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Validating Drug Targets through Inhibition of Protein-Protein Interactions in Mycobacterium Tuberculosis

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VALIDATING DRUG TARGETS THROUGH INHIBITION OF PROTEIN-PROTEIN INTERACTIONS IN *MYCOBACTERIUM TUBERCULOSIS*

by

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Abstract

Tuberculosis is the leading cause of death by single infectious disease worldwide; novel antibiotics are needed to continue to treat this disease. To goal of this project is to provide proof-of-principle support for the idea that targeting protein-protein interactions (PPI) is an appropriate course for the discovery of new drugs. This study optimized the M-PFC assay, which allows detection of PPI in Mycobacteria, through the use of stronger promoters and inducible expression of a peptide blocker by riboswitch. To accomplish this, promoter induction studies were used to find stronger promoters for the M-PFC, optimization of the riboswitch as a method for inducible protein expression within this system, and the addition of both elements to the existing version of the M-PFC. This M-PFC targets DosR homodimerization; this process is known to be essential for survival within the host. This study optimizes a system that may be used to screen for drugs that are capable of interrupting this interaction.

Acknowledgements

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Table of Contents

List of Figures

List of Tables

Abbreviations

BCG: Bacille Calmette-Guerin, *M. bovis* attenuated strain

DHFR: Dihydrofolate Reductase

Hyg: Hygromycin

Kan: Kanamycin

M-PFC: Mycobacterial Protein Fragment Complementation

Msm: *Mycobacterium Smegmatis*

Mtb: *Mycobacterium tuberculosis*

TRIM: Trimethoprim

Introduction

Tuberculosis (TB) disease is the leading cause of death caused by a single infectious agent worldwide. The organism responsible for this disease, *Mycobacterium tuberculosis,* is estimated to infect one third of the world's population, through either latent or active infection [1]. TB disease is responsible for approximately 2 million deaths annually, and 10 million new infections occur each year [2]. Tuberculosis is a disease that primarily affects the lungs and respiratory tissues; however, other organ systems may be affected; these organs include the liver, spleen, and bones, as well as neurological tissues and urogenital tissues. TB is difficult to treat due primarily to poor accessibility to drugs inside of granulomas, the persistence of dormant bacteria, and inducible drug resistance mechanisms including efflux pumps [1]. Treatment commonly requires a cocktail of isoniazid, rifampin, ethambutol, and pyrazinamide over the course of 6 to 9 months [1].

TB is becoming increasingly difficult to treat; drug resistant *Mtb*, multi-drug resistant *Mtb* (MDR-TB) even extremely multi-drug resistant *Mtb* (XDR-TB), are an increasing threat globally [3]. MDR-TB is defined as a TB infection that is resistant to both isoniazid and rifampicin, and XDR-TB is resistant to isoniazid, rifampicin, and fluoroquinolone [3]. In 2012, the World Health Organization estimated 450,000 cases of MDR-TB worldwide, with an average of 9.6% of MDR cases being XDR-TB [3]. MDR-TB and XDR-TB are emerging due to inadequate management of cases; patient non-compliance is often an issue due to the length of treatment and side effects of available drugs [4]. Improper management of HIV/TB co-infection is also a possible complication to proper

treatment [4]. Because TB is difficult to treat and becoming increasingly drug-resistant, novel therapeutic agents are needed to treat this disease. Before the antibiotic era, TB disease carried a mortality rate of 50% [3]. With drugs available today, the mortality rate is 17.3% [1]. The development of new drugs is important, then, to ensure we are able to continue to treat TB disease.

Current antibiotics target several cellular processes. Isoniazid targets the synthesis of lipids that are essential components of the cell wall [5]. Rifampicin targets core subunits of RNA polymerase [6]. Fluoroquinolones target topoisomerases and gyrases, inhibiting DNA replication in the pathogen [7]. As resistance to these drugs becomes more prevalent, new targets must be sought.

Commonly used drug discovery methods mostly fall into two categories. Phenotypic screens use a non-targeted approach; drugs are introduced to cells in media, and loss of viability is used to study effectiveness of drugs. This method is limited because conditions in culture are unlike conditions experienced within the host. The other approach is target based screening, in which drugs are screened in a biochemical assay for their ability to disrupt the function of essential targets in the cell. This approach is limited, because the target must first be identified. Additionally, because these screens are often conducted *in silico* or on purified target proteins, membrane permeability of drugs is also a concern. To limit these concerns, new screening methods have been developed. One such screening tool was developed by Russell *et al*. using a cell-based phenotypic approach in which cells were subject to high-throughput screening while exposed to the conditions of a macrophage phagosome [8]. Such screens mitigate limitations of traditional phenotypic screening,

which occurs in culture under conditions that are not necessarily similar to conditions during infection.

The approach to drug screening used in this study is a hybrid approach to drug screening. The Mycobacterial Protein Fragment Complementation (M-PFC) assay is a cell-based screening tool that uses a target-based approach. In this system, protein interactions may be selectively targeted. The assay is conducted in cells, avoiding limitations of a traditional target based approach that is conducted outside of a cell. For these reasons, the use of the M-PFC is a novel method to study and target important protein-protein interactions in *Mycobacterium tuberculosis.*

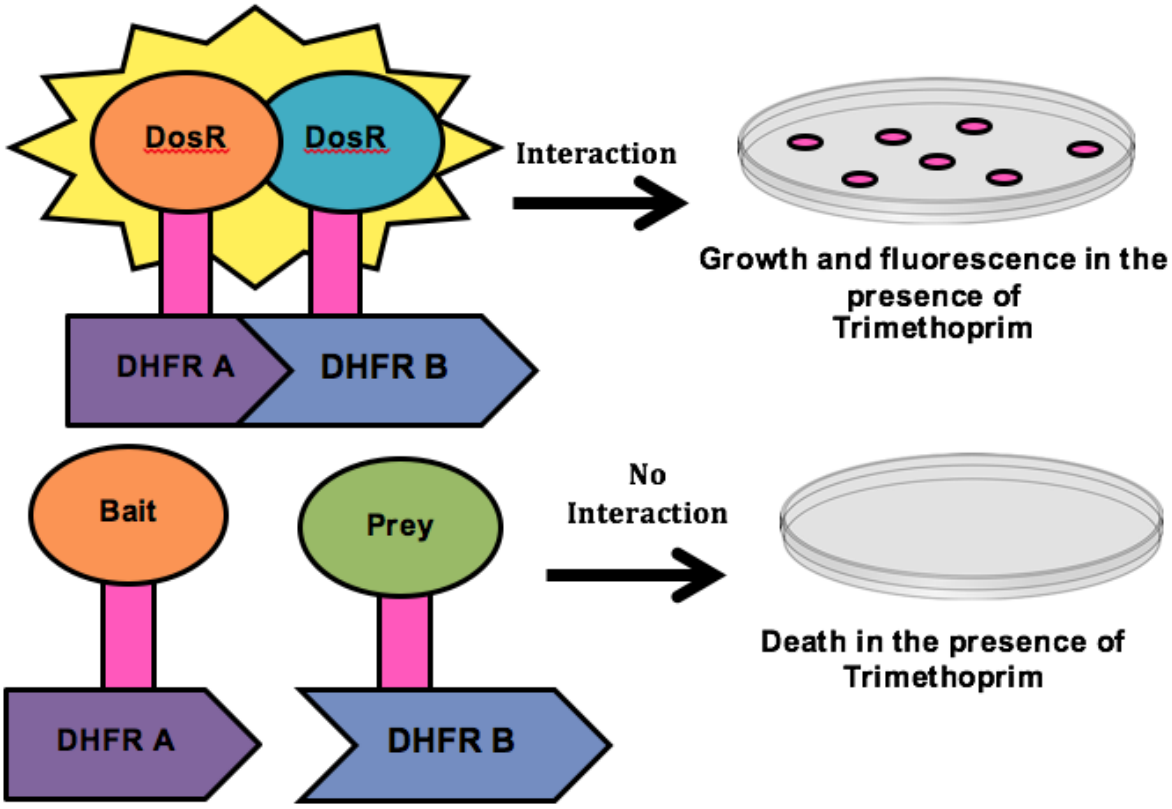
Background

Protein-Protein Interactions

Inhibition of protein-protein interaction is a promising area for discovery of new drugs. This approach has been validated in other studies, and allows for high-throughput screening of a selective target [9]. Another possibility for targeting essential protein-protein interactions is the use of proteomimetics to create small molecules that mimic the structure of the interaction interface, allowing competitive inhibition of the interaction [10]. A screen for proteomimetic inhibitors of p53/*h*DM2 and other PPIs involved in cancer pathogenesis revealed that proteomimetic compounds could inhibit these interactions and show promise for use as anticancer agents [10].

By blocking such interactions in pathogenic organisms, bacteria can be inhibited through a targeted and specific pathway. The M-PFC (*Mycobacterial* protein fragment complementation) assay is commonly used to study protein-protein interactions (PPI) in *Mtb* and other mycobacteria. By preventing the interaction of proteins such as essential regulators, a drug could be discovered to selectively inhibit *Mtb* specifically, resulting in decreased pathogenesis and death of *Mtb*. This project focuses on specific *Mtb* protein-protein interactions thought to be important for virulence and viability. One such regulator, DosR, is part of a two-component system, which regulates an essential dormancy regulon of approximately 50 genes. It forms a homodimer that is essential for *Mtb* survival under hypoxic conditions, which occur during infection within the host. A drug capable of blocking this interaction would result in decreased viability.

Figure 1: M-PFC Mechanism



The M-PFC system was developed by Steyn *et al*. to study and quantify protein-protein interactions in vivo in *Mycobacteria* [11]. This system utilizes *Mycobacterium smegmatis* as a model organism; this is ideal for drug screening, because it is closely related to *Mtb* but is considered to be safe and non-pathogenic; *M. smegmatis* can be grown outside of a BSL-3 facility and additionally grows faster than *Mtb.* The mycobacterial cell wall can present difficulty for the antibiotic permeability, and *M. smegmatis* is similar in structure [11]. For these reasons, *M. smegmatis* is an ideal model organism. In the M-PFC system, proteins of interest are linked to domains of a split dihydrofolate reductase (DHFR) protein. When the two proteins interact, the domains of DHFR are brought into close enough proximity to interact, recreating the active site of the protein and enabling survival

on trimethoprim (TRIM) proportional to the strength and quantity of the protein interaction. Figure 1 illustrates the mechanism through which proteins interact, bringing DHFR domains into contact. The system is similar to the Yeast Two Hybrid assay; however, it has been adapted for use in mycobacteria. Post-translational modifications of bacterial proteins are not always appropriate in yeast, and the high GC content of *Mycobacteria* DNA is not always well tolerated in yeast [11]. Although the assay is traditionally used to quantify the strength of an interaction between two proteins, it may be used to study a disruption of normal interactions between two proteins [6]. Once protein interactions are quantified with the M-PFC, a disruption to the interaction can be identified using the M-PFC as a tool for high throughput screening. This assay allows a hybrid approach to drug screening, in which an interaction may be targeted within a whole cell screen [12]. The M-PFC system uses two plasmids, pUAB100 and pUAB200 [11, 12]. Each plasmid expresses one protein of interest linked to one half of a split DHFR. pUAB100 is an episomal plasmid, while pUAB200 lacks OriM, the mycobacterial origin of replication, and is incorporated into the host chromosome [11].

The M-PFC system is limited as a drug-screening tool in certain areas. Certain analogs of sulfa drugs act on tetrahydrofolic acid synthesis and interfere with the DHFR component of the assay; these drugs cannot reliably be screened using the M-PFC system [12]. Another possible limitation arises from solubility of drugs. To avoid solubility issues, drugs may be used in 10% DMSO in the M-PFC assay[12]. Another limitation of this method is that the interaction interface must be small enough to be disrupted by a small molecule; the molecule must be able to enter the cell and block the interaction [12]. The interaction

must also not be too robust to be effectively blocked *in vivo*. Another important caveat of this method is the need to identify and verify an essential PPI in advance; the use of a riboswitch inducible blocker to address this requirement is introduced in this study.

Protein Interaction Target: DosR Homodimerization

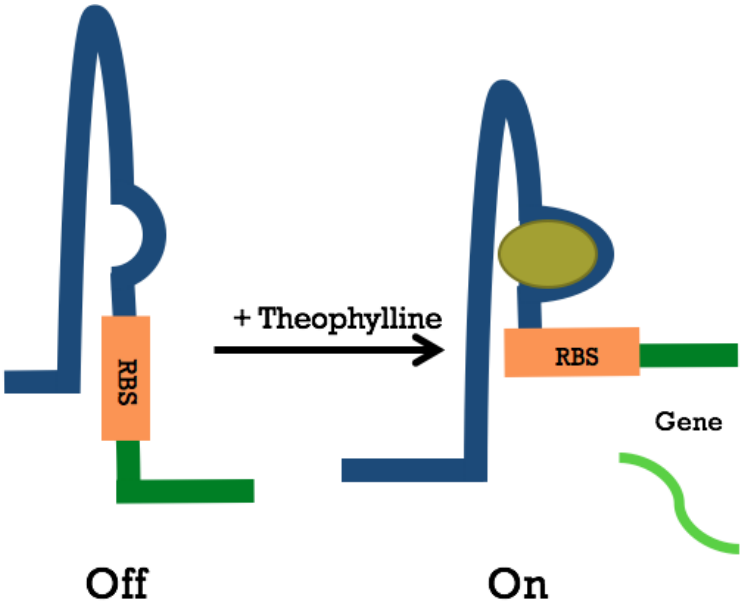
DosR, also known as DevR, is involved in the hypoxia response of *Mycobacterium tuberculosis*. DosR expression is induced in response to low oxygen tension, nitric oxide, carbon monoxide, and citric acid [13]. These conditions are experienced during *Mtb* infection; bacteria react to hypoxic conditions they encounter within the phagolysosomes macrophages and within granulomas [13]. It is part of the atypical tripartite DosRST two component system. DosS and DosT are histidine kinases that respond to hypoxic conditions by phosphorylating DosR, leading to dimerization. Under such conditions, it forms a homodimer that is involved in the regulation of 48 genes thought to contribute to *Mtb*'s ability to survive under hypoxic conditions encountered during infection. DosR was chosen as a target for this project because it is essential under hypoxic conditions and its role is well documented in literature [14-16]. Preventing DosR-DosR homodimerization would decrease expression of genes that allow *Mtb* to survive in low oxygen environments, decreasing cell viability in response to *in vivo* stresses [16]. In DosR knockouts, DosR has been seen to be essential for survival under hypoxic conditions [14, 15]. In macaque models, DosR knockout mutants showed significant attenuation after initial infection; under hypoxic conditions, attenuation allowed for the elimination of *Mtb* [16]. For this

reason, an agent capable of selectively disrupting this interaction would be effective for treating an active or dormant *Mtb* infection [15].

Riboswitch

The riboswitch is optimized in this study as an addition to the M-PFC assay. It is a genetic, mRNA construct that allows for inducible expression of a protein. Adding the riboswitch to the M-PFC system allows for the expression of proteins/peptides that will act as competitive inhibitors to disrupt protein interactions in the M-PFC assay. This system can be used to validate competitive inhibitors; once validated, the system can be used to truncate proteins and determine the dimerization domain. Then, riboswitch:inhibitor constructs may be introduced into *M. bovis* BCG or *Mtb* and expressed to study effects of blocking interactions in culture.

Figure 2: Simplified Illustration of Riboswitch Mechanism



The riboswitch system of controlling protein induction at the translational level is a new addition to the M-PFC system. A riboswitch is a sequence at the start of an mRNA that forms a hairpin structure. Figure 2 shows a simplified diagram of the riboswitch mechanism used in this study. Depending on the system, the addition of an inducer will bind to the aptamer region of the RNA hairpin altering its structure to either expose or obstruct the ribosome-binding site (RBS). A synthetic riboswitch for use in *Mycobacteria* has been identified and developed by Seeliger *et al.* [8]. In this system, a riboswitch forms a secondary structure that obstructs the RBS. Theophylline, the inducer, binds the aptamer, leading to a conformational change that exposes the RBS, allowing translation to occur. The level of protein induction is proportional to the concentration of the inducer. For this reason, the system can be used to control the level of protein induction within a system [8]. A DosR-DosR M-PFC expressing a riboswitch-truncated DosR construct will be created in *Mycobacterium smegmatis* to observe the competitive inhibition of the interaction. After this approach is validated, in future work, the riboswitch-controlled DosR fragment will be expressed in *Mtb* surrogate *M. bovis* (BCG) to demonstrate proof of principle for this as a new potential drug target through decreased expression of downstream genes by qRT-PCR or by impaired survival under hypoxia.

A high throughput drug screen using the M-PFC and DosR as a target was conducted as a collaboration between Dr. Rohde and Sanford Burnham Prebys Medical Discovery Institute through the Florida Translational Research Program. This screen utilized the *hsp60* version of the assay rather than the updated assay in this study. In this study, 433,014 compounds were screened for their ability to inhibit DosR:DosR

homodimerization in the DosR:DosR M-PFC strain. Of these compounds, 1,072 revealed activity of greater than 30%. Through counter screening, 206 compounds were found to target DosR specifically rather than other essential cellular processes. This was indicated through survival in the absence of TRIM. 83 of these compounds were selected for their structure as promising targets, and future testing will be conducted on these compounds. This area of drug discovery is promising because it allows for the screening and identification of novel compounds; in the future, more M-PFC strains will be created. Optimization will improve our ability to screen compounds in this way, and will hopefully lead to the discovery of novel therapeutic agents for the elimination of *Mtb.*

Objectives

The proposed objectives for this project are:

1. To optimize the M-PFC system for greater protein induction through the use of stronger promoters, increasing signal to background detection.
2. To demonstrate riboswitch inducible competitive inhibition of protein targets by development and optimization of an M-PFC DosR screening strain with the addition of a riboswitch-inducible protein.

Methods

Fastcloning

M-PFC strains and other constructs were created using Fastcloning [17]. Fastcloning is a method that allows cloning without the use of restriction enzymes or ligases; this method uses overhanging primers to insert a gene of interest at any site in a plasmid. Primers with overhanging nucleotides that are reverse complements of the ends of the plasmid into which the insert gene will be placed were used to create insert DNA fragments through polymerase chain reaction (PCR). PCR reactions used 0.25 μL (1 unit) Phusion polymerase, 0.2 mM dNTPs, 0.6 μg/μL template DNA, 0.2 μg/μL forward primer, 0.2 μg/μL reverse primer, 5 μL 1x GC buffer, and 16.5 μL deionized water. PCR conditions are as follows:

Table 1: PCR Reaction Cycle Procedure

Cycle	Time (s)	Temperature (°C)	Repeats
Initial Melting	30	98	1x
Melting	10	98	18x
Annealing	30	65	
Extension	15/kilobase of DNA to be replicated	72	
Final Extension	180	72	1x

Amplification of the vector was conducted with divergent primer pairs that define the site of insertion. Vector and insert DNA were combined and subjected to Dpn1 digestion for 1 hour, to digest methylated DNA including unamplified original plasmid DNA. Then, 2 μ t of this mixture was introduced to chemically competent *E. coli* and heat shocked

for 30 seconds before 200 μn of SOC media was added. Cells were then shaken at 37° C for 1 hour before plating on appropriate antibiotic. To allow for selection for colonies containing the plasmid of interest, plasmids contain antibiotic selection makers. For plasmids used in this study, pST5552, pVVRG, and pUAB200 base plasmids contain a region that confers kanamycin resistance, and pUAB100 base plasmid contains hygromycin resistance. Strains were plated on appropriate antibiotics after transformation, and PCR was used to confirm the presence of the gene of interest. pUAB200, pST5552, and pVVRG in *E. coli* were plated on LB agar with 50μg/ml kanamycin (K50), and pUAB100 in *E. coli* was plated on LB agar with 250μg/ml hygromycin (LB H250). Colony PCR was performed using primers flanking the region of insertion. Cells are picked from each colony with a sterile toothpick and boiled for 5 minutes in 20 μL of sterile, deionized water. The lysate is used as a PCR template, and PCR band size is used to confirm the presence of an appropriately sized band. Once confirmed with PCR, plasmid DNA was purified and sent for sequencing by Eurofins.

Promoter Induction Assay

Stronger promoters were sought to improve expression of protein partners to enhance the signal to background ratios in the M-PFC assay. *Hsp60* is a commonly used promoter in mycobacteria, and is the promoter that drives expression of protein partners in the M-PFC as well as in the pST5552 plasmid. To find new promoters, microarray data obtained by Dr. Rohde was analyzed to find promoters of consistently high activity. Promoters of Rv1197, Rv1038, and *cspA* were chosen as strong promoters with

constitutive expression levels and were cloned in front of GFP in the PVVRG plasmid (Figure 3). The activity of these promoters was compared to the activity the *smyc* promoter based on levels of GFP fluorescence. For this assay, the PVVRG plasmids containing promoter:GFP constructs were electroporated into *M. smegmatis* competent cells. Transformed cells were plated on LB agar with 25 μg/ml kanamycin (K25), then selected colonies were grown up in 5 mL of 7H9 media with kanamycin for selection. Cells of OD 0.2 were incubated in a 96 well plate, 100 μL total volume. GFP and mCherry induction were measured by fluorescence in a Synergy H4 plate reader.

Riboswitch

Riboswitch plasmid pST5552 was obtained from Seeliger *et al* [18]. The plasmid contains a riboswitch-inducible GFP driven by the *hsp60* promoter. See Appendix A, Figure S1 for features of the riboswitch plasmid construct. This plasmid was introduced into *Mycobacterium smegmatis* (*Msm*) through electroporation. 200-500 μg of DNA was added to 100 μL of *Msm* competent cells, which were electroporated, added to 250 μL of LB Tween 0.05%, and shaken for 4 hours before being plated on LB Kanamycin 25 μg/mL. A 6 hour assay was conducted where 100 μL total volume with cells of OD 0.1 and 0.2 were incubated for 6 hours with theophylline concentrations of 0, 0.25, 0.5, 1, 2, 4, 6, and 8 mM. To achieve these concentrations, a 10 mM theophylline stock was diluted; this stock was made using sonication to increase solubility of theophylline. The plate was read at an excitation of 485 nm and emission at 528 nm for GFP fluorescence after 6 hours.

To understand differences between maximum expression under the riboswitch system and under standard expression under the control of a promoter alone, an assay was conducted to compare GFP expression under each system. *Msm* strains containing PVVRG and pST5552 were compared for GFP induction. PVVRG contains *hsp60*::GFP, and pST5552 contains *hsp60*::riboswitch:GFP, allowing for comparison of the system with and without the riboswitch. This assay was conducted in a 96-well black bottom plate with 100 μL total volume with cells of OD 0.1. The cells were incubated for 6 hours with theophylline concentrations of 0, 0.25, 0.5, 1, 2, 4, 6, and 8 mM theophylline and 7H9 media. After 6 hours, the plate was read at an excitation of 485 nm and emission at 528 nm.

Optimizing Riboswitch for M-PFC

To optimize this assay for the M-PFC assay, a 48-hour assay, we sought to determine optimal lower concentrations of theophylline. *Msm* containing either pST5552 (original plasmid containing *hsp60*::Riboswitch:GFP), pST5552smyc (*smyc*::Riboswitch:GFP), and PVVRG (a control containing *hsp60*::GFP) were added to each well of a 96-well plate. The plate was read at an excitation of 485 nm and emission at 528 nm for GFP fluorescence after 6 hours. This experiment was adapted from a study by Seeliger et al, and a similar study was conducted to optimize theophylline levels for the 48 hour M-PFC assay. Concentrations of 2, 1, 0.5, 0.25, 0.125, and 0 mM of theophylline were created in each well using a serial dilution from a stock of 10 mM theophylline. The plate was read at an excitation of 485 nm and an emission of 528 nm for GFP fluorescence after 48 hours. After

optimization of theophylline levels, fastcloning was used to replace GFP with DosR, so that the riboswitch-controlled DosR construct could be added to M-PFC strains.

Construction of M-PFC Strains

The next goal was to construct an M-PFC strain in which a riboswitch-DosR construct was added to pUAB100. This pUAB100 features both the DosR-DHFR construct and the riboswitch-DosR construct, which is expected to competitively inhibit the interaction between the two DosR-DHFR halves. Positive clones are confirmed through PCR screening and through sequencing. Using fast cloning, DosR was added to the riboswitch plasmid pST5552 downstream of the riboswitch. Positive clones were selected for using Kanamycin resistance of the plasmid in *E. coli*. Positive clones are confirmed through PCR screening and through sequencing. The riboswitch-DosR construct was then added to a region of the pUAB100 plasmid distinct from the DosR-DHFR cassette. Both this plasmid and pUAB200 containing the DosR-DHFR construct were introduced into *Msm* through electroporation, and colonies containing both plasmids were selected for with LB K25 H50 agar plates. See Appendix A Figure S3 for illustration of M-PFC plasmid constructs.

M-PFC Alamar Blue Assay

Three cultures are used for the M-PFC assay. The positive control strain contains a pair of M-PFC plasmids both expressing GCN4, a zinc finger domain known to undergo strong homodimeric interactions, fused to DHFR domains. The negative control contains pUAB100 only, which should show no signs of interaction because only one half of the

DHFR protein is present. The test strain contains both M-PFC Plasmids pUAB100 and pUAB200, each containing DosR linked via glycine linker to DHFR-A or DHFR-B, respectively. 135 μ L of culture at an OD 0.0005 was added to each well of a sterile black bottom 96-well plate. 15 μL of TRIM is added to each well in decreasing concentration; trimethoprim concentrations are prepared by serial dilution in 10% DMSO to create a gradient from 200 μg/mL to 0 μg/mL for each strain. Wells that receive no trimethoprim are given 15 μL of 10% DMSO instead. The plate is incubated at 37° C for 48 hours.

After 48 hours, an Alamar blue assay is used to measure cell viability. If proteins interact in the M-PFC assay, DHFR halves will be brought together, and TRIM resistance will result. Therefore, cells with stronger protein interactions will survive higher concentrations of TRIM, and cell viability can be used to measure strength and presence of protein interactions. To measure cell survival, 15 μL of 10x Resazurin is added to each well. Resazurin is a dye used to measure cell viability; in the presence of live cells, blue resazurin dye is reduced to pink resorufin. After addition of resazurin, the plate is incubated for 4 hours at 37°C, then read on a Synergy 4 plate reader using monochromators with an excitation of 530 nm and an emission of 590 nm.

Conducting M-PFC of Test Strains

M-PFC strains containing DosR were previously constructed. In this project, these strains will be optimized and updated. The first update was the addition of a "riboswitchable" blocker DosR to the pUAB100 plasmid containing the DosR-DHFR fusion. This updated pUAB100DosRRiboDosR was added with pUAB200DosR to *M. smegmatis* by

electroporation. M-PFC Alamar blue assay was conducted with this strain. Once M-PFC strains are assembled, the M-PFC and Alamar blue assay will be completed. Theophylline levels were optimized for the assay using results from GFP riboswitch induction studies described above. For early versions of this assay, theophylline levels were optimized at 4 mM based on data from the 6 hour GFP inductance assay.

Improvement of M-PFC Strains

Fastcloning was used to replace the *hsp60* promoter that drove expression of DosR-DHFR fusions with the *smyc* promoter in pUAB100 and pUAB200. The *smyc* promoter was chosen as a result of the Promoter Induction Assay described above; it is a promoter taken from *Mtb* that shows constitutive high expression in microarray data. The *smyc* promoter was amplified from *Mtb* DNA, and added to pUAB100DosR and pUAB200 DosR in place of the *hsp60* promoter that controlled DosR expression. Both plasmids were introduced into *M. smegmatis* through electroporation. M-PFC Alamar blue assays were conducted using *smyc*:DosR M-PFC strains.

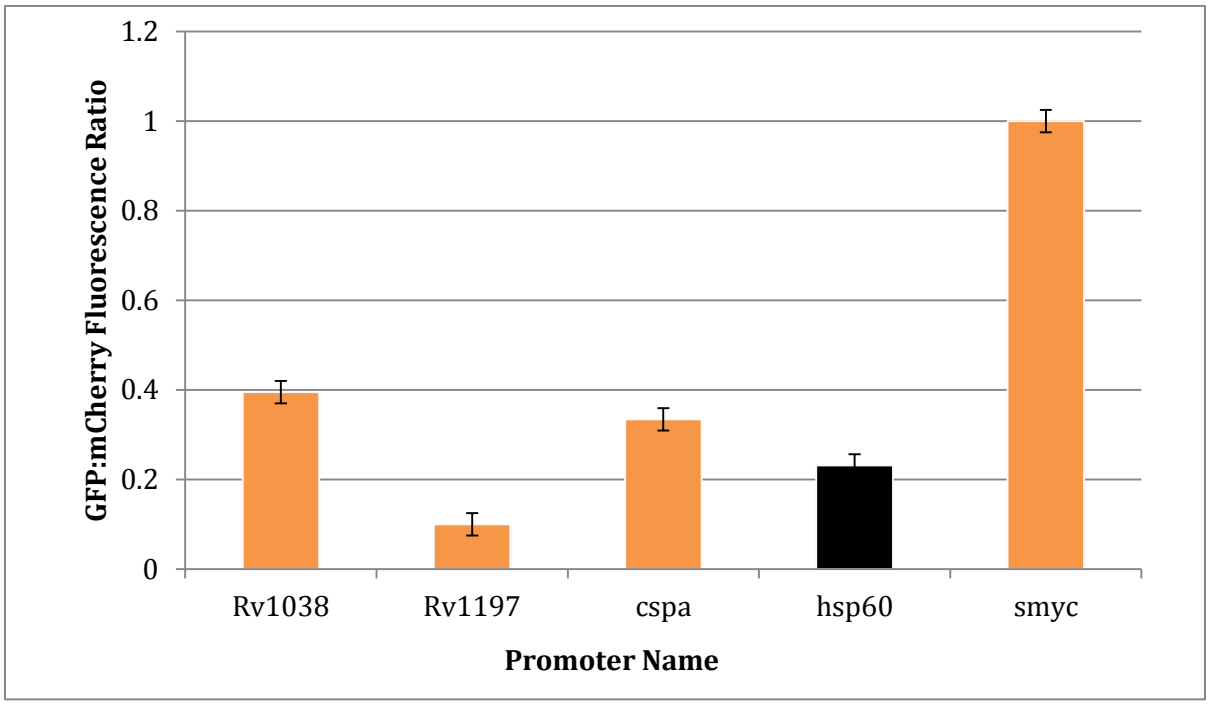
Finally, an M-PFC strain was constructed in which *smyc* was used to replace the *hsp60* promoter that drove expression of the riboswitch DosR construct, which was added to pUAB100 as a riboswitch inducible competitive inhibitor. Both plasmids were introduced into *M. smegamtis* through electroporation. M-PFC Alamar blue assays were conducted using *smyc*:DosR M-PFC strains.

Results

Promoter Induction and Reporter Strain Assays

The *hsp60* promoter originally controlled expression of DosR – DHFR fusions on M-PFC strains and RiboDosR. Because stronger promoters could enhance detection of weak interactions, new promoters were sought. Promoters were identified from microarray data of *Mtb* promoters. GFP reporter assay was used to assess the relative activity of promoters. Reporter strains were created for this assay using fastcloning in the PVVRG plasmid. The *hsp60* controls GFP expression in this plasmid, and promoters of Rv1197, Rv1038, and *cspA* were chosen and induction was compared to strong promoters identified from microarray data or literature (*smyc*). GFP signal was normalized to fluorescence of constitutive mCherry. Results are seen below in Figure 3. All promoters exhibited activity, and 3 of 4 exhibited greater activity than *hsp60*. The *smyc* promoter was chosen for improvement to the assay because *smyc* yielded the greatest GFP fluorescence in the assay.

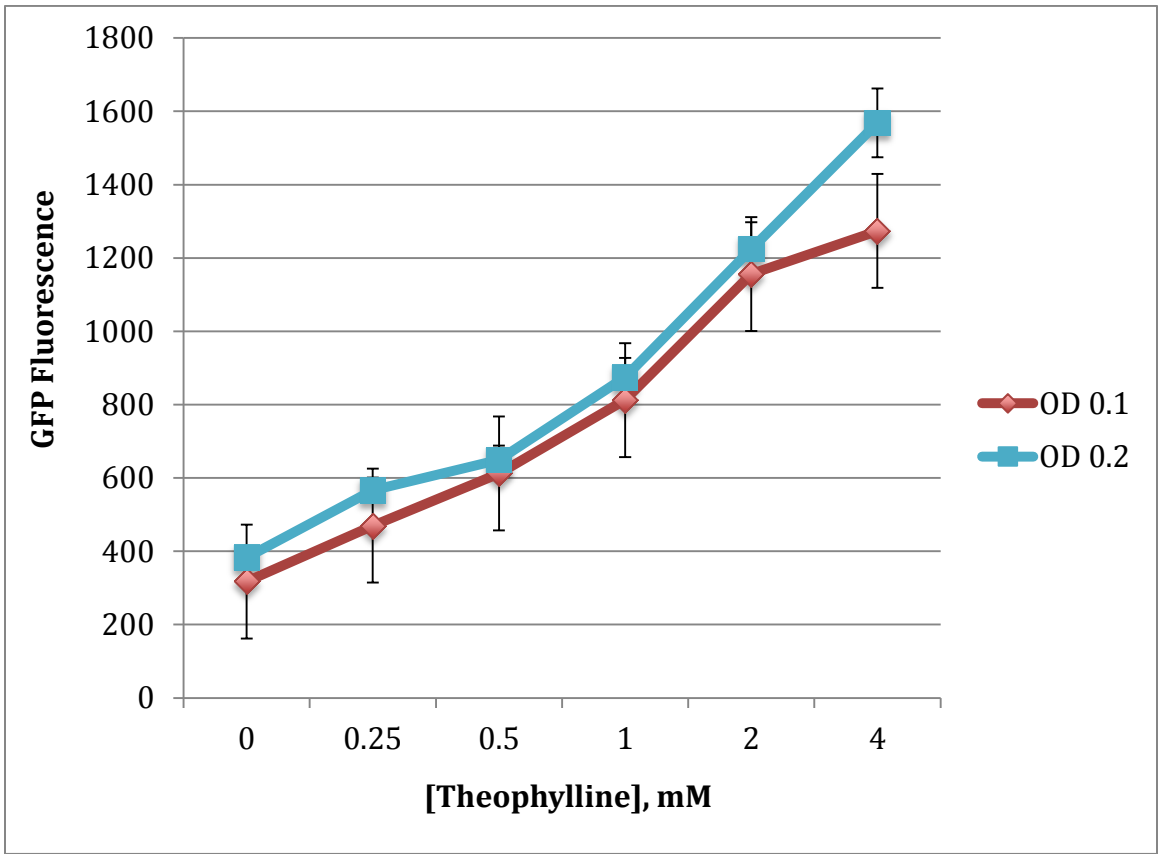
Figure 3: GFP Expression By Promoter Reporter Strain



Riboswitch Optimization

An *Msm* strain containing an *hsp60::*riboswitch:GFP construct expressed from a plasmid was created. A 6-hour assay was conducted where 100 µL of cells at either OD 0.1 or 0.2 were incubated for 6 hours with different theophylline concentrations. This assay replicates an assay performed by Seeliger *et al*. [18]. An initial study of protein expression under riboswitch control, this assay was updated to study theophylline concentrations for induction over 48 hours, as required in the M-PFC alamar blue assay. See Figure 4 for the results of this assay. Between a starting OD of 0.1 and 0.2, a significant difference in theophylline dose-dependent induction was not observed. This study provided initial information about the dose-dependent range of induction through the use of the riboswitch expression system.

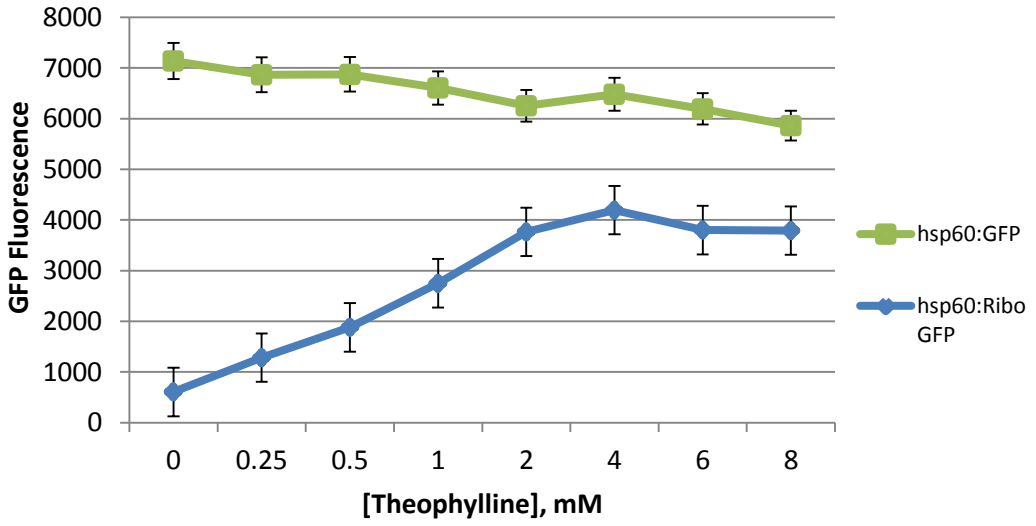
Figure 4: Theophylline Induction of GFP by Riboswitch



An assay was conducted in which GFP induction under control of the riboswitch was compared to GFP expression under the control of a promoter alone. This assay was conducted to determine possible expression range when compared with expression from the same promoter without the riboswitch. In this assay, *Msm* containing PVVRG and pST5552 were compared. PVVRG contains *hsp60*::GFP, while pST5552 contains *hsp60*::Riboswitch:GFP. A 6 hour assay was conducted where 100 μ L total volume with cells of OD 0.1 were incubated for 6 hours with different theophylline concentrations in a 96-well black bottom plate with 7H9 growth media. Results are shown below in Figure 5. The riboswitch allows for controlled protein expression; however, protein expression is

lower under riboswitch control, and does not reach the same levels of expression as a standard promoter expression system alone. At 4 mM theophylline, GFP fluorescence under the riboswitch system is 64.8% of fluorescence under the *hsp60* promoter alone. A gradual decline is seen in fluorescence of the non-riboswitch strain which may be due to the presence of increasing levels of theophylline.

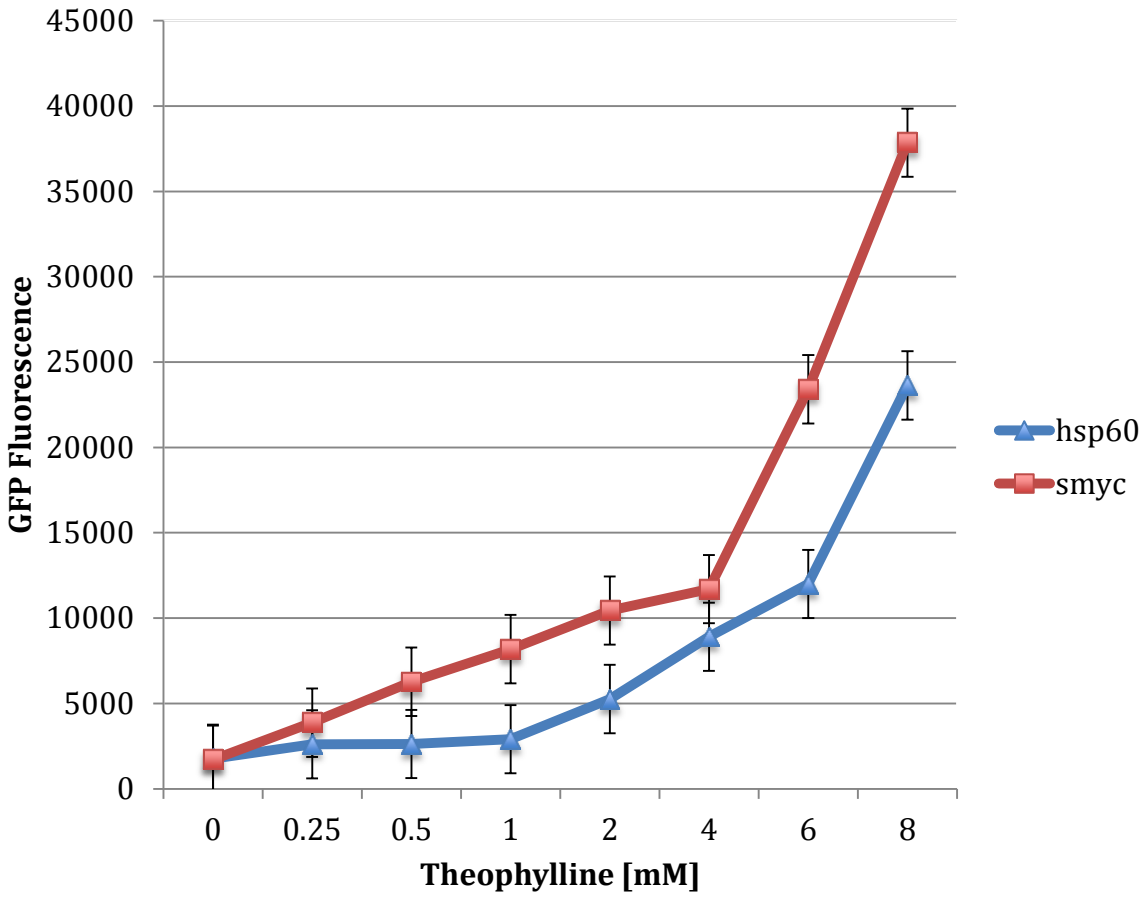
Figure 5: Riboswitch Regulated Expression of Protein



An *Msm* strain containing an *hsp60::*Riboswitch:GFP construct expressed from a plasmid was created and compared to an *Msm* strain containing *smyc::*Riboswitch:GFP. A 48 hour assay was conducted where 100 µL total volume with cells of OD 0.2 were incubated for 48 hours with different theophylline concentrations as seen in Figure 2. This assay was conducted to optimize theophylline levels for the M-PFC assay, which requires a 48-hour incubation period. From this assay and the previous assays, a concentration of 4 mM theophylline was chosen for M-PFC screening. 4 mM Theophylline reaches the highest level of expression. This assay also allows comparison between promoters *hsp60* and *smyc*

as promoters of the riboswitch. Results are shown below in Figure 6. Protein expression measured through GFP fluorescence was approximately 2 times greater at all data points with the promoter *smyc*.

Figure 6: *hs60* and *smyc* Riboswitch Induction of GFP



M-PFC and M-PFC Optimization

Results of a DosR-DosR M-PFC protein interaction study are included below in Table 2 for baseline comparison. This data was collected by Zina Versfeld, a former undergraduate researcher in the Rohde lab. This M-PFC was conducted using pUAB100 and pUAB200 with

an *hsp60::DosR-DHFR* construct. The positive control is GCN4-GCN4, a known strong interaction. The negative control includes only the pUAB100 plasmid with DosR-DHFR, so no interaction should occur because both halves of DHFR are not present. In this assay, the *hsp60* promoter controls DosR expression. When replaced with *smyc*, DosR levels should increase, increasing potential for interaction and increasing the signal to background ratio as a result.

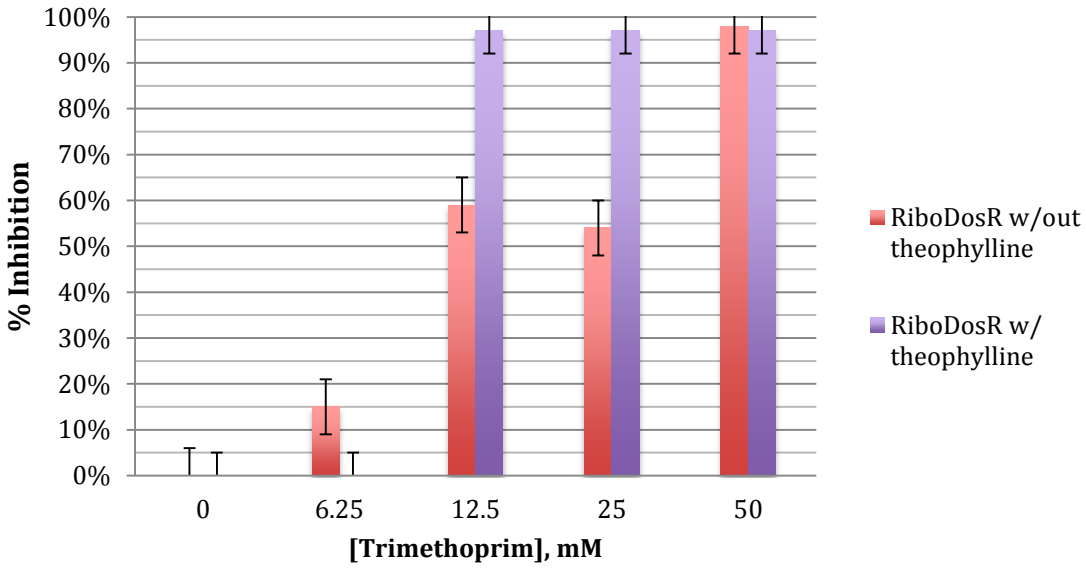
Table 2: Preliminary M-PFC Alamar Blue

[TRIM]	+ control	DosR:DosR	- control
100	100.0	6.9	5.2
50	100.0	8.7	5.7
25	100.0	10.4	5.6
12.5	100.0	18.8	5.1
6.25	97.9	41.3	6.7
3.125	94.6	100.0	22.0
0	100.0	100.0	100.0

Induction of DosR was predicted to block DHFR assembly by inhibiting DosR dimerization, thus increasing TRIM sensitivity. To test this, an M-PFC was conducted using pUAB200DosR and pUAB100DosR with the riboswitch:DosR blocker expressed on pUAB100. As predicted, we noted RiboDosR induction in the presence of theophylline led to increased cell death. This effect was most pronounced in the mid-range of trimethoprim concentration, the difference in cell viability is most significant at 12.5 mM trimethoprim and 25 mM trimethoprim. This assay was conducted to compare M-PFC strains with and without the riboswitchable DosR blocker; results from this assay are seen in Figure 7.

These results show proof of principle for the concept of the induction of a blocker peptide by riboswitch as a method of validating inhibition of protein interaction. Demonstration that expression of effective blocker peptides in *M. tuberculosis* leads to loss of viability or virulence would serve to validate the chosen PPI as a drug target. Results from this assay may also be used to optimize trimethoprim concentrations for future screens with greatest difference between strains with and without the expressed blocker. In future studies, these results may be used to optimize expression of truncated protein blockers, which is ultimately the goal of the riboswitch expression system as an addition to the M-PFC.

Figure 7: Theophylline Induction of RiboDosR Sensitizes DosR:DosR M-PFC to TRIM



An M-PFC comparing the *smyc*:DosR updated strain was compared to the original *hsp60*:DosR strain constructed by Zina Versfeld. As seen in Table 3, the *smyc* strain yields greater survival at higher levels of trimethoprim. At 3.125 mM TRIM, this difference is particularly evident. This assay supports the idea that increasing expression of protein partners yields a greater signal to background ratio in the M-PFC, producing more robust results.

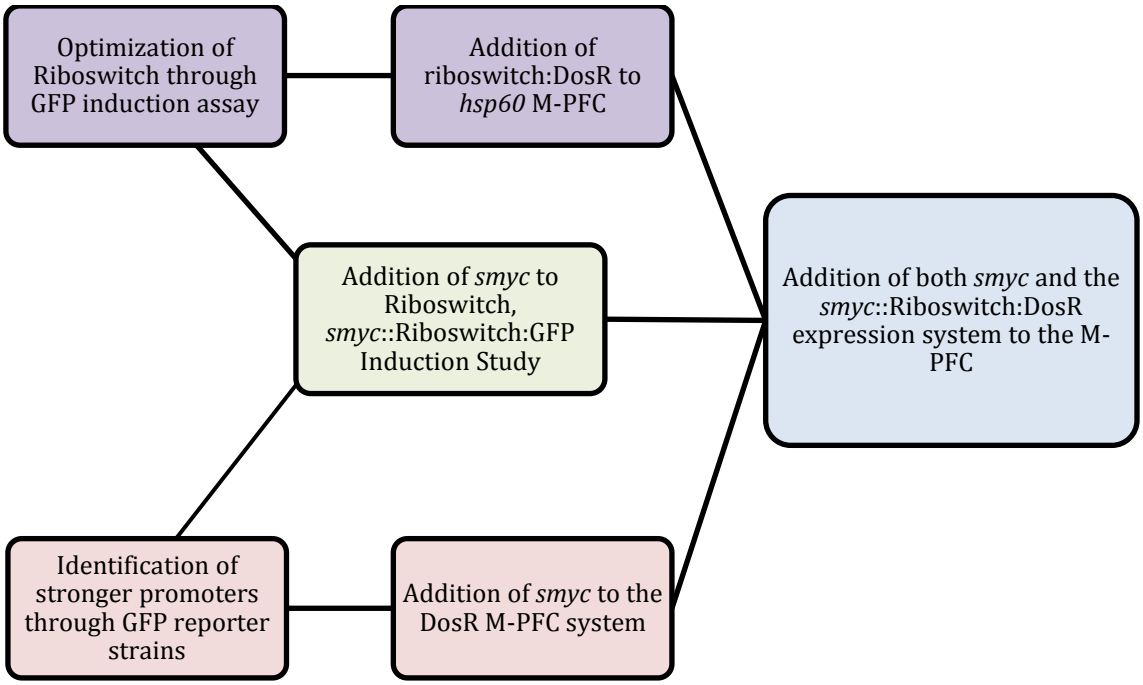
Table 3: M-PFC of *smyc*:DosR and *hsp60*:DosR

[TRIM]	+ control	*smyc*	*hsp60*	— control
100	93.0	8.5	7.8	4.9
50	100.0	8.5	7.3	5.0
25	100.0	9.5	8.4	5.1
12.5	100.0	9.3	7.8	5.1
6.25	97.9	20.1	10.0	6.7
3.125	98.8	94.1	66.8	10.1
0	100.0	100.0	100.0	100.0

Discussion

This project hopes to address the greater problems of *Mtb* infection and growing antibiotic resistance by providing a novel approach to drug screening. New therapies are needed for the treatment of tuberculosis, and the use of the M-PFC as a tool for screening inhibitors of protein-protein interactions is a promising new area for drug discovery. This project serves a dual purpose in this area; through the use of new promoters, the M-PFC system can be optimized to increase expression of protein partners, reducing signal to background ratios of the assay. GFP reporter strains were constructed to identify stronger promoters for the M-PFC system, and the strongest promoter, *smyc*, was used to replace the *hsp60* promoter in the DosR-DosR M-PFC. The other addition is the use of the riboswitch as a method of inducing protein expression at a controlled level. Through this project, three new M-PFC strains were constructed. These include the *hsp60* DosR M-PFC with the addition of the riboswitch controlled blocker, and the *smyc* DosR M-PFC with and without the riboswitch controlled blocker. The flow of this study is described in the flow chart figure below.

Figure 8: Flowchart of Study Design



From the promoter GFP expression study, data indicated that *smyc* resulted in greater protein expression over *hsp60* and other *Mtb* promoters tested. For this reason, *smyc* was chosen to replace *hsp60* as the promoter driving the M-PFC and riboswitch expression systems. By increasing induction through *smyc*, signal to background ratios are seen to be decreased. In the 48-hour assay, at 1 and 2 mM of theophylline, GFP fluorescence approximately doubles under *smyc* control over *hsp60*. This is first seen in the pST5552smycRiboGFP constructs in Figure 6; at every concentration of theophylline in the study, GFP fluorescence is greater under control of *smyc* than *hsp60*.

Initial results from the addition of the inducible blocker peptide to the M-PFC as seen in Figure 7 suggest that the blocker is capable of producing a measurable interference in the M-PFC system. This provides support for the use of the M-PFC as a drug screening

technique. In the future, this system will be used to truncate the blocker. Truncation of the protein will allow for the discovery of the minimal domain necessary to block the interaction. Identifying this minimal region of the peptide will allow for the expression of this non-functional peptide fragment *in vivo* to study viability and downstream effects. Identification of this domain also opens the possibility for drug discovery through peptidomimetic inhibition, in which a small molecule that mimics the shape of the peptide may be designed to block the interaction. Truncated proteins will be introduced into the M-PFC with the riboswitch, allowing the study of their ability to block the protein interaction of interest. Once the riboswitch-truncated-protein cascade is constructed, it may be introduced in BCG to study transcription of downstream products and survival under hypoxia. The *smyc*::DosR + *smyc*::Riboswitch:DosR M-PFC may also now be used for additional protein targets.

It is important to recognize possible limitations of this study. Solubility of theophylline was, at first, a weakness. Theophylline is a crystalline solid that is difficult to dissolve in water. Sonication of the solution for 1 hour allowed for the creation of a 10 mM stock solution. Theophylline toxicity was a concern as well in early studies; however, with optimization using a 48 hour induction assay, these conditions were mitigated through the use of a lower dose. Another possible limitation is the inability of the riboswitch to completely eliminate expression of the target protein in the absence of theophylline. In the 6-hour assay, GFP fluorescence is 318 for OD 0.1 and 382 for OD 0.2. In the 48-hour assay, fluorescence values were 1768 for *hsp60* and 1700 for *smyc*. Little difference between the two promoters was seen at this lowest level of expression, however, indicating that smyc

does not lead to greater unintended expression. This result is supported by Seeliger *et al* in their use of riboswitch expression in *Msm* [18]. Figure 3 also illustrates the upward limits of the riboswitch induction system. While it is important to note that the riboswitch does not allow for expression levels that are as high as those accomplished through traditional expression, the riboswitch system is valuable over traditional expression because protein levels can be controlled.

For the final two assays, repeats of the study have not yet been conducted. Repeating studies is optimal, however, time did not allow for a repeat assay. The assay is conducted in replicate to mediate some of these limitations. In the future, more replications will be conducted with all strains constructed as a part of this study, and future optimization will occur.

In future work, the *smyc*::DosR M-PFC strains could be used for additional high throughput screening studies. The *smyc*::DosR M-PFC strain will now be used to validate hits from the Sanford Burnham Prebys DosR screen through the Florida Translational Research Program. This screen was conducted to find inhibitors of the DosR-DosR interaction; if inhibitors are validated, they may then be tested *in vitro* against BCG or *Mtb*. These optimized plasmids will also be used to screen additional protein targets, allowing for the identification of possible therapeutic agents. Greater signal to background ratios will improve this assay by enhancing survival of bacteria where an interaction is present, allowing for greater confidence in the identification of hits.

With high throughput screens like the screen conducted through collaboration with the Sanford Burnham Medical Discovery Institute as well as other screens in the lab and

future collaborations, studies like this hope to provide the tools necessary to discover novel therapeutic agents in the interest of eradicating tuberculosis. Continued development of an optimized screening platform for PPI inhibitors and methods to identify and validate essential and validate essential PPIs could have a significant impact on efforts to discover novel treatments for TB.

Appendix A: Plasmid Maps

Figure S1: PVVRG Plasmid

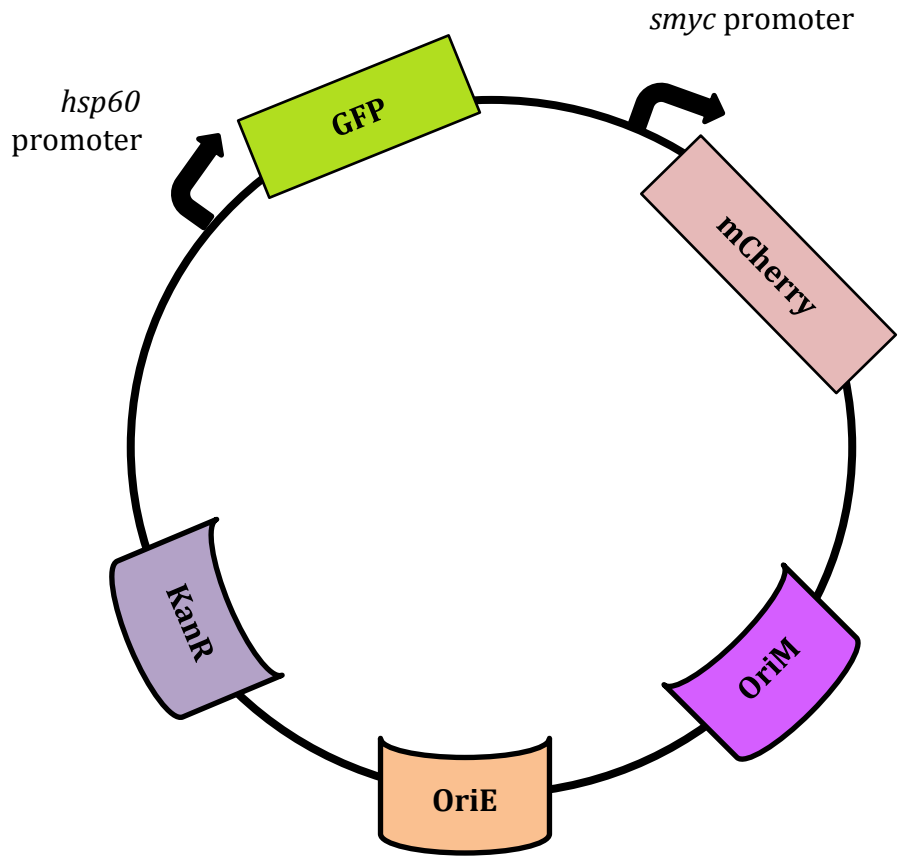


Figure S2: pST5552 Riboswitch Plasmid

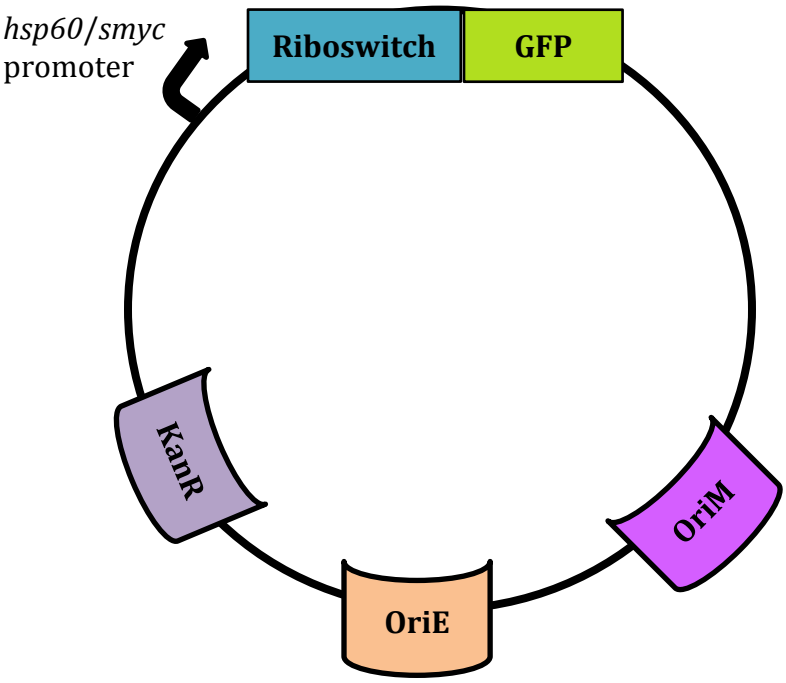
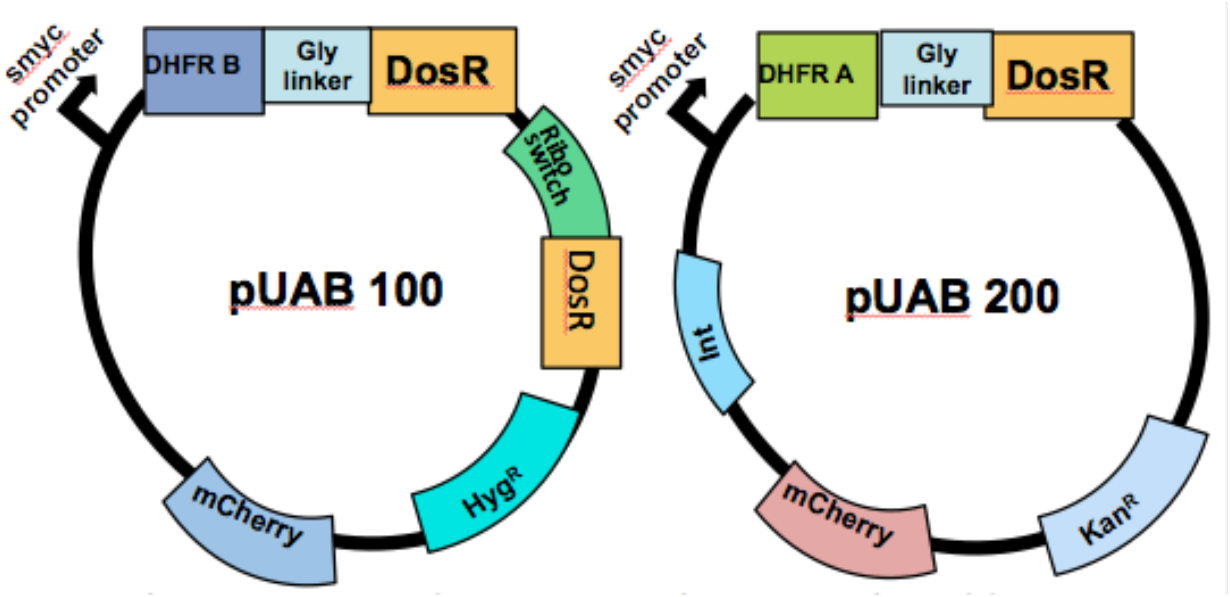


Figure S3: M-PFC DosR-DosR plasmid set with *smyc* promoter:



Appendix B: Primer Table

Table S1: Fast Cloning Primers

Primer Name	Primer	Function
PVVRG_esat6_FC_F	CTGGATGATGGGGCGAgcaggtgacgtcgttgttc	Forward Primer esat6 (3875) promotor
PVVRG_esat6_FC_R	CCAGTGAAAAGTTCTTCTCCTTTACTCATgctggactactttctctcttaccttcc	Reverse Primer esat6 (3875) promotor
PVVRG_1038_FC_F	CTGGATGATGGGGCGAcctagcaaatgccctgaacag	Forward Rv1038 primer promotor
PVVRG_1038_FC_R	CCAGTGAAAAGTTCTTCTCCTTTACTCATgctgctgtctccttgtctcg	Reverse Primer Rv1038 promotor
PVVRG_Rv1197_FC_F	CTGGATGATGGGGCGAgatgttgaagggctttgct	Rv1197 Forward Primer promotor
PVVRG_Rv1197_FC_R	CCAGTGAAAAGTTCTTCTCCTTTACTCATgttgcctgtctccttgttgaac	Rv1197 Reverse Primer promotor
PVVRG_cspa_FC_F	CTGGATGATGGGGCGAtgataggccaacgactttcc	Rv3648c Forward Primer promotor *cspa*
PVVRG_cspa_FC_R	CCAGTGAAAAGTTCTTCTCCTTTACTCATttctcgatctttccttttcttctg	Rv3648c Reverse Primer promotor *cspa*
smycRibo_FC_5552-F	CGCGGTACCAGATCTTTAAATCTAGA GGATCGTCGGCACCG	To copy *smycRiboDosR* cascade from pST5552 to add to pUAB100smycDosR
smycRibo_FC_5552-R	TCACCGGTACCTATAGTGAGTCGTAT GGATCCGGATCGTGCTC	To copy smycRiboDosR cascade from pST5552 to add to pUAB100smycDosR
smycRibo_FC_5552-F	CGCGGTACCAGATCTTTAAATCTAGAGGATCGTCGGCACCG	F insert primer to clone *smyc* in place of *hsp60* driving riboS-GFP in pST5552
smycRibo_FC_5552-R	TCACCGGTACCTATAGTGAGTCGTATGGATCCGGATCGTGCTC	R insert primer to clone *smyc* in place of *hsp60* driving riboS-GFP in pST5552
5552_pro_sub_F	ATACGACTCACTATAGGTACCGGTGA	F vector primer in pST5552 to insert *smyc* - downstream of *hsp60*
5552_pro_sub_R	TCTAGATTTAAAGATCTGGTACCGCG	R vector primer in pST5552 to insert *smyc* - upstream of *hsp60*
smycDosR_FC_100-F	CCGCGGTACCAGATCTTTAAAGGATCGTCGGCACCG	F primer to insert the *smyc* promoter in front of DosR in pUAB100-mCh

smycDosR_FC_100-R	ATCGACCAAGAAGACCTTTAC CACGGATCCGGATCGTGCTC	R primer to insert the *smyc* promoter in front of DosR in pUAB100-mCh
100_FC_smycDosR-F	GTGGTAAAGGTCTTCTTGGTC GAT	F vector primer for *smyc* promoter in front of DosR in pUAB100-mCh
100_FC_smycDosR-R	TTTAAAGATCTGGTACCGCGG	R vector primer for *smyc* promoter in front of DosR in pUAB100-mCh
RibosmycDosR_100_ F	gcaacgcgtgagcccGGATCGTCGG CACCG	To copy the smycDosRRibo cascade to add to pUAB
RiboDosR_100_R	cgccatcgacggcgcgTCATGGTCC ATCACCGGG	To copy the DosRRibo cascade to add to pUAB
RiboDosR_100_R_2	ggcgcgccgaaggccttTCATGGTC CATCACCGGG	To copy the DosRRibo cascade to add to pUAB
pUAB100_DosR_mC h_Ribo_F_2	AAGGCCTTCGGCGCGCC	To copy the pUAB plasmid to insert the DosRRibo cascade
smycDosR_FC_200-F	CGTTCCCGCCAGAAATCTGGA TCGTCGGCACCG	F primer to insert the *smyc* promoter in front of DosR in pUAB200-mCh
smycDosR_FC_200-R	CGAACAATTGTCTTGGCCAGG ATCCGGATCGTGCTC	R primer to insert the *smyc* promoter in front of DosR in pUAB200-mCh
200_FC_smycDosR-F	CGAACAATTGTCTTGGCCA	F vector primer for *smyc* promoter in front of DosR in pUAB200-mCh
200_FC_smycDosR-R	AGATTTCTGGCGGGAACG	R vector primer for *smyc* promoter in front of DosR in pUAB200-mCh
200_FC_smycDosR-F.2	TGGCCAAGACAATTGTTCG	F vector primer for *smyc* promoter in front of DosR in pUAB200-mCh
smycDosR_FC_200-F.ext	CATTGCCGTTCCCGCCAGAAA TCTGGATCGTCGGCACCG	R primer to insert the *smyc* promoter in front of DosR in pUAB200-mCh
smycDosR_FC_200-R.2	TGGCCAAGACAATTGTTCGGG ATCCGGATCGTGCTC	R primer to insert the *smyc* promoter in front of DosR in pUAB200-mCh
smycDosR_FC_200-R.ext	ACCACTCGAACAATTGTCTTG GCCAGGATCCGGATCGTGCTC	R primer to insert the *smyc* promoter in front of DosR in pUAB200-mCh

Table S2: Sequencing and Screening Primers

Primer Name	Primer	Function
pUAB100_smyc_screen_F	CCGCGGTACCAGATCTTTAA	To screen pUAB100DosR for *smyc*
pUAB100_smyc_screen_R	TCGACCAAGAAGACCTTTACCA	To screen pUAB100DosR for *smyc*
pUAB200_smyc_screen_F	CGTTCCCGCCAGAAATCT	To screen pUAB200DosR for *smyc*
pUAB200_smyc_screen_R	CGAACAATTGTCTTGGCCA	To screen pUAB200DosR for *smyc*
pST5552_smyc_screen_F	CGCGGTACCAGATCTTTAAATCTAGAG	To screen pST5552 for *smyc*
pST5552_smyc_screen_R	TCACCGGTACCTATAGTGAGTCGTAT	To screen pST5552 for *smyc*
smycDosRscreen_200	CCGGATCCGTGGTAAAGG	To sequence for the *smyc* DosR overlap
smycDosRscreen_100	CGGATCCTGGCCAAGAC	To sequence for the *smyc* DosR overlap
smyc_seq100_F	GTGCTTGTGGTGGCATCC	To sequence for DosR promoter in pUAB 100
smyc_seq200_F	GCCGCCAGGAGCATT	To sequence for DosR promoter in pUAB 200
smyc_seq5552_F	GTGGTTGTGGTGATGTACGTG	To sequence for DosR promoter in pst5552
smyc_screen_F	GATCGTCGGCACCGT	Forward primer at the start of *smyc* promoter for screening
GFPProSeq-F	CCGACAACGCAGACCGT	To sequence promoter driving GFP on PVVRG
GFPProSeq-R	GCATCACCTTCACCCTCTCC	To sequence promoter driving GFP on PVVRG
smyc_screen_F	GATCGTCGGCACCGT	Forward primer at the start of smyc promoter for screening
pUAB200smycSeqF	CAGTACGCGAAGAACCACGC	To confirm *smyc* in pUAB200 by sequencing
pUAB200smycSeqR	GACATCTGGGCGCGC	To confirm *smyc* in pUAB200 by sequencing
pST5552ProRiboSeqF	GGAGCTCACCGCCAGAAT	To confirm *smyc* in pST5552 by sequencing

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