, 2000) in order to provide stability and a predictable plasmid copy number. The native copy of *pth* remains, making this a merodiploid strain (Figure 1B). The promoter is again regulated by ATC, although in this case the loss of ATC will induce over-expression of *pth*. RT-PCR will be used to confirm that *pth* is expressed at higher levels in the mutant strain as compared to wild-type.

The depletion and over-expression mutants will be used to investigate the minimum inhibitory concentration (MIC) of the drugs of interest listed in Table 1. The MIC assay will use a chemical dye called resazurin or alamar blue to detect live versus dead bacteria at varying drug concentrations. The microtiter method described by Banfi, Scialino, and Monti-Bragadin, (2003) will be used. A shift in MIC (predicted MIC's are listed in Table 2) for any of the mutant strains exposed to translation-targeting drugs will provide information about a relationship between Pth

and prokaryotic translation and some information about how a drug targeting this particular Pth enzyme may augment current TB treatment plans.



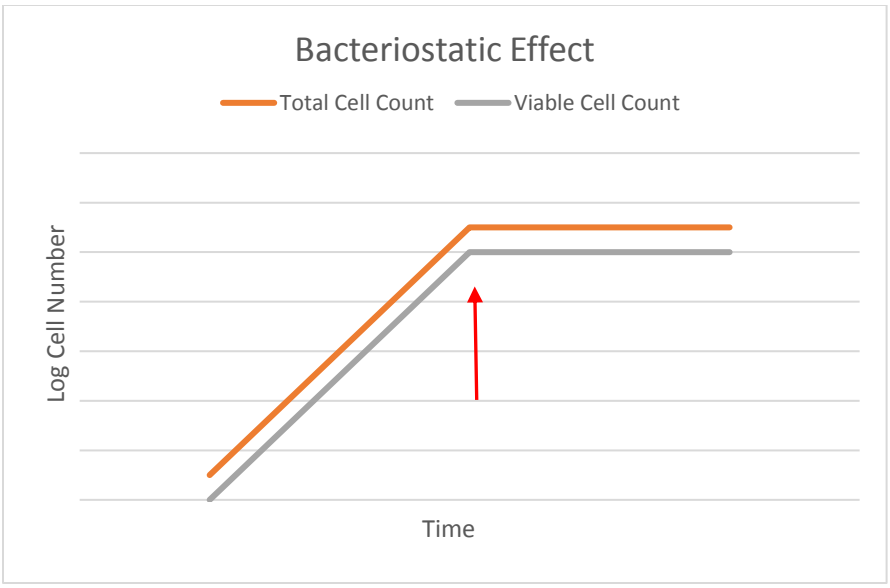
A.



B.

Figure 1. **Mutant Construction.** A simplified graphical representation of the mutant strain construction plan for depletion (A) and over-expression (B) mutants. Figure 1A shows the replacement of the native *pth* stop codon with contiguous flag and DAS tags complete with a new (opal) stop codon. Antibiotic selection marker and lox sites not shown. Figure 1B shows an L5 integrating vector with zeocin selection marker, tet repressor, promoter, and *pth* gene.

A.



B.

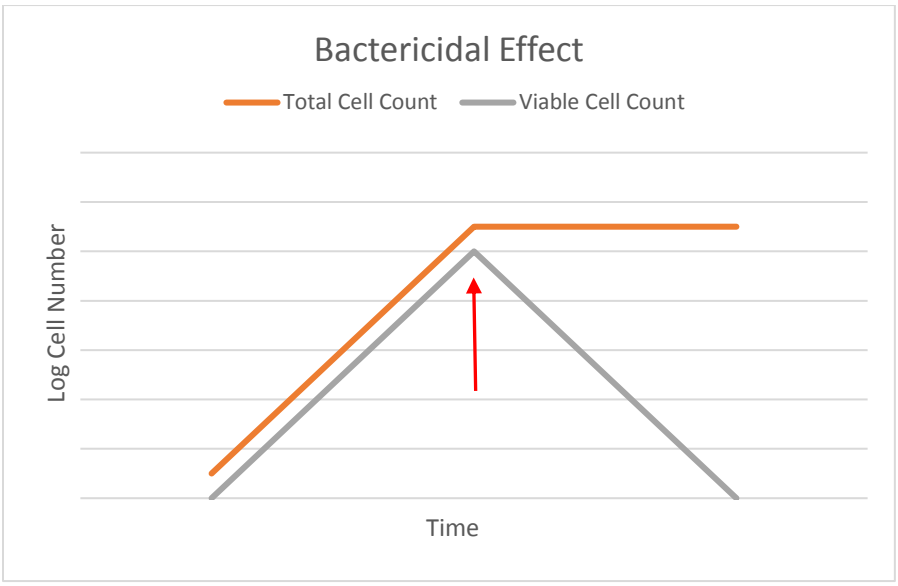
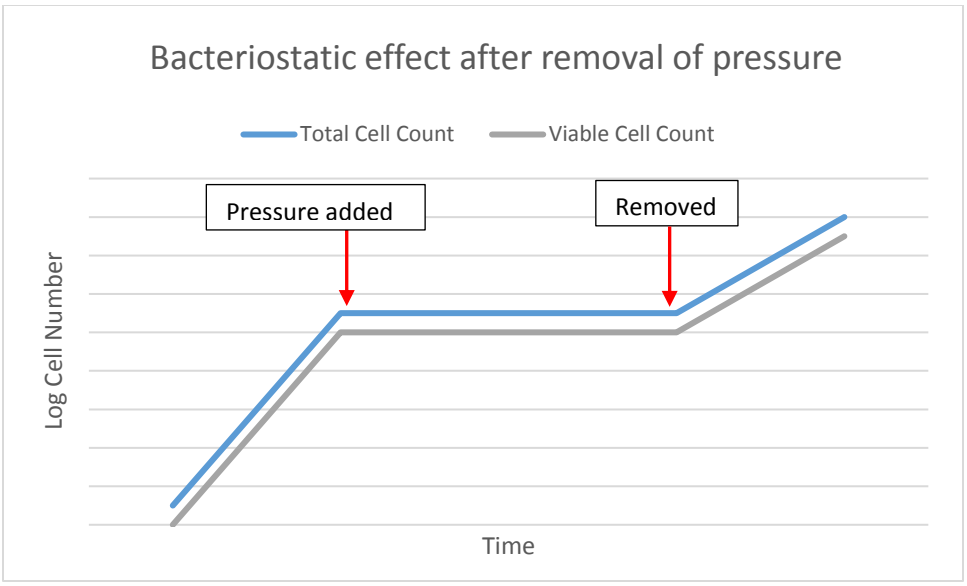


Figure 2. An illustration of two types of antimicrobial effects (A) bacteriostatic and (B) bactericidal on the observable total cell and viable cell numbers, reworked from a diagram found in Brock Biology of Microorganisms (Madigan, Martinko, & Parker, 2003). The red arrow indicates the time at which an antibiotic has been added to the culture.

A.



B.

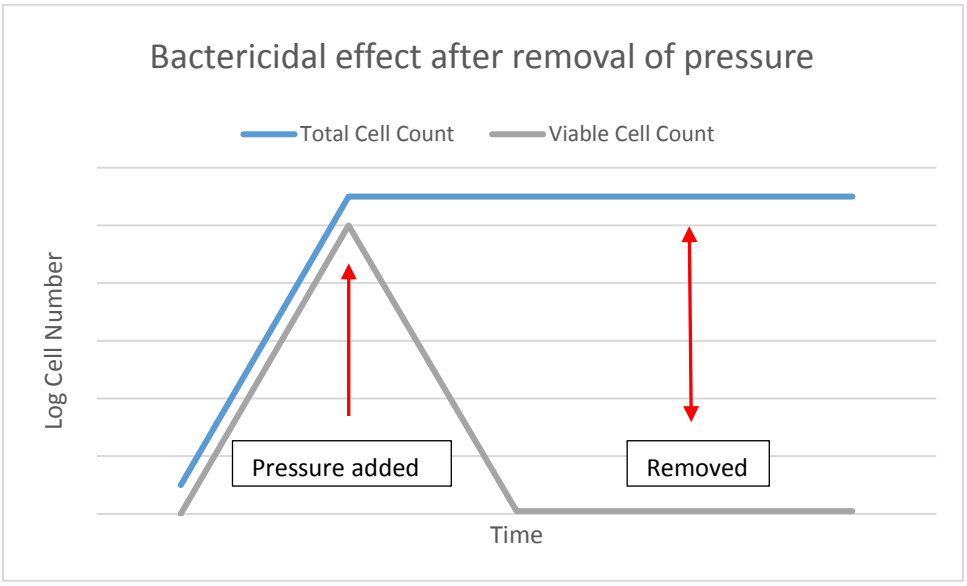


Figure 3. Illustration based on the previous Figure 2 showing recovery of bacteriostatic (A) and complete loss of viability in bactericidal (B) treatment after the removal of antibiotic pressure.

Table 1. Mechanism of Action of Antibiotics Targeting Prokaryotic Protein Synthesis

Drug Name	TB Drug?	Mechanism of Action	Gene Target	Reference
Isoniazid *	Yes	inhibits mycolic acid synthesis	*inhA*	Boshoff 2004
Ethambutol *	Yes	disrupts arabinogalactan synthesis, cell wall permeability	*embB*	Boshoff 2004
Pyrazinamide *	Yes	binds ribosomal protein S1, inhibits trans-translation	*rpsA*	Shi et al. 2011
Streptomycin	Yes	inhibits initiation, causes miscoding	*rpsL, rrs*	Moazed 1987
Kanamycin	Yes	binds 30S ribosomal subunit, causes mistranslation	*Rrs, eis*	Palomino 2014
Linezolid	Yes	inhibits initiation, binds 50S subunit		Swaney 1998
Hygromycin	No	stabilizes A-site, inhibits translocation, induces misreading		Moazed 1987
Chloramphenicol	No	domain V of the 23S rRNA, inhibits peptidyl transferase		Richter 2007, Sohaskey 2004
Erythromycin	No	Binds to 50S subunit, blocks peptidyl transferase activity	*erm*	Rodrigues 2013
Fusidic acid	No	Inhibits EF-G at ribosome		Collignon 1999

Table 2. Estimated MIC Against *M. tuberculosis* H37Rv

Drug Name	Abbrev.	Phenotype	Estimated MIC ug/ml	Reference
Isoniazid *	INH	bactericidal	0.25	Rodrigues 2013
Ethambutol *	EMB	bacteriostatic	1.25	Rodrigues 2013
Pyrazinamide	PZA	Low *in vitro*	100 (+?)	
Streptomycin	SM	bactericidal	0.125 - 1.0	Banfi 2003
Kanamycin	KAN	bactericidal	0.15	Rodrigues 2013
Hygromycin	HYG	bactericidal	Try range 0.1 - 25	(couldn't find ref.)
Chloramphenicol	CAM	bacteriostatic	5.0	Sohaskey 2006
Erythromycin	ERM	bacteriostatic	25.0	Rodrigues 2013
Fusidic acid	FD	bacteriostatic	16.0	Fuursted 1992
Linezolid	LZD	bacteriostatic	0.5	Ahmed 2013

* Antibiotics to be used as controls

VI.

Research Limitations

With any research there are limitations and that goes double when working with *M. tuberculosis*. The main drawback of any work with this organism is it's doubling time. It is very slow growing (approximately 24 hours doubling time under optimal *in vitro* culture conditions), and therefore difficult to produce genetically manipulated strains and to perform experiments. Another drawback is the infectious nature of the disease. *M. tuberculosis* is classified as a BSL3 microorganism and strict safety precautions must be taken whenever working with live strains. Extensive training and specific facilities must be used. Working in a BSL3 laboratory carries an increased cost of both time and money. Many researchers use faster-growing non pathogenic mycobacteria strains such as *M. smegmatis* instead of *M. tuberculosis*, but the drug susceptibilities differ between these strains so it will be most useful to use the more clinically relevant *M. tuberculosis* for our experiments.

There are potential issues with methods used to produce these mutant strains. Activity of the *pth* over-expression mutant can be confirmed by RT-PCR to validate that expression levels have increased, but the important thing is whether the amount of protein has increased. A higher expression level generally correlates with an increased amount of protein, but there are other post-transcriptional and post-translational regulatory factors that the cell might use to overcome increased expression. This is especially true if over-expression of *pth* has a detrimental effect on the cell. Therefore it is imperative that time be put aside to use Western Blotting techniques to confirm that true over-expression can be observed with this strain.

Another issue is that the most stable and effective selection antibiotics used for genetic manipulation in *M. tuberculosis* (hygromycin and kanamycin) are also the drugs that will be tested against the *pth* mutant strains. We will need to limit the strain's antibiotic marker usage to zeocin, which, according to the manufacturer (Invitrogen, 2012) cleaves DNA instead of targeting protein synthesis, or to use Cre-Lox recombination (Sauer, 1987) to remove the undesirable antibiotic resistance cassette from the strain entirely.

Experimentally, there are three main limitations. The first is that a clear answer to the question of whether Pth depletion in *M. tuberculosis* is bactericidal or bacteriostatic may not be achieved. Because Pth is essential for growth and the depletion occurs over some time, the bacteria may be able to overcome the depletion system through spontaneous mutation. This could make the strain appear to recover from Pth depletion by mutation. This could lead to a false-bacteriostatic result. The second limitation is the fact that if a clear result does come from the bacteriostatic versus bactericidal experiment and the result is that depletion is bactericidal, it may be difficult to proceed with MIC experiments. Although, it may be possible to titer the amount of depletion by changing ATC concentration in the media such that the cells do not die immediately. Lastly is the question of how much one can infer from the MIC experiments. Although a relationship between intracellular Pth levels and activity of a translation-targeting drug may be recorded, the results will not indicate the specifics of that relationship. It is likely that there would be several confounding factors or intermediate pathways involved in any relationship that may be observed.

VII.

Tentative Schedule

Submission of proposal to research advisor……...December 15, 2014

Proposal returned for revision……..….January 2, 2015

Submission of final proposal……..….January 15, 2015

Proposal accepted by research advisor……..…….….....February 1, 2015

Thesis director agrees to serve……..….February 2, 2015

First draft returned by thesis director……...……..….June 15, 2015

Revised draft completed…….……July 1, 2015

Revised draft returned by thesis director……..……..……..July 15, 2015

Final text submitted to thesis director and research advisor……....…….August 1, 2015

Final text approved…….……..…September 1, 2015

Final approved thesis uploaded to ETD…….……...….October 15, 2015

Graduation……...November, 2015

VIII.

Bibliography

Works Annotated

Ahmed, I., Jabeen, K., Inayat, R., & Hasan, R. (2013) Susceptibility testing of Extensively drug-resistant and pre-extensively drug-resistant Mycobacterium tuberculosis against levofloxacin, linezolid, and amoxicillin-clavulanate. *Antimicrobial Agents and Chemotherapy*. 57 (6): 2522-2525.

- Study of local resistance to drugs used to treat XDR-TB in Pakistan using H37Rv as a control
- Linezolid (LZD) MIC was 0.5ug/ml, and was the most effective drug in the study

Banfi, E., Scialino, B., & Monti-Bragadin, C. (2003) Development of a microdilution method to evaluate Mycobacterium tuberculosis drug susceptibility. *Journal of Antimicrobial Chemotherapy*. 52: 796-800.

- Some MIC data for INH, RIF, SM, EMB using resazurin
- Good protocol for using resazurin/alamar blue in 96-well plates

Bertram, R., & Hillen, W. (2008) The application of Tet repressor in prokaryotic gene regulation and expression. Microbial Biotechnology. 1(1): 2-16.

- Very helpful review about TetOn and TetOFF systems. Great figures.

Boshoff, H. I. M., Myers, T. G., Copp, B. R., McNeil, M. R., Wilson, M. A., & Barry, C. E. (2004) The transcriptional responses of Mycobacterium tuberculosis to inhibitors of metabolism: novel insights into drug mechanisms of action. *The Journal of Biological Chemistry*. 279 (38): 40174-40184.

- Used transcriptional responses to drug treatment to find mechanism of action of anti-tubercular drugs
- Confirmed that INH and ETH inhibit enzymes in the pathway that elongates mycolic acids
- EMB thought to inhibit arabinosylotransferases, but may touch FAS-II pathway.
- EMB-treated cells contained less arabinose and rapidly lost acid-fastness (may indicate cell wall permeability)

Brites, D., & Gagneux, S. (2012) Old and new selective pressures on Mycobacterium tuberculosis. *Infection, Genetics and Evolution.* 12 (4).

- Interesting discussion of TB's possible co-evolution with the human population throughout history
- New selective pressures include emergence of HIV/AIDS and drug treatments

Collignon, P., & Turnidge, J. (1999) Fusidic acid in vitro activity. *International Journal of Antimicrobial Agents*. Aug; 12 Supplemental 2: S45-58.

- EF-G inhibited by fusidic acid which inhibits protein synthesis

Das, G., & Varshney, U. (2006) Peptidyl-tRNA hydrolase and its critical role in protein biosynthesis. *Microbiology*. 152: 2191-2195.

- Less up-to-date than one would like but a nice review about pth which summarizes works done regarding substrate specificity, mechanism of action, potential genetic interactions and essentiality across several bacterial species

Falzon, D., Jaramillio, E., Schünemann, H.J., Arentz, M., Bauer, M., Bayona, J., ... Zignol, M. (2011) WHO guidelines for the programmatic management of drug-resistant tuberculosis: 2011 update. *European Respiratory Journal*. 38 (3): 516-28.

- Good description of WHO-recommended treatment plans for TB
- Outlines the need for a faster, easier drug regimen

Feaga, H.A., Viollier, P.H., & Keiler, K.C. (2014) Release of nonstop ribosomes is essential. *MBio*. 5(6).

- Study outlining the recovery mechanism for "nonstop" ribosomes which is tmRNA-mediated trans-translation for most bacteria
- Trans-translation is not essential for all bacteria so these authors used tn-Seq to discover what other genes might be taking over that role in those cases
- They found an *ArfB* homolog which the authors felt suggested that the release of nonstop ribosomes may be essential in most or all prokaryotes
- In addition, *rluD, lepA, efp* and *pth* were found in the Tnseq screen, in Δ*ssrA* strain there were no *pth* insertions but there were only 24 wild type insertion sites

Fuursted, K., Askgaard, D., & Faber, V. (1992) Susceptibility of strains of the Mycobacterium tuberculosis complex to fusidic acid. APMIS*: Acta Pathologica, Microbiologica, et Immunologica.* 100 (7): 663-667. (only abstract was available)

- MIC determined by BACTEC method, for Mtb about 16ug/ml MIC90, and 250ug/ml MBC90
- No cross-resistance to EMB, INH, RIF, SM, PZA, ofloxacin or Cipro observed

Giudice, E. & Gillet, R. (2013) The task force that rescues stalled ribosomes in bacteria. *Cell*. 38 (8): 403-411.

- Good review outlining non-stop and no-go ribosome stall situations
- Describes a large network of recovery factors including tmRNA-SmpB, AfrA/ArfB, EF4 and EF-P, and Pth

Ivanova, N., Pavlov, M. Y., Felden, B., & Ehrenberg, M. (2004) Ribosome rescue by tmRNA requires truncated mRNAs. *Journal of Molecular Biology*. 338: 33–41.

- The length of mRNA downstream from the P-site codon has an effect on tnRNA activity, shorter is better (more than 15 is bad)
- Implies that tm-RNA is for stalled & truncated mRNA, not full-length mRNA which induces pause because of mistranslation

Lewis, J. A., & Hatfull, G. F. (2000) Identification and characterization of mycobacteriophage L5 excisionase. *Molecular Microbiology*. 35(2): 350-360.

- Highly directional phage integration is a powerful molecular genetics tool

Madigan, M. T., Martinko, J. M., & Parker, J. Brock Biology of Microorganisms. Upper Saddle River, NJ: Prentice Hall, 2003. Print

- Good starting place for background on translation pages 194-204
- Figure on the types of action of antimicrobials page 704

Mdluli, K., Kaneko, T., Upton, A. (2014) Tuberculosis drug discovery and emerging targets. *Annals of the New York Academy of Sciences.* Vol. 1323 (56–75).

- Overview of recent efforts in drug discovery (bedaquiline, delamanid) for anti-tubercular medicines and current regimen's drawbacks
- Recommends certain types of drug targets and methods that would bolster the hit rates of more effective drugs

Menninger, J. R. (1979) Accumulation of peptidyl tRNA is lethal to Escherichia coli. *Journal of Bacteriology*. 137(1): 694-696.

- Temperature sensitive Pth studies
- used erythromycin to enhance production of peptidyl tRNA while depleting Pth and observed enhanced killing

Moazed, D., & Noller, H. F. (1987) Interaction of antibiotics with functional sites in 16S ribosomal RNA. *Nature.* 327: 389- 394.

- Streptomycin induces misreading and inhibits translation initiation step, targets RpsL and rrs
- Hygromycin induces misreading as well, but in a different fashion, and inhibits translocation

Murphy, K. C. (2012) Phage recombinases and their applications. Advances in Virus Research. 83: 367-414.

- Excellent and thorough background and update of recombineering systems

Nuermberger, E., Tyagi, S., Tasneen, R., Williams, K.N., Almeida, D., Rosenthal, I., Grosset, J.H. (2008) Powerful bactericidal and sterilizing activity of a regimen containing PA-824, moxifloxacin, and pyrazinamide in a murine model of tuberculosis. *Antimicrobial Agents and Chemotherapy*. 52 (4).

- Mouse studies done with combinations of RIF, INH, PZA, moxifloxacin (MXF) and experimental drug PA-824 show that PZA and MXF cannot be replaced in the drug treatment in combination with PA-824.
- However, RIF shown to be dispensable when replaced by PA-824
- Most effective combination was Pa-MXF and PZA in preventing relapse

Palomino, J. C., & Martin, A. (2014) Drug resistance mechanisms in Mycobacterium tuberculosis. *Antibiotics*. 3: 317-340.

- KAN and AMI inhibit translation at 16S RNA
- High-level resistance correlated with mutations in *rrs*, low level with *eis* promoter region.

Personne, Y. & Parish, T. (2014) Mycobacterium tuberculosis possesses an unusual tmRNA rescue system. *Tuberculosis*. 94 (1): 34-42.

- *ssrA* is essential but SmpB is Not essential for growth in M tuberculosis,
- tmRNA both rescues stalled ribosomes and marks nascent polypeptide for clearance, but the rescue function is the essential one
- they postulate that tmRNA in *M tuberculosis* may have additional functions
- Δ*smpB* mutant was sensitive to chloramphenicol and erythromycin (but not RIF)
- Mutations in SmpB or ssrA did not lead to PZA resistance
- They propose that ssrA is the only pathway available to rescue stalled ribosomes in *M. tuberculosis* and that this may indicate that there is a limited number of ribosomes or that ribosome stalling is more serious in *M. tuberculosis*

Raju, RM., Jedrychowski, M.P., Wei, J.-R., Pinkham, J.T., Park, A.S., O'Brien, K., ...Rubin, E.R. (2014) Post-translational regulation via Clp protease is critical for survival of Mycobacterium tuberculosis. *PLoS Pathogens.* 10 (3).

- Fig 1. Explains construction of DAS-tag via merodiploid in TB
- Clp proteases are different in *M. tuberculosis*, and essential

Richter, E., Rüsch-Gerdes, S., Hillemann, D. (2007) First linezolid-resistant clinical isolates of Mycobacterium tuberculosis. *Antimicrobial Agents and Chemotherapy*. 51 (4); 1534-1536.

- Chloramphenicol binding site is domain V of the 23S rRNA, similar to linezolid
- Lack of cross-reaction may indicate unique mechanisms of action

Rodrigues, L., Villellas, C., Baila, R., Viveiros, M., and Ainsa, J. A. (2013) Role of the Mmr efflux pump in drug resistance in Mycobacterium tuberculosis. *Antimicrobial Agents and Chemotherapy.* 57(2): 751-757.

- EMB MIC is 1.25ug/ml in H37Rv, and 0.15 ug/ml for KAN
- Mmr is mainly pumping ethidium bromide and CTAB, may not be related to INH resistance in Mtb.

Sauer, B. (1987) Functional expression of the cre-lox site-specific recombination system in the yeast Saccharomyces cerevisiae. *Molecular and Cellular Biology*. 7 (6): 2087-2096.

- Excision recombination at integrated lox sites shown to be effective in yeast, excellent tool for molecular biology

Selvaraj, M., Roy, W., Singh, N.S., Sangeetha, R., Varchney, U., Vijayan, M. (2007) Structural plasticity and enzyme action: crystal structures of Mycobacterium tuberculosis peptidyl-tRNA hydrolase. *Journal of Molecular Biology*. 372: 186-193.

- Proposed model for structural changes during Pth enzyme activity
- *E. coli* and *M. tuberculosis* Pth are homologous, sequence identity of 38%
- *M. tuberculosis* Pth can rescue depletion of *E. coli* Pth.

- The major difference between the two is in the region supposedly involved in peptide and tRNA binding, these are structural changes associated with enzyme action

Schmeing, T.M., & Ramakrishnan, V. (2009) What recent ribosome structures have revealed about the mechanism of translation. *Nature*. 461, 1234-1242.

- Review of ribosome structure studies and what they reveal about protein synthesis in both prokaryotes and eukaryotes

Sharma, S., Kaushik, S., Sinha, M., Kushwaha, G. S., Singh, A., Sikarwar, J., ...Singh, T.P. (2014) Structural and functional insights into peptidyl-tRNA hydrolase. *Biochimica et Biophysica Acta*. 1844: 1279-1288.

- Great review of Pth as an example of a member of the stalled ribosome rescue task force
- *M. smegmatis* and *M tuberculosis* Pth structures show subtle difference which may indicate differential substrate binding
- Pth activity is ubiquitous, compares variations among different species
- Active site: Conserved residue His138 is changed to Arg139 *M. tuberculosis* and *M. smegmatis* may account for conformation difference in Pth and likely accommodates different substrate binding specificities
- CCA site: glycine140 to proline substitution, narrower conformation, Lys142 substituted to arginine, Mtb Pth especially different
- Acceptor site: Msm, and Mtb very divergent from other species

Shi, W., Zhang, X., Jiang, X., Ruan, H., Barry, C. E., Wang, H., ... Zhang, Y. (2011) Pyrazinamide inhibits trans-translation in Mycobacterium tuberculosis: a potential mechanism for shortening the duration of tuberculosis chemotherapy. *Science*. 333(6049): 1630–1632.

- Identified the target of activated PZA (POA) as RpsA
- Confirmed by resistance when over-expressed and by biochemical means to find POA and RpsA bound to each other
- POA inhibited trans-translation

Sohaskey, C. D. (2004) Enzymatic inactivation and reactivation of chloramphenicol by Mycobacterium tuberculosis and Mycobacterium bovis. *FEMS Microbiology Letters*. 240: 187-192.

- Chloramphenicol MIC about 5ug/ml, not used to treat Mtb
- Chloramphenicol acts upon the peptidyl transferase centers of ribosomes

Swaney, S. M., Aoki, H., Ganoza, M. C., & Shinabarger, D. L. (1998) The oxazolidinone linezolid inhibits ignition of protein synthesis in bacteria. *Antimicrobial Agents and Chemotherapy.* 42 (12): 3251-3255.

- Oxazolidinones inhibit initiation by preventing the formation of $tRNA^{fMet}$-mRNA-30S complex

Velayati, A. A., Masjedi, M. R., Farnia, P., Tabarsi, P., Ghhanavi, J., ZiaZarifi, A. H., & Hoffner, S. E. (2009) Emergence of new forms of totally drug-resistant tuberculosis bacilli. *Chest.* 136:420–425.

- Totally Drug-Resistant (TDR) strains of *M. tuberculosis* reported in Iran, many were Afghani immigrants (43% of XDR and TDR)
- TDR strains are a major health problem for both the infected patient and TB control programs
- TDR TB strains resistant to INH, RIF, SM, ETB, PZA, CAP, KAN, ETH, PAS, CYC, OFX, AMK, & Cip

Vivanco-Dominguez, S., Cruz-Vera, L. R., & Guarneros, G. (2006) Excess of charged $tRNA^{Lys}$ maintains low levels of peptidyl-tRNA hydrolase in *pth*(Ts) mutants at a non-permissive temperature. *Nucleic Acids Research.* 34 (5).

- Created a temperature sensitive pth strain in *E. coli*
- Showed that over-production of $tRNA^{Lys}$ was able to rescue the cell from Pth depletion for a significant time by stimulating synthesis of Pth(Ts)

World Health Organization (WHO). Tuberculosis Fact sheet. Updated October 2014. Available at http://www.who.int/mediacentre/factsheets/fs104/en/ . Accessed January 17, 2015.

- TB second only to HIV/AIDS as greatest infectious killer, background, etc