

Partial depletion of Pth Increases Susceptibility to Macrolide
Drug Treatment in *M. tuberculosis*

A Thesis in the Field of Biotechnology
for the Degree of Master of Liberal Arts in Extension Studies

Harvard University

March 2016

Abstract

The goal of this work was to investigate whether a ubiquitous bacterial protein, peptidyl-tRNA hydrolase (Pth), would be an appropriate candidate for target-based drug discovery research directed against pathogenic *M. tuberculosis*. Many successful antibiotics target protein synthesis machinery to arrest bacterial growth. Hydrolysis of peptidyl-tRNA by Pth is an important action during recovery from stalled protein synthesis. To determine whether an attack on Pth would damage *M. tuberculosis*, several Pth depletion strains were created. A tightly regulated knockdown strain confirmed essentiality of the protein for normal growth. Loosely regulated partial knockdown (hypomorph) strains were created for use in drug-susceptibility testing which showed that depletion of Pth induced hypersensitivity to macrolide antibiotics erythromycin, clarithromycin, and azithromycin. These drugs are comparatively ineffective against *M. tuberculosis* and are not used in first line treatment regimens. Pth was shown to be an attractive candidate for drug discovery research not only because it is essential but because abrogation of Pth function may sensitize this pathogen to an entire class of well-characterized drugs not typically used to combat *M. tuberculosis*.

Dedication

To ■■■

Acknowledgements

I'd like to thank

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Chapter I

Introduction

Roughly 9 million people contracted the respiratory disease tuberculosis (TB) in 2013 and of those infected, 1.5 million died (WHO, 2014). The absence of effective antibiotic treatment contributed to many of these deaths. Combined factors of HIV/AIDS prevalence and sub-lethal exposure to antibiotics are further driving the evolution and spread of drug-resistant *Mycobacterium tuberculosis* (Brites & Gagneux 2012), the causative agent of TB. The growing problem of multiple drug resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) infections absolutely requires that new drugs and treatment plans be made available to the global population. This goal of this work was to investigate alternate targets for drug therapy to perturb tuberculosis infections.

Treatment of Tuberculosis

TB drug therapies have not changed significantly in the past several decades even though this treatment regimen is long and complicated (Table 1). The established regimen requires multiple drugs be taken simultaneously; increasing the patient's risk of 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 to combat TB will need to be effective against emerging MDR/XDR TB strains, must shorten the treatment time, and use novel mechanisms of action to reduce the incidence of disease in an impactful way on a global scale (Mdluli, Kaneko, & Upton, 2014).

Table 1. Tuberculosis treatment regimen.

Regimen	Initial Phase			Continuation Phase			Total
	Drugs	Interval	Doses	Drugs	Interval	Doses	
1	INH	7 Days/Week	56 doses	INH	7 Days/Week	126 doses	182-130 doses, 26 weeks
	RIF	5 Days/Week	40 doses	RIF	7 Days/Week	126 doses	
	PZA	5 Days/Week	40 doses				
	EMB						
2	INH	7 Days/Week	56 doses	INH	7 Days/Week	217 doses	273-195 doses, 39 weeks
	RIF	5 Days/Week	40 doses	RIF	7 Days/Week	217 doses	
	EMB	5 Days/Week	40 doses				

Note: Two different drug regimens for treatment of culture-positive pulmonary tuberculosis caused by drug-susceptible organisms as recommended by the Centers for Disease Control and Prevention (CDC, 2003).

Studying Genes Essential for Replication

One of the best ways to discover new anti-TB drugs is to study factors which are essential for the survival of *M. tuberculosis*. However, studying essential genes can be a double-edged sword in many ways. To learn the most about a gene's function and its importance to a bacterium we must disrupt it in some way; unfortunately, the bacterium

may not survive long if an essential factor is abrogated. This limits our ability to study the effect of the disruption. Most genetic research aims to discover a gain or loss of function in the absence of a particular gene, but many important experiments, such as drug-screening assays, are impossible to conduct with dead or non-replicating bacteria. For these reasons it is desirable to use techniques that allow the study of partial, rather than complete loss of a gene.

Regrettably, many of the genetic tools used to easily manipulate genomes of the average model organism do not work well, or at all in mycobacteria. Mycobacteria are quite different from most Gram positive and Gram negative bacteria in that they possess a uniquely complicated acid-fast cell wall and are genetically very GC rich. Another difficult factor is their long doubling time: *M. tuberculosis* doubles its population only once about every day in nutrient-rich culture medium, which means research in this area is also time consuming. For these reasons, our ability to study the effect of partial loss of gene function in *M. tuberculosis* has been historically limited.

However, progress has been made in this area. Multiple techniques employing regulated protein degradation have been developed recently (Wei *et al.* 2011, Kim *et al.* 2011). Traditional approaches relied on lowering gene transcript levels through promoter replacement. This method relied on dilution of the protein target through successive rounds of cell division. One benefit of regulated protein degradation is that the protein target is depleted directly and rapidly, with the potential to modestly titrate that effect. In addition, the use of regulated proteolysis allows for native expression of the target gene, an advantage which may be critical for study of essential genes operating within a narrow window of expression.

Some protein degradation techniques rely on tetracycline regulatory systems to induce proteolysis. In 2009, significant improvements were made to the tetracycline regulatory system for use in *M. tuberculosis*. Klotzche, Ehrt, and Schnappinger mutated tetracycline repressor (TetR) sequences to provide a 50-fold better repression in mycobacteria, and improved the reverse TetR (revTetR) sequences by altering the codon usage and structure of revTetR so that this system could be used in a single copy plasmid integrated onto mycobacterial chromosomes. These advances have greatly improved the genetic tools available to the mycobacterial researcher, but highly essential proteins can still be difficult to study without unintentionally killing the cell.

In this study, tetracycline regulated inducible proteolysis was used to create regulated knockdown mutants of the essential *M. tuberculosis* gene, *pth*. Pth is an esterase which breaks peptidyl-tRNA apart into its tRNA and polypeptide components. It is ubiquitous and essential in most prokaryotes, including such divergent species as *E. coli* and *M. tuberculosis*. Its function supports one of the most essential processes in living cells – translation – which has been a successful target for antibacterial drug discovery for many decades.

Studying Translation through the Lens of Antibiotics

Translation, the process of synthesizing proteins, is an exceptionally important activity in any living cell. Most of the factors related to this process are essential for growth in bacteria. 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. This chain of peptides should go on to form a functional protein upon completion. There are still many details about this process which are unknown, even in an organism as relatively simple as a bacterium.

The study of ribosome processes has been aided by the discovery of antibiotics which inhibit different aspects of translation in certain microbes (Figure 1). Some of these drugs have the ability to effectively target and disable 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 site (P-site) of the ribosome (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 an 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 ribosome.

Several antibiotics can throw a wrench into elongation machinery. Hygromycin stabilizes the A-site, inhibiting translocation and inducing misreading of the mRNA transcript (Moazed & Noller, 1987). While chloramphenicol and erythromycin bind to the 50S subunit of the ribosome and inhibit peptidyl transferase activity (Richter, Rüscher-Gerdes, & Hillemann 2007) so 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, which may also inhibit ribosome recycling (Collignon & Turnidge, 1999, Wilson, 2014).

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. Release factors also help the ribosome disassemble and recycle so that the individual factors which once made up the former 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 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 often leads to frame shifts which can drastically

alter polypeptide formation. Mistranslation and frame shifts can even lead to the appearance of a stop codon long before the end of the transcript causing ribosome stall and the production of toxic peptides. In some cases the ribosome will stall and remain locked, unable to dissociate. This removes that ribosome from the cell's essential pool of translation machinery, which is also harmful to the cell.

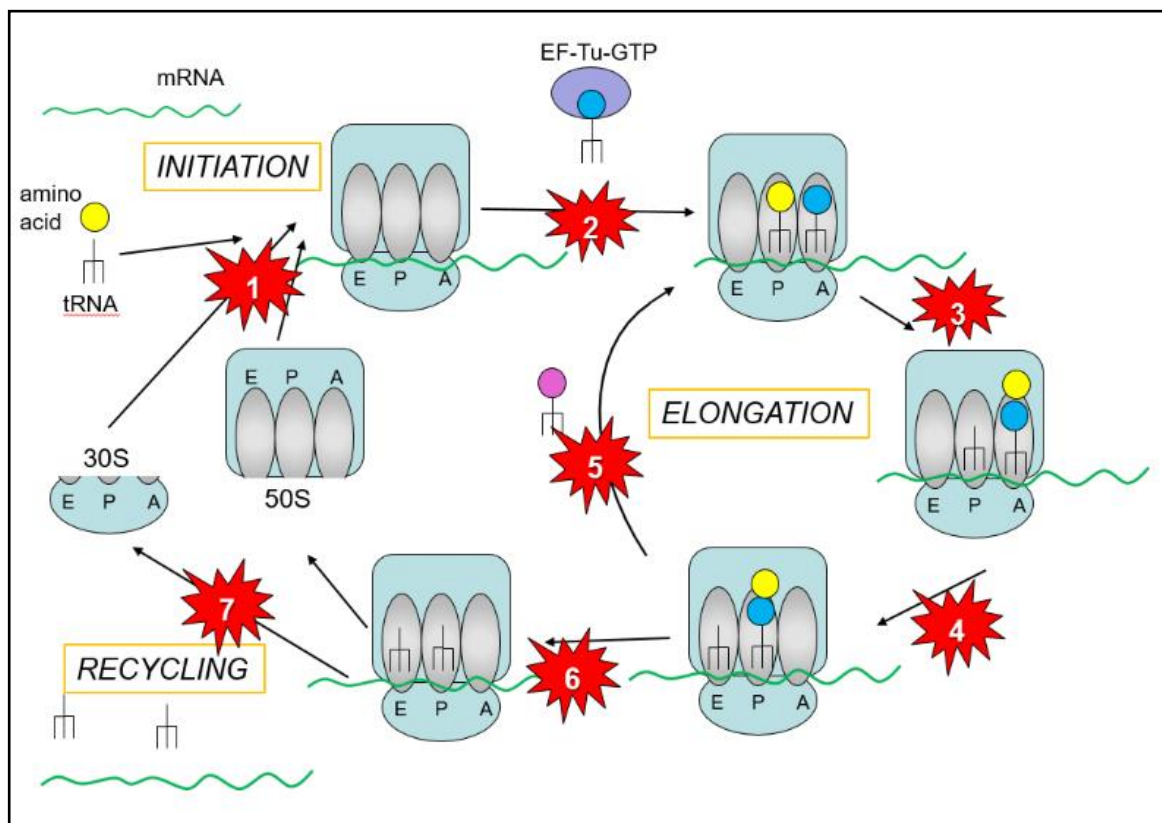


Figure 1. A schematic representation of the translation cycle at the 70S ribosome. Antibiotics can inhibit processes at each numbered step: (1) Initiation: kasugamycin (2) Amino-acylated tRNA delivery: streptomycin, tetracycline (3) Peptide-bond formation: chloramphenicol, clarithromycin, linezolid (4) Translocation: capreomycin, fusidic acid, hygromycin B (5) Elongation: erythromycin (6) Termination: chloramphenicol, kanamycin, and (7) Recycling: fusidic acid. Figure modeled after Wilson, D.N., (2014) Nature Reviews

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 mechanisms designed to help recover stalled ribosomes. These recovery systems are themselves just as essential to a bacterium's viability as many 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 levels of 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.

Another type of ribosome stall, called non-stop, is 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 recovery from non-stop mRNA stall (Guidice & Gillet, 2013). Trans-translation is ubiquitous in bacteria and has two main components: transfer-mRNA (tmRNA), and small basic protein (SmpB). Both tmRNA and SmpB work together with other ribosome-associated factors to recognize and alleviate the non-stop stalled ribosome.

Unfortunately for bacteria relying on trans-translation, in addition to all of the drugs that target translation and cause ribosome stall, there is also a chemical that deters trans-translation. A drug called pyrazinamide (PZA) inhibits trans-translation in *M. tuberculosis*

by binding 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 a newer addition 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 treatment regimen *in vivo*. PZA is now a first-line drug that has had the powerful effect of shortening the TB treatment course from nine or twelve months to just six months. New drug combinations tested against TB infection that did not involve the use of PZA were found to be significantly inferior to regimens including PZA (Nuermberger *et al.*, 2008).

It is not known exactly why PZA is so effective only *in vivo* and in combination with other drugs, but some researchers think it may have to do with attacking latent tuberculosis infections. Trans-translation, the system inhibited by PZA, may play a role in recycling ribosomes and rescuing stalled ribosomes in latent, or non-replicating bacteria. Bacteria in a 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)

Given the success of drugs targeting both translation and ribosome recovery I have focused my work on a protein which may be involved in both processes. The ribosome stall recovery process mediated by trans-translation requires that mRNA be truncated (Ivanova *et al.*, 2004) but sometimes this doesn't happen. In that case, the stalled ribosome must be processed in another way. An alternative recovery mechanism is premature drop-off and involves cleavage of peptidyl-tRNA by a peptidyl tRNA hydrolase (Pth).

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) also showed enhanced killing with erythromycin treatment while depleting Pth. The author postulated that the depletion of Pth during erythromycin treatment increased the concentration of peptidyl tRNA in the cell which was toxic in *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 increase in charged tRNA actually stimulated over-production of Pth so this finding may have been an artifact. However, Bal *et al.* found that overexpression of *M. tuberculosis pth* in *E. coli* increased cell viability in the presence of lethal concentrations of erythromycin (2007).

More recent studies have 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 the mycobacterial model organism, *Mycobacterium smegmatis* Pth (MsPth) especially in the CCA site (Figure 2). These structural studies indicate different substrate specificity or some unique function may be carried out by MtPth.

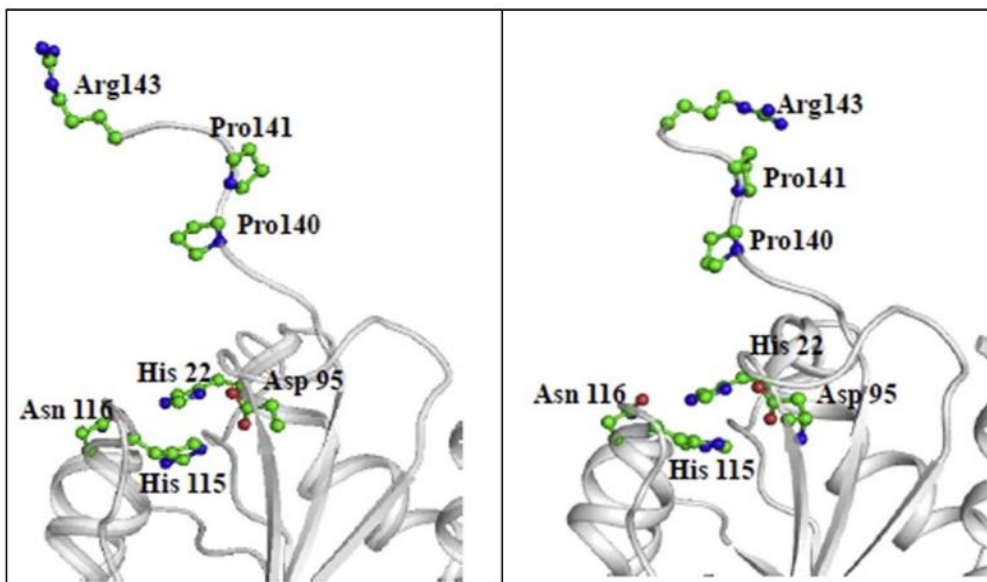


Figure 2. MsPth versus MtPth Structures. Schematic figures of the conformation of the loop 139-146 (CCA site) of MsPth compared to MtPth. Sharma *et al.* (2014) *Biochimica et Biophysica Acta*

Additional differences between *E. coli* and *M. tuberculosis* ribosome stall recovery systems were recently revealed by Personne and Parish (2014) who suggested 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 certainly 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 non-mycobacterial species that did not require it for growth. This group used a transposon based genetic screen 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 screen was *pth*. It was also shown that deletion of *smpB* sensitized *M. tuberculosis* to treatment with erythromycin (Figure 3, Personne & Parish, 2014), which was also observed in *E.coli* studies with EcPth (Meninger, 1979) and MtPth (Bal *et al.*, 2007). This could indicate that Pth has a role linked to *ssrA* and/or *smpB* and the release of non-stop ribosomes.

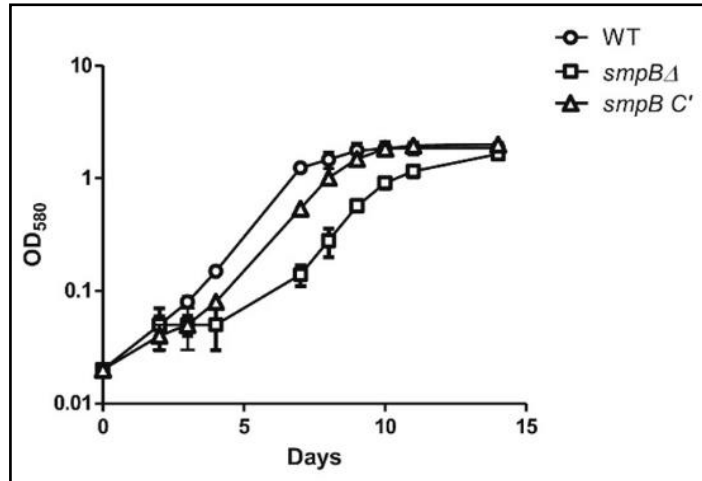


Figure 3. Erythromycin sensitivity in $\Delta smpB$ strain. Deletion of *smpB* leads to erythromycin sensitivity in *M. tuberculosis* strains grown in the presence of sub-lethal concentrations (here, 2.5ug/mL) of erythromycin. *Personne & Parish (2014) Tuberculosis*

For all of these reasons it is plausible that MtPth could be a very good candidate for target-directed drug discovery aimed at TB treatment. A drug targeting ribosome stall recovery could enhance the activity of translation inhibitors which may or may not be in use currently against TB. Investigation of MtPth may also reveal a novel approach to ribosome stall recovery that has not yet been described, which could lead to a better understanding of both Pth and ribosome stalling activity at large in *M. tuberculosis*.

Chapter II

Materials and Methods

The strain construction techniques and experimental protocols are outlined within this section. Advanced molecular biology techniques were used to create the genetic constructs that were used to manipulate the genome of pathogenic *M. tuberculosis*. All experiments were conducted in a biosafety cabinet within a BSL-3 level containment facility.

Strain Construction

All manipulations of live *M. tuberculosis* cultures were performed under BSL-3 conditions. The author has several years of BL3 experience and was qualified to handle this pathogenic strain of *M. tuberculosis*.

FLAG and DAS tagged *pth* in *M. tuberculosis* H37Rv

A tagged *pth* mutant was created in the *M. tuberculosis* H37Rv background. Two tags were inserted into the genome by means of a genetic manipulation technique known as recombineering. Recombineering was mediated by che9c mycobacteriophage recombinase genes called *recET*. These were first described and successfully used to

manipulate the genome of mycobacteria by van Kessel and Hatfull (2007). This technique was further expanded and employed by several groups to make different types of mutant strains at relatively high efficiency in *M. smegmatis* and *M. tuberculosis* (Murphy, Papavinasundaram, & Sasseti 2015). The *recET* genes used in this work were located on an episomal plasmid and regulated by a nitrile-inducible promoter. The plasmid carried a kanamycin resistance marker and contained *sacBR* genes to aid in removal of the plasmid upon completion of recombineering. This plasmid was a gift from the Sasseti Lab at UMASS Medical School in Worcester, MA and will be referred to as pNitET-SacB-kan.

Two hundred nanograms (ng) of pNitET-SacB-kan plasmid was electroporated in 350 microliters (ul) of competent *M. tuberculosis* H37Rv culture and electroporated cells were recovered in 1mL of 7H9 media for 24 hours at 37°C. After recovery, the cells were plated on 7H10 agar plates containing 25ug/mL kanamycin to select for the plasmid and incubated at 37°C for two and a half weeks. Single colonies were picked and grown in 7H9 broth with kanamycin and plasmid-specific PCR was used to confirm the presence of pNitET-SacB-kan.

During this time, the insertion construct was designed and cloned. The insertion consisted of double stranded linear DNA and was created using the primers listed in Table 2. Three overlapping fragments were created and “stitched” together using a common PCR amplification and cloning technique. The insertion required at least 500 base pairs of homologous sequence on either side of the tags, and an antibiotic selection marker in order to place the sequence in the correct location. For clarification, the insertion plan is diagrammed in Figure 4. The entire PCR product used to create this insertion was 2,491

base pairs long with the final insertion adding 1,387 base pairs into the area of the genome directly following the native locus of *pth*.

Two consecutive tags were inserted onto the C-terminal end of the *pth* gene: a FLAG epitope tag followed by a DAS+4 tag (Kim *et al.*, 2010). The FLAG tag was inserted in order to quantify the amount of protein present in a particular strain using a commercially available antibody. This tag could also be used in future studies to purify Pth from *M. tuberculosis* bacterial lysates and find any interacting partners with high purity and low background using single-step affinity purification (Plocinski *et al.*, 2014). The purpose of the DAS tag was to regulate proteolysis of Pth under the inducible system described by Kim *et al.* (2010). In order to use both tags, the stop codon was removed from both *pth* and the flag tag sequences, allowing transcription and translation to proceed through the end of the DAS tag.

Once the insertion sequence was cloned and amplified, 50mL of the pNitET-SacB-kan *M. tuberculosis* strain was grown up to an OD of 0.8. At this OD, 50 ul of 1 millimolar (mM) isovaleronitrile was added to the growing culture to induce *recET* expression. After 8 hours of induction at 37°C, 5 mL of 2M Glycine was added to the culture. The culture was incubated overnight at 37°C. The next day, about 800ng of the insertion DNA was electroporated with 350ul of prepared culture into the strain using the transformation and recombineering techniques described previously (Murphy, Papavinasasundaram, & Sasseti 2015). This was done in triplicate due to the fact that recombineering efficiency is low. The electroporated cultures were allowed to recover at least 24 hours in antibiotic-free media and were then plated on 7H10 plates containing 50ug/mL hygromycin B. Single colonies were picked, grown, and analyzed by PCR approximately two and a half to three

weeks later. The PCR technique used to confirm successful recombineering probed the location of each end of the insertion as well as the full length of the insertion (Figure 5). A successfully recombinant clone was cured of pNitET-SacB-kan, and saved. This strain will be referred to as the “parent” strain because it was used to construct further modified mutant strains.

Inducible Degradation via SspB and ClpXP

Proteolysis of DAS-tagged Pth was regulated as described by Kim *et al.* (2010). To summarize this work, the DAS tag is designed to be recognized by a protein encoded by a gene called *sspB*. When the SspB protein locates a DAS-tagged protein it cleaves the DAS sequence. The cleaved sequence becomes a signal to mycobacteria’s native ClpXP proteolysis system to degrade the protein. ClpXP machinery quickly depletes the cell’s supply of that protein. This system is perfect for investigating proteins which are essential for growth because it is inducible and robust. Degradation of the target protein continues even when the effects are deleterious to the cell. This may be because Clp-mediated proteolysis is itself an essential factor for growth and survival in *M. tuberculosis* (Raju *et al.*, 2014).

Tightly Regulated Knock-Down

After construction of the tagged strain, the regulatory element was introduced on an integrating plasmid. This plasmid, named pGMCtZq19-TSC10M1-*sspB*, was marked with a zeocin resistance cassette, integrated into the Tweety mycobacteriophage site in the genome (Pham *et al.*, 2007) and carried *sspB* under a tetracycline regulated promoter.

Addition of anhydrotetracycline (atc) released the tetracycline repressor (TetR, or, more specifically TSC10), stimulating expression of *sspB*, which caused Pth depletion. The kanamycin resistant form of this plasmid was described and created by Blumenthal, Trujillo, Erht, and Schnappinger (2010) and was gifted to the Rubin Lab by the Schnappinger Lab at Weill Cornell Medical College, New York, NY. I used *E. coli* recombineering to exchange the kanamycin resistance marker for a zeocin resistance marker in order to preserve the ability to test the *pth* mutant strain against kanamycin. The zeocin resistant form of this plasmid was transformed into the *pth*-FLAG-DAS mutant strain and single colonies were analyzed by PCR for successful integration and presence of *sspB*.

A Variety of Hypomorph Strains

Several plasmids were designed by the Schnappinger Lab to demonstrate variable levels of *sspB* expression. The variation was created by generating select point mutations in the tet promoter sequence. A fluorescence reporter assay was used to assess the depletion levels of the strains containing promoters with different combinations of point mutations in *M. smegmatis* (Dirk Schnappinger, personal communication, 2015). Promising plasmids were chosen based on the *M. smegmatis* experiments, and transformed into five different DAS-tagged gene target strains in *M. tuberculosis*. These were tested for growth defects related to induction of SspB (with help from Shoko Minami). The revTetR/TetR38 (“Tet-ON”) promoter/repressor combination worked better than the TetR/TSC10 (“Tet-OFF”) promoters and optimal expression levels varied according to the gene target. Four of the

nine plasmids tested worked quite well in the *M. tuberculosis pth*-FLAG-DAS strain, providing an array of partial knockdown-dependent growth defects.

All of these plasmids integrated at the Giles mycobacteriophage site (Morris *et al.* 2008), and carried a streptomycin selection marker. A control, or “empty” plasmid was included with the variable promoters which also integrated at Giles and carried a streptomycin resistance marker but lacked the tetracycline regulatory system and *sspB*. All five of these plasmids were created by and gifted to our lab by the Schnappinger lab. The successful transformation and integration of these plasmids were confirmed by PCR with the plasmid-specific primers listed in Table 2.

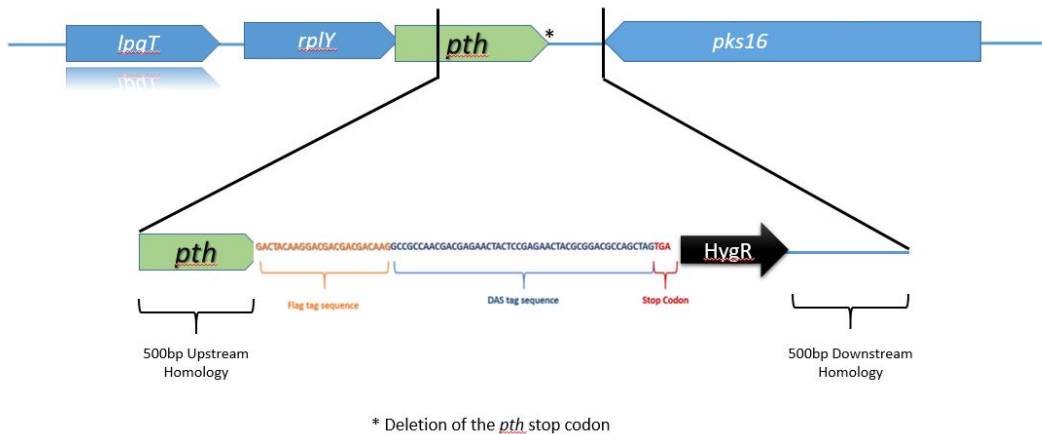


Figure 4. FLAG and DAS tag insertion. Construct includes 500bp of homology upstream, deletion of *pth* stop codon, FLAG and DAS tags, Hygromycin B resistance cassette and another 500bp of homology downstream from the insertion site.

Table 2. Primer sequences.

	Sequence	Purpose
1	atggccgagccggttgctc	Amplify Upstream Homologous Region (F)
2	ctcgtcgttggcggccttgctcgtcgtccttg tagtcccaggcgtggacgcggtttt	Amplify Upstream Homologous Region, append flag and partial DAS tag (R)
3	ctacttgctcgtcgtcctttcactagctggcg tccgcgtagttctcggagtagttctcgtcgttgg cggccttg	Complete DAS tag and append HygR overlap sequence (R)
4	gactacaaggacgacgacga	Amplify HygR cassette (F)
5	tccttcctaggcccaaaaa	Amplify HygR cassette (R)
6	gttttttgggctagggaggataaacgacccg cgtgagcaga	Amplify Downstream Homologous Region and append overlap (F)
7	agtcgctaactcccggctac	Amplify Downstream Homologous Region (R)
8	gagtccgagtagacggcttg	To confirm recombineering into the genome (F) (Figure 5)
9	aactgcatctcaacgccttc	To confirm recombineering into the genome (R) from HygR cassette (Flank 1) (Figure 5)
10	gcgtaggaatcatccgaatc	To confirm recombineering into the genome (F) from HygR cassette (Flank 2) (Figure 5)
11	gacgaacagggcgatgtcat	To confirm recombineering into the genome (R) (Figure 5)
12	gccagatcgtgctgaacat	To confirm transformation of an sspB- containing plasmid (F)
13	ttttgtgatgctcgtcagg	To confirm transformation of an sspB- containing plasmid (R)

Note: These primers were designed and used for the creation of pth mutant strains

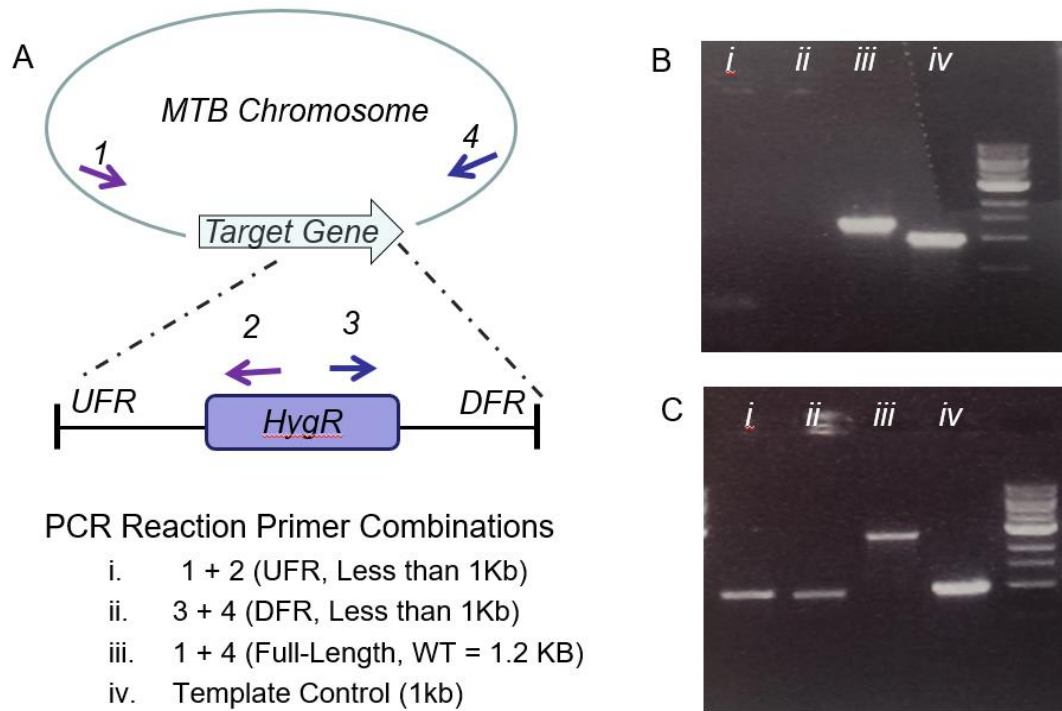


Figure 5. Diagram of PCR clone verification technique. The location of primers inside of the HygR cassette and outside of the insertion's upstream flanking region (UFR) and downstream flanking region (DFR) (A) were designed to confirm whether illegitimate recombineering (B) or successful recombineering (C) had taken place.

Experiments

Growth Curves and Depletion

The parent, control, and all knock-down strains were grown to mid-log phase in 7H9 complete broth and back-diluted to an optical density (OD) at A600 of 0.005. The strains were grown with and without atc added to the growth medium, in triplicate, for 10-14 days. The OD of each culture was recorded at least every two to three days. In the case of the tightly regulated knock-down strain, 100 ul samples from each culture were taken every two to three days. The samples were serially diluted and plated in duplicate on 7H10

agar plates. The plates were grown at 37 °C for two to three weeks. The number of colony forming units (CFU/mL) were recorded for each dilution that could be counted. Figure 6 shows two possible outcomes of this experiment.

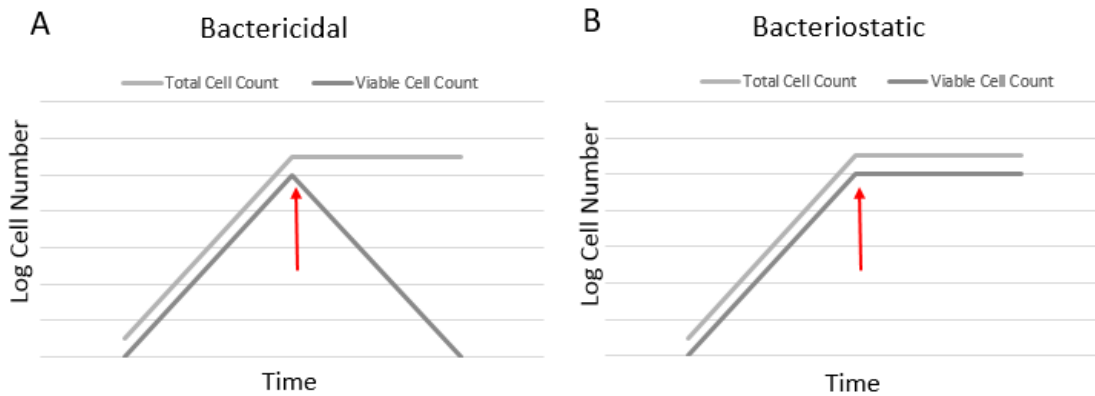


Figure 6. An illustration of two types of antimicrobial effects. Bacteriostatic (A) and bactericidal (B) graphs describing the observable total cell and viable cell numbers, reworked from a diagram found in Brock Biology of Microorganisms (Madigan, Martinko, & Parker, 2003). The arrow indicates the time at which an antibiotic has been added to the culture.

Minimum Inhibitory Concentrations

To determine the minimum inhibitory concentration (MIC) of drugs known to target some part of the translation process on strains depleted of Pth, the parent, control, knockdown, all hypomorph strains, and a wild-type H37Rv strain were grown to mid-log phase in 7H9 complete broth. The microtiter method was used in order to test the different strains and drugs efficiently (Banfi, Scialino, & Monti-Bragadin, 2003, and Collins & Franzblau, 1997). The OD of each strain was recorded and used to back-dilute all cultures

to an OD of 0.006 in 7H9 broth. One hundred microliters of culture was added to each well in a 96 well plate containing one hundred microliters of the drugs of interest (Table 3) making the final concentration of bacteria approximately 1×10^5 cells per well. In order to assess the reactivity of the drug with the assay chemicals, two rows were left un-inoculated as “Drug and Media Only” controls. The final column in all plates were growth positive controls containing no drug, only bacteria and media.

Each drug was stored according to the manufacturer’s instruction and prepared at a concentration of 10 or 20 mg/ml on the same day that the bacteria were to be plated. Erythromycin and clarithromycin were dissolved in ethanol, and azithromycin in dimethylsulfoxide (DMSO), while every other drug tested was solvent in water. Each drug was filter sterilized using a 0.2 μ M syringe filter, except in the case of azithromycin, due to the DMSO. Working stocks of each drug were made in sterile 7H9 media. Dilutions of each drug of interest were dispensed in 100 μ l volumes into 96 well plates, in duplicate for each strain. The starting concentration of the drug varied according to the predicted MIC (Table 4) of that particular drug against wild type *M. tuberculosis*. The starting concentration was diluted two-fold across the plate ten times.

After bacteria were added to the plates, they were sealed with breathable film and incubated in a shaking incubator at 37 °C for six days. On the sixth day, ten microliters of 0.02% resazurin/alarazar blue (Collins & Franzblau 1997) was added to every well. The plates were re-sealed with new films and incubated at 37 °C. The plates were photographed and color changes from blue to pink were recorded 24 and 48 hours later.

Table 3. Antibiotic mechanism of action.

Drug Name	TB Drug?	Mechanism of Action	Gene Target
Isoniazid *	Yes	inhibits mycolic acid synthesis	<i>inhA</i>
Ethambutol *	Yes	disrupts arabinogalactan synthesis, cell wall permeability	<i>embB</i>
Pyrazinamide *	Yes	binds ribosomal protein S1, inhibits trans-translation	<i>rpsA</i>
Azithromycin	No	Binds to 50S subunit, blocks peptidyl transferase activity	
Capreomycin	Yes	Binds ribosome subunit interface (70S), inhibits translocation	<i>tlyA</i>
Chloramphenicol	No	domain V of the 23S rRNA, inhibits peptidyl transferase and termination	
Clarithromycin	Yes	Binds to 50S subunit, blocks peptidyl transferase activity	
Erythromycin	No	Binds to 50S subunit, blocks peptidyl transferase activity and prevents nascent chain elongation	<i>erm</i>
Fusidic acid	No	Targets EF-G at ribosome, inhibits elongation and ribosomal recycling	
Kanamycin	Yes	binds 30S ribosomal subunit, causes mistranslation	<i>Rrs, eis</i>
Linezolid	Yes	binds 50S subunit, inhibits initiation at the peptidyl-transferase center	
Nourseothricin	No	comparable to that of other aminoglycosides	<i>nat</i>
Streptomycin	Yes, historically	inhibits initiation, causes miscoding	<i>rpsL, rrs</i>

Note: This table lists the known mechanisms of action of antibiotics used in this study

Table 4. Estimated minimum inhibitory concentration.

Drug Name	Phenotype	Estimated MIC ug/ml	Reference
Isoniazid *	bactericidal	0.25	Rodrigues 2013
Ethambutol *	bacteriostatic	1.25	Rodrigues 2013
Pyrazinamide *	Low in vitro	>100	estimates vary
Azithromycin	bacteriostatic	>128	Watt 1996
Capreomycin	bactericidal	2.00	Heifets 2005
Chloramphenicol	bacteriostatic	5.00	Sohaskey 2006
Clarithromycin	bacteriostatic	< 1.00	Doucet-Populaire 1998
Erythromycin	bacteriostatic	25	Rodrigues 2013
Fusidic acid	bacteriostatic	16	Fuursted 1992
Kanamycin	bactericidal	0.15	Rodrigues 2013
Linezolid	bacteriostatic	0.50	Ahmed 2013
Nourseothricin			not found

Note: A list of the predicted MIC against *M. tuberculosis* H37Rv of drugs used in this study according to previous published works.

Chapter III

Results

Mutant strains were successfully created and individual clones were verified to show they contained the expected sequences. The experiments conducted with these strains helped describe Pth activity in *M. tuberculosis*. The data collected from these experiments are described in further detail in this section.

Pth Essentiality

Transposon based genetic screens indicate that *pth* (Rv1014c) is required for growth in *M. tuberculosis* (Sasseti, Boyd, & Rubin, 2003, and Zhang *et al.* 2012). However these data do not show whether loss of essential Pth function elicits a bactericidal or bacteriostatic effect. Proteins that cause death when depleted are assumed to invoke the same response when inhibited by drugs; these make better drug targets than those that only result in cessation of growth. To determine the effect of Pth depletion, a tightly regulated knockdown strain was made in *M. tuberculosis*. Via recombineering, a DAS tag was appended to the C-terminal end of Pth to create *M. tuberculosis pth*-FLAG-DAS. The addition of *atc* to bacterial growth medium caused production of an enzyme called SspB,

which cleaves any DAS tag, marking the tagged protein for proteolysis. Therefore, addition of *atc* induced depletion of Pth in this strain.

The depletion strain was grown (uninduced) to mid-log phase, back-diluted to an optical density (OD) of 0.005, split into six small cultures, and *atc* was added to half of the cultures. As the culture grew, or in the case of the depleted cultures, failed to grow, samples were plated for colony forming units (CFU/mL) to show the proportion of live bacterial cells while OD was used to estimate the total number of cells. Figure 7 shows the results of this experiment comparing the uninduced cultures and the *atc* regulated, depletion-induced cultures.

The Pth depleted cultures showed modest but significant killing by day three of the experiment, indicating a bactericidal effect, but this was followed by a slow recovery (Figure 7A). It is possible that the recovery was actually caused by escape mutants; a population of cells which become mutated to overcome *atc*-regulated depletion and out-compete regulated cells. Overall, the trend observed in this study, implies that Pth depletion is bacteriostatic. However, this does not exclude the possibility that drug-based Pth impairment could have a bactericidal effect on the cell.

The data also show that this tightly regulated knockdown strain removed enough functional Pth from the cell to significantly prevent growth (Figure 7B). This confirms the essentiality of Pth for normal growth in broth culture. Unfortunately, this also indicates that there will be limitations to the types of experiments one might perform with this strain.

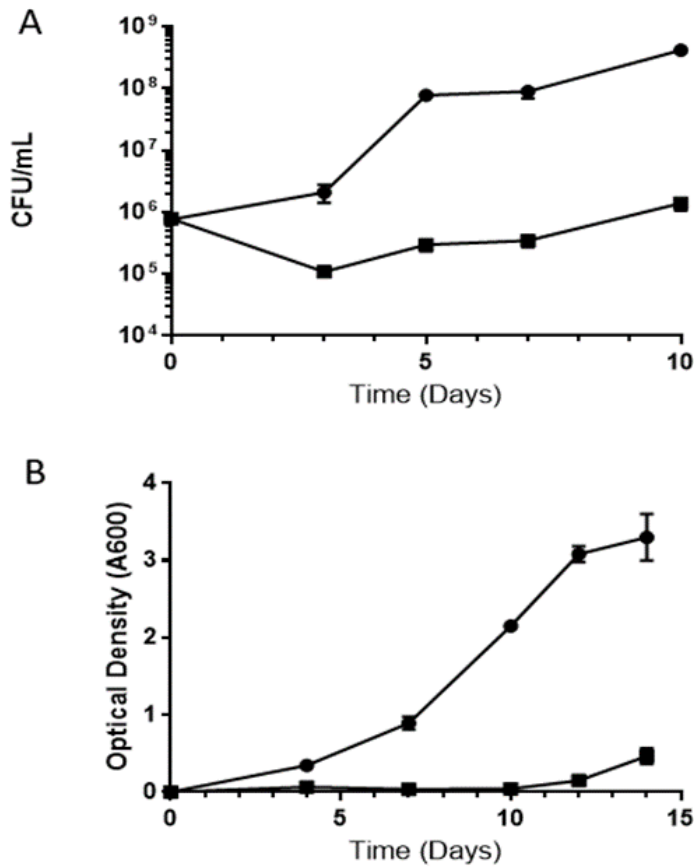


Figure 7: Growth curve and depletion of tightly regulated Pth knock-down strain. The tightly regulated knockdown strain of *pth* (Rv1014c) shows a bacteriostatic effect in comparing uninduced (●) and induced (■) cultures for colony forming units (A) and optical density (B).

Proteolysis: Regulated and Varied

For this reason, new strains were created. Integrating *sspB*-expressing plasmids created by the Schnappinger lab (unpublished), were tested to see whether a less stringent, yet more stable, partial depletion phenotype could be attained. Four plasmids and an empty vector control were transformed into *M. tuberculosis pth*-FLAG-DAS. The cultures were

grown for a period of 15 days either uninduced (Figure 8A) or induced (Figure 8B) for Pth depletion. These strains were regulated using the reverse TetR, therefore depletion was induced upon removal of atc.

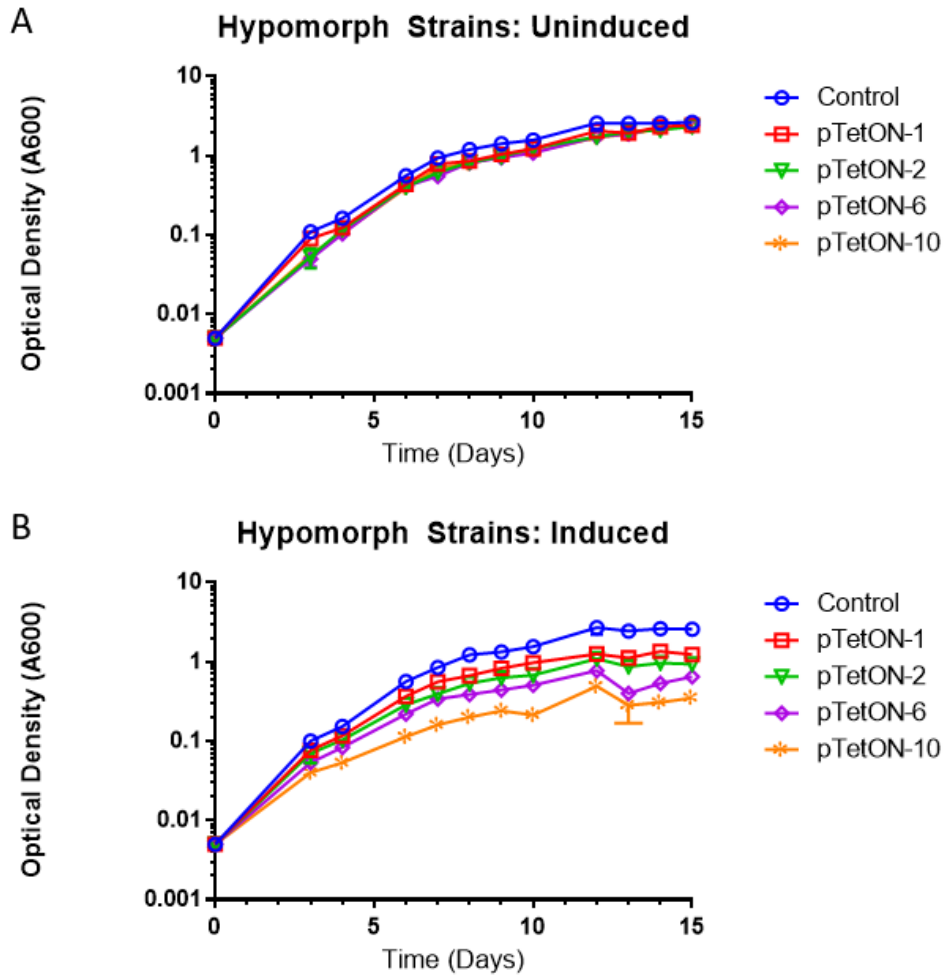


Figure 8. Growth curves of hypomorph strains. Variably induced proteolysis displays a range of growth defects without completely stalling growth when induced (B) yet relatively normal growth when uninduced (A).

The growth of all uninduced strains was largely similar, although the control strain (*pth*-FLAG-DAS with an “empty” integrated plasmid lacking the tet regulatory system and *sspB*) grew slightly better. This difference can be attributed to low levels of background *sspB* expression in the regulated strains. The induced strains showed varied levels of growth defect when induced (Figure 9), yet were still able to replicate.

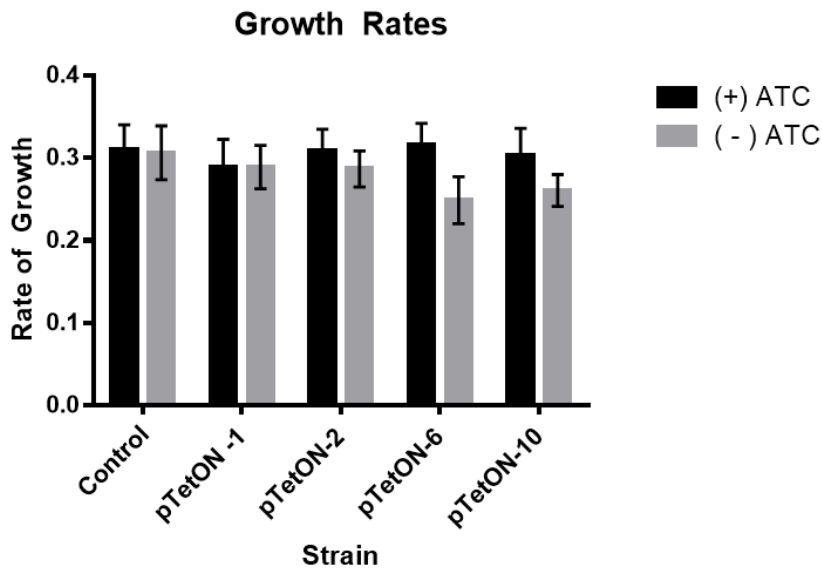


Figure 9. Growth rates of hypomorph strains. Nonlinear regression analysis was used to calculate the growth rates of each strain in the presence (uninduced) or absence (depletion induced) of atc.

Minimum Inhibitory Concentrations of Translation Inhibitors

Wild-type *M. tuberculosis* H37Rv and all *pth* mutant strains were tested against chemical translation inhibitors. The tightly regulated knockdown strain did not grow at all

when Pth depletion was induced, whether in the presence or absence of any drug (Figure 10). As expected, that strain was not useful for drug screening.

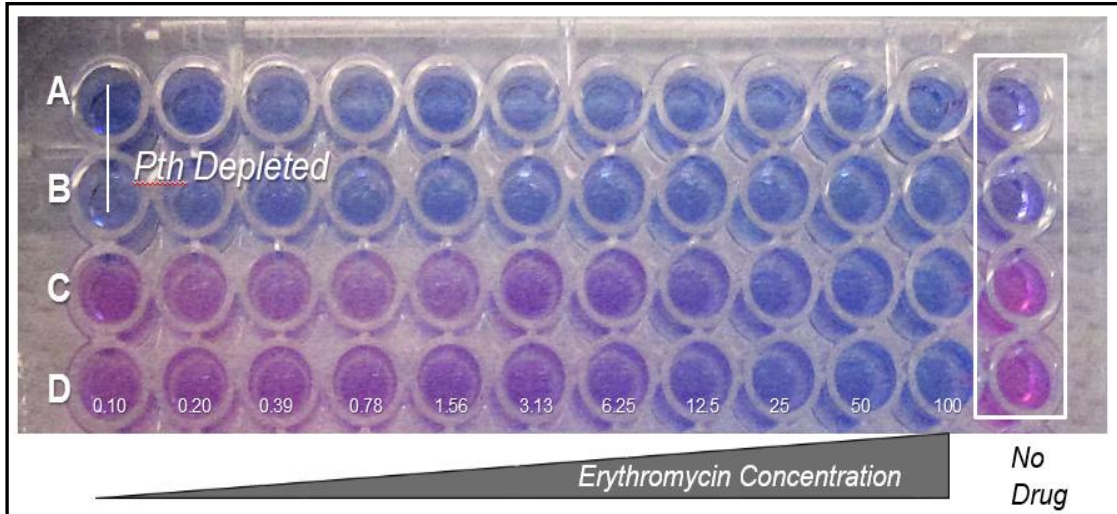


Figure 10. MIC results of tightly regulated Pth depletion strain. The tightly regulated knockdown strain did not grow when induced (rows A/B), although the uninduced strain (rows C/D) had an MIC similar to the control strain.

Fortunately, the hypomorph strains survived induction during drug susceptibility testing. Table 5 shows the results of strains tested against control drugs that do not target translation. Isoniazid and ethambutol both target the cell wall instead of translation. There was no significant difference observed between control, induced, or uninduced strains with isoniazid treatment, although the MIC was generally lower than expected across all strains. The same observation was made in ethambutol treated wells, including wild type *M. tuberculosis* and the parental *pth*-flag-DAS mutant strain from which all of the knock down strains were constructed.

Table 5. Control MIC results.

Strain	Isoniazid	Ethambutol
Anticipated WT MIC	0.25	1.25
Experimental WT H37Rv	-	0.39 - 0.78
Parental Pth tagged	-	0.39 - 0.78
Control	0.04 - 0.08	0.39 - 0.78
pTetON-1 (+) atc	0.04 - 0.08	0.39 - 0.78
pTetON-1 (-) atc	0.04 - 0.08	0.39 - 0.78
pTetON-2 (+) atc	0.04 - 0.08	0.39 - 0.78
pTetON-2 (-) atc	0.04 - 0.08	0.39 - 0.78
pTetON-6 (+) atc	0.04 - 0.08	0.39 - 0.78
pTetON-6 (-) atc	0.04 - 0.08	0.39 - 0.78
pTetON-10 (+) atc	0.04 - 0.08	0.78 - 1.56
pTetON-10 (-) atc	0.02 - 0.04	0.39 - 0.78

Note: The MIC of Pth depletion strains with (+) or without (-) ATC grown in the presence of control drugs. (concentrations in $\mu\text{g/ml}$)

The results of drug susceptibility testing with translation inhibitors showed more variation (Table 6). Kanamycin, chloramphenicol, fusidic acid and linezolid showed small but insignificant fluctuations from strain to strain (Figure 11). Pyrazinamide, nourseothricin, and Clp inhibitors were also tested but did not show appreciable differences (data omitted). Capreomycin showed a slight change in MIC when comparing wild type to induced pTetON-10 but it was not significant when compared to the parental, control, or uninduced strain.

The more remarkable phenomenon was that every regulated *pth* mutant strain was hyper-sensitive to erythromycin. Even the unregulated parental strain showed a two- to four-fold increase in sensitivity as compared to wild type, though this may be a function of the tags alone affecting Pth activity. The most sensitive strain of the group was pTetON-

10, where the phenotype in this strain was so drastic that even the lowest concentration of erythromycin tested here prevented growth.

Table 6. Translation drug MIC results.

	KAN	CAM	ERY	FUS	LZD	CAP
WT H37Rv	1.56 - 3.13	0.78 - 1.56	25 - 50	0.39 - 0.78	0.20 - 0.39	3.13
Parental Pth	1.56 - 3.13	0.78 - 1.56	12.50	0.39 - 0.78	0.10 - 0.20	1.56
Pth-DAS Ctl.	1.56	0.78-1.56	25	0.39 - 0.78	0.10 - 0.20	1.56
pTetON-1 (+)	1.56	0.39 - 0.78	0.78 - 1.56	0.20 - 0.39	0.10 - 0.20	0.78
pTetON-1 (-)	1.56	0.39 - 0.78	0.78 - 1.56	0.20 - 0.39	0.10 - 0.20	0.78
pTetON-2 (+)	1.56 - 3.13	0.39 - 0.78	0.78 - 1.56	0.20 - 0.39	0.10 - 0.20	0.78
pTetON-2 (-)	1.56 - 3.13	0.39 - 0.78	0.78 - 1.56	0.20 - 0.39	0.10 - 0.20	0.78
pTetON-6 (+)	1.56 - 3.13	0.39 - 0.78	0.78 - 1.56	0.20 - 0.39	0.10 - 0.20	0.78
pTetON-6 (-)	1.56 - 3.13	0.39 - 0.78	0.78 - 1.56	0.20 - 0.39	0.10 - 0.20	0.78
pTetON-10 (+)	1.56 - 3.13	0.39 - 0.78	0.78 - 1.56	0.2	0.10 - 0.20	1.56
pTetON-10 (-)	1.56 - 3.13	0.39 - 0.78	< 0.10	0.2	0.10 - 0.20	0.78

Note: MIC of Pth depletion strains with (+) or without (-) ATC grown in the presence of translation inhibitors: kanamycin (KAN), Chloramphenicol (CAM), erythromycin (ERY), fusidic acid (FUS), linezolid (LZD), and capreomycin (CAP). All MIC's are listed

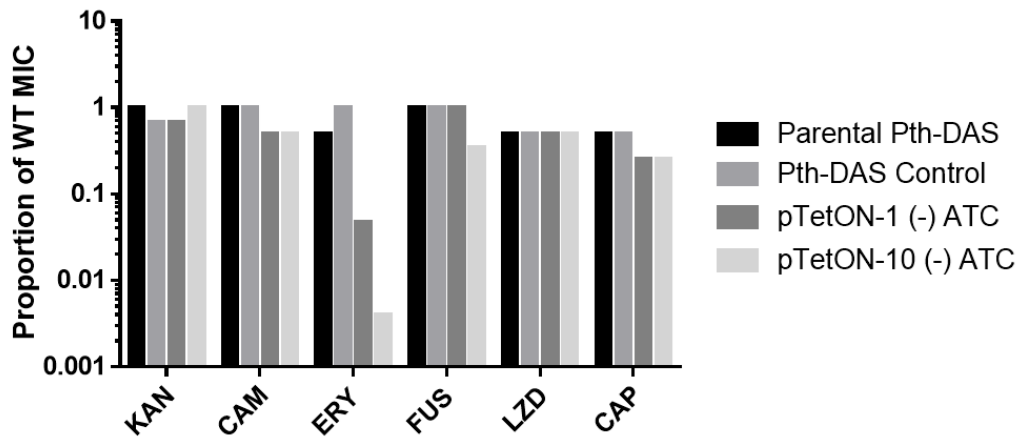


Figure 11. MIC values of depletion strains as a proportion of wild type MIC. A significant change in sensitivity is observed in depletion strains treated with erythromycin. This reaction appears to be specific to macrolide drugs, this phenotype was not observed in other translation inhibitors.

A Closer Look at Macrolide Antibiotics

To further explore the erythromycin sensitivity phenotype and discover a more precise MIC estimate, the assay was repeated. The increased erythromycin sensitivity observed in the induced pTetON-10 strain as compared to wild type *M. tuberculosis* was greater than 1,000 fold (Table 7 and Figure 12). Again, the parental strain was hypersensitive to erythromycin, as was every other strain except the unregulated control. This round also showed a greater difference between uninduced and induced (no atc) strains, displaying a dose-dependent phenotype based on the level of Pth depletion (Figure 13).

Table 7. Macrolide MIC results.

	Azithromycin	Clarithromycin	Erythromycin
Anticipated WT MIC	> 128	2.00	25.00
Exp. WT H37Rv	> 250	5.00	50.00
Parental Pth tagged	15.63	1.25	6.25
Control	> 250	2.50 - 5.00	50.00
pTetON-1 (+) atc	1.95	0.04	0.39
pTetON-1 (-) atc	0.98	0.04	0.20
pTetON-2 (+) atc	1.95	0.04	0.39
pTetON-2 (-) atc	0.49	0.04	0.10
pTetON-6 (+) atc	1.95	0.08	0.39
pTetON-6 (-) atc	< 0.24	0.02	0.05
pTetON-10 (+) atc	0.98	0.04	0.39
pTetON-10 (-) atc	< 0.24	< 0.01	0.012

Note: MIC with erythromycin and erythromycin-derived compounds. (concentrations in µg/ml)

Two compounds derived from erythromycin– azithromycin and clarithromycin, were also tested. A similar pattern was observed, with greater than 1,000 and 500-fold increased sensitivity to azithromycin and clarithromycin, respectively, in Pth-depleted strains. In the case of azithromycin treated strains, both induced pTetON-10 and pTetON-6 strains did not grow at all in the presence of the lowest concentration of azithromycin tested. Again, the parental strain was significantly more sensitive than wild type *M. tuberculosis* to these drugs.

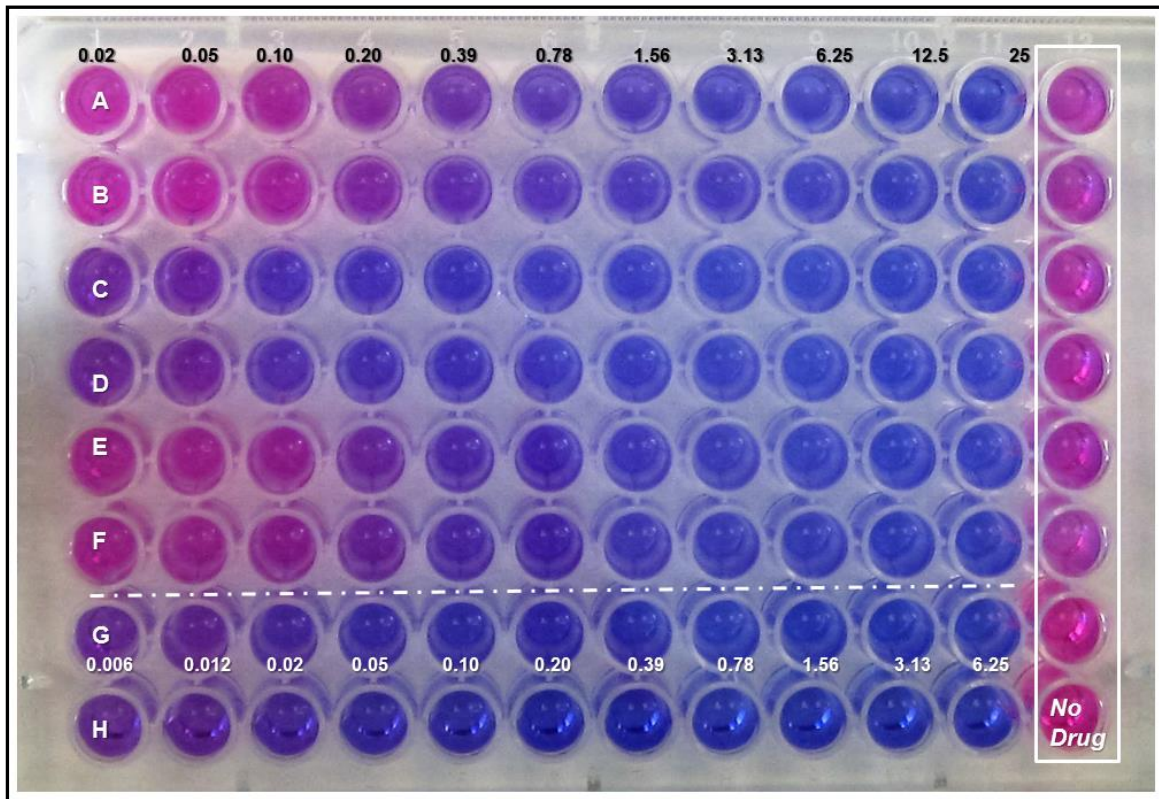


Figure 12. Erythromycin sensitivity in Pth hypomorph strains. MIC of pTetON-6 uninduced (row A/B) pTetON-6 induced (row C/D) pTetON-10 uninduced (row E/F) and pTetON-10 induced (G/H) where the pTetON-10 drug-treated wells started at a lower concentration of erythromycin. Drug concentration are listed in black at the top for rows A through F and white for rows G and H, all concentrations in $\mu\text{g/ml}$.

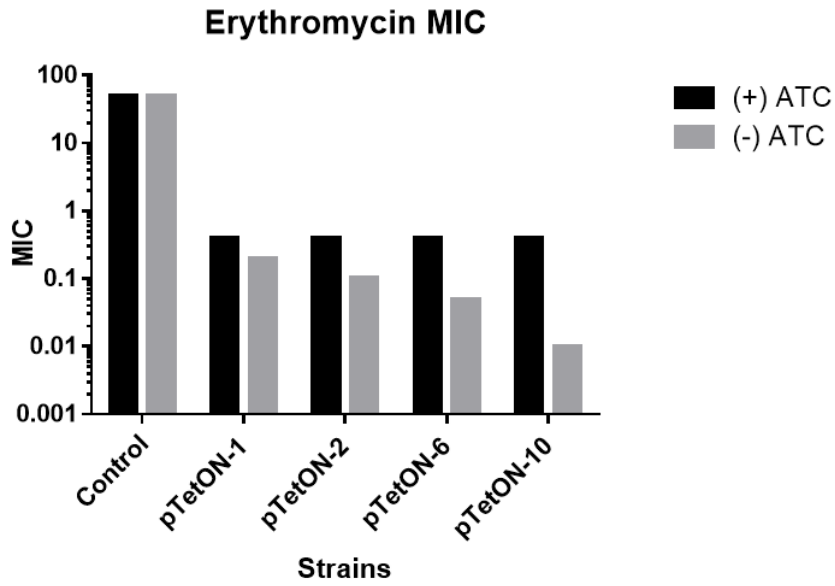


Figure 13. A dose-dependent response to erythromycin. Treatment of Pth depletion strains with erythromycin shows a dose-dependent correlation between sensitivity and Pth depletion levels

Chapter IV

Discussion

The aim of this study was to discover if Pth, the protein encoded by *pth* (Rv1014c) could be a strong candidate for target-directed drug discovery intended to combat TB. Pth is essential in *M. tuberculosis* and ubiquitous among prokaryotes. It has been shown that depletion of EcPth sensitized *E. coli* to erythromycin treatment (Menninger, 1979) and when MtPth complemented EcPth in *E. coli*, that phenotype was reproduced (Bal *et al.*, 2007). Although MtPth complements EcPth, structural studies have shown MtPth possesses subtle, yet distinct features when compared to other prokaryotic organisms, including closely related nonpathogenic *M. smegmatis* (Selvaraj, M. *et al.*, 2007 and Sharma, *et al.* 2014). Pth seems to play a role in ribosome stall recovery, as does RpsA, the target of a highly successful anti-tubercular drug, PZA (Shi *et al.* 2011). To be more specific, synthetic lethality of *pth* in an *ssrA* knock-out in *C. crescentus* indicated that *pth* may act in non-stop ribosome stall release in the absence of other recovery factors (Feaga, Viollier, & Keiler, 2014). Work by Personne and Parish (2014) indicated that ribosome recovery in *M. tuberculosis* may be more vulnerable to disruption than other bacterial species. Therefore, a drug which upsets this system may be highly effective against *M. tuberculosis*.

The literature cited above indicate that Pth plays an essential role in bacterial growth, possibly related to ribosome recovery, and that an attack on Pth could augment the

antimicrobial activity of erythromycin in *M. tuberculosis*. In this study, depletion of MtPth in *M. tuberculosis* caused drastic shifts in the MIC of erythromycin, azithromycin, and clarithromycin. These macrolide antibiotics inhibit bacterial translation by binding the 23S rRNA very near the upper part of the exit tunnel through which nascent polypeptide leaves the ribosome (Mankin, 2008). Macrolides likely interfere with ribosome assembly in a general sense, decreasing ribosome assembly and activity. However, these drugs may also cause premature peptidyl-tRNA drop-off from the ribosome by hindering exit of the polypeptide chain (Mankin, 2008). This causes the ribosome to stall before the peptide chain completes elongation to be released in a fashion that promotes normal dissociation of the ribosome.

This activity leads to an accumulation of peptidyl-tRNA in the cell (Menninger, 1979). There is some debate as to whether the accumulation of peptidyl-tRNA itself is toxic, or if the loss of uncharged tRNA and subsequent amino acid starvation is more detrimental to the cell, but there is no dispute that Pth is a hydrolase capable of breaking down peptidyl-tRNA, which improves both conditions. Menninger's work indicates that Pth can mitigate the harm done by macrolide-induced overproduction of peptidyl-tRNA. Fascinatingly, erythromycin binding causes the ribosome to overproduce peptidyl-tRNA with peptide chains about five amino acids in length (Macvanin *et al.*, 2007), while structural studies have concluded that the ideal nascent peptide length of a Pth substrate contains three to four peptide bonds, which is equal to four or five amino acids (Das & Varshney, 2006). Perhaps this coincidence helps explain the specific nature of the relationship between Pth depletion and erythromycin sensitivity.

Interestingly, certain macrolides, such as azithromycin, are more effective against non-tuberculous mycobacteria (NTM) infections than TB (Watt, Rayner, & Harris, 1996), yet depletion of Pth brings the MIC in *M. tuberculosis* down to a respectable inhibitory concentration of less than 0.24 ug/mL. The disparity between TB and NTM sensitivity to azithromycin could be related to variations in Pth expression or function between mycobacterial species, and would be an exciting area of study to pursue.

Mycobacteria at large possess one of the most well-characterized native inducible resistance mechanisms, the *erm* genes, which confer resistance to erythromycin. Methylation of the 23S rRNA by an *erm* enzyme impairs the ability of macrolides to bind (Nash, Brown-Elliott, & Wallace, 2009), yet activity of this system among non-tuberculous mycobacteria can cause discrepancies between *in vitro* and *in vivo* drug susceptibility testing (van Ingen *et al.*, 2012). Future studies might aim to discover if the macrolide sensitivity caused by Pth depletion occurs *in vivo* to the same degree as it does *in vitro* for *M. tuberculosis*.

Another future direction of study may be to repeat the MIC experiments with ketolides, a group of antibiotics which are synthetically derived from macrolides. Telithromycin and celithromycin are two such chemicals which have been synthesized in recent decades. These antibiotics have a greater binding affinity than the older macrolides and, more importantly, possess the ability to bind to an alternate site, domain II of the 23S rRNA, which preserves their antimicrobial effects to some degree in the face of macrolide resistance (Zuckerman, Qamar, & Bono, 2009). Although Pth hydrolyzes peptidyl-tRNA *in vitro* in the absence of the ribosome, it is not known whether Pth can interact with the

ribosome. If Pth interferes with erythromycin by competitively out-binding it, one might observe a different phenotype when treating Pth hypomorph strains with ketolide drugs.

It has also been shown that telithromycin allows for the production of longer peptidyl-tRNA's before forcing peptidyl-tRNA drop-off. Compared to erythromycin's enrichment of peptidyl-tRNAs with very short amino acid chains, telithromycin-treated ribosomes produce peptidyl tRNA's which are longer: 9 or 10 amino acids in length (Tenson, Lovmar & Ehrenberg, 2003). Experiments with telithromycin may indicate whether the amino acid length of the peptidyl-tRNA affects the macrolide sensitivity observed in this study. Further studies would be necessary to determine whether peptide length or the presence of an alternate binding site caused a change in phenotype.

There are many exciting avenues of pursuit in the area of research, now that the work described in this thesis has definitively shown that depletion of Pth causes arrest of growth in pathogenic *M. tuberculosis*, and that any depletion of this vital protein sensitizes the pathogen to macrolides, a well-characterized set of antibiotics. This work indicates that a drug which abrogates Pth function would not only discourage growth of *M. tuberculosis*, but could enhance the activity of an entire group of approved antibiotics which are not currently in use as first-line TB drugs.

Macrolide drugs have been in use since the mid-1900's and are well tolerated in long-term treatments. This class of antibiotics accounts for 11% of the total world antibiotic production and use (Madigan, Martinko & Parker, 2003), being fairly common; they are also relatively inexpensive to produce, making them well suited in the treatment of a global TB patient population. A drug targeting Pth which could also increase the effectiveness of macrolide antibiotics in human patients could drastically change the current TB treatment

regimen, and also challenge the current approach to how these regimens are assembled. Systematic studies of favorable drug interactions based on biological data confirmed in *in vitro*, rather than *in vivo*, could lead to faster discovery of more effective drug combinations. This new approach could save time and money, in addition to expanding the availability and uses of known antibiotics. For all of these reasons, Pth could be a highly valuable nominee for target-based drug discovery research against TB and other pathogenic bacteria.

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