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Development of a Fluorescent Drug Screening Platform for Inhibitors of Mycobacterium Tuberculosis Protein-Protein Interactions

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DEVELOPMENT OF A FLUORESCENT DRUG SCREENING PLATFORM FOR INHIBITORS OF *MYCOBACTERIUM TUBERCULOSIS* PROTEIN-PROTEIN INTERACTIONS

by

ZINA C. VERSFELD

A thesis submitted in partial fulfillment of the requirements
for the Honors in the Major Program in Biomedical Sciences
in the Burnett School of Biomedical Sciences
and in The Burnett Honors College
at the University of Central Florida
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ABSTRACT

Tuberculosis (TB) is a respiratory disease caused by *Mycobacterium tuberculosis* (*Mtb*) that kills around 1.3 million people annually. Multi-drug resistant TB (MDR-TB) strains are increasingly encountered, in part resulting from shortcomings of current TB drug regimens that last between six to nine months. Patients may stop taking the antibiotics during their allotted regimen, leading to drug resistant TB strains. Novel drug screening platforms are therefore necessary to find drugs effective against MDR-TB. In order to discover compounds that target under-exploited pathways that may be essential only *in vivo*, the proposed screening platform will use a novel approach to drug discovery by blocking essential protein-protein interactions (PPI).

In *Mtb*, PPI can be monitored by mycobacterial protein fragment complementation (M-PFC). This project will re-engineer the M-PFC assay to include the red fluorescent mCherry reporter for increased efficiency and sensitivity in high-throughput screening applications. To optimize the mCherry assay, we have developed fluorescent M-PFC reporter strains to monitor distinct PPI required for *Mtb* virulence: homodimerization of the dormancy regulator DosR. A drug screen will then identify novel compounds that inhibit this essential PPI. The screen will involve positional-scanning combinatorial synthetic libraries, which are made up of chemical compounds with varying side chains. This work will develop novel tools for TB drug discovery that could identify new treatments for the emerging world threat of MDR-TB.

DEDICATION

For my sister, Gera,

for always being there for me.

“The mind is not a vessel to be filled but a fire to be ignited.” – Plutarch

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1. Introduction

Tuberculosis (TB) is a respiratory disease caused by *Mycobacterium tuberculosis (Mtb)*. According to the World Health Organization, 8.6 million people became infected with TB and 1.3 million people died from TB infection in 2012 [1]. TB is characterized by a prolonged cough, chest pain, fatigue, and weight loss [2]. The disease is contracted by inhalation of particles or aerosols from coughing or sneezing from someone that has TB [3, 4]. TB can be treated with antibiotics, such as isoniazid and rifampin, which are two of the first-line drugs that are prescribed to patients with the disease [5]. The drug regimen for those diagnosed with TB can last between six and nine months [6]. Due to the length of treatment, patients may sometimes choose to stop taking the antibiotics during their allotted regimen, contributing to the development of drug resistant TB strains [6]. Patients are often prescribed multiple drugs to treat *Mtb* in granulomas. Granulomas are a cluster of immune cells surrounding the *Mtb*, generally in the lungs. The *Mtb* are dormant in this cluster of encasing immune cells, "hibernating" in a metabolically inactive state [7-9]. *Mtb* that are dormant or "latent" in granulomas are difficult to treat because they become phenotypically drug tolerant [10].

Multi-drug resistant TB (MDR-TB) has become a worldwide issue, and drug screening approaches are necessary to find novel drugs in order to treat patients with MDR-TB. The proposed drug screening platform will utilize an underexploited approach to drug discovery based on blocking essential protein-protein interactions, unlike more common approaches which identify drugs that inhibit bacterial DNA synthesis, RNA synthesis, cell wall synthesis, or protein synthesis [11].

In order to develop a safer and faster drug screening platform for *Mtb*, the non-pathogenic, faster growing relative of *Mtb*, *Mycobacterium smegmatis (Msm)*, will be used. *Msm* has been used as a substitute for *Mtb*, and has been tested for its efficacy and safety in numerous experiments. A study done by the Institute of Medical Microbiology in Hanover, Germany, shows the minimal harmful effects of *Msm* containing *Mtb* DNA that was injected into mice. Infection cleared very rapidly in the mice, and the safety of using *Msm* was confirmed, both through this study and many others [12]. The legitimacy of using *Msm* as a surrogate for *Mtb* has also been investigated. *Msm* expresses *Mtb* proteins ectopically due to similar high G+C content of around sixty-five percent [13]. It has been found that *Msm* has close homologs of many *Mtb* virulence genes, making it a good fit for studying the functions of these genes [14].

2. Literature Review

In order to better understand the need to find new drugs to inhibit *Mtb*, it will be important to evaluate the magnitude of the problem of drug resistance, the targets of current drugs, and the approach that will be taken to find novel drugs that inhibit protein-protein interactions (PPI).

2.1. Problem of Drug Resistance

The problem of drug resistant TB is a worldwide threat, especially in developing countries, such as in southern Africa and Southeast Asia [15-17]. The number of MDR-TB worldwide cases has increased by 51% from 2011 to 2012 [18]. MDR-TB cases have been rising in developing countries, although detection and reporting of these cases may be underestimates. Surprisingly, around 88% of reported MDR-TB cases have been documented in middle or high income countries [18].

Drug resistant strains can emerge in patients in whom the course of antibiotics they are taking is interrupted, either because the patient forgets to take their medication, or because of shortages of antibiotics in countries that provide free or cheap medications to those who cannot afford the medication [4]. Drug resistant *Mtb* strains can also be spread through normal means of transmission; air-borne transmission of bodily fluids from sneezing and coughing [3, 4]. These drug resistant strains are becoming nearly impossible to treat, and in some cases there are currently no treatment options available.

There are different categories of drug resistance depending on the resistance profile of the causative *Mtb* strain. Multi-drug resistant TB (MDR-TB) is resistant to both isoniazid and rifampin, the best drugs currently available to treat TB [17]. Extensively drug-resistant

tuberculosis (XDR-TB) is resistant to both isoniazid and rifampin in addition to any fluoroquinolones, and at least one of the injectable of second-line drugs that are used when the best antibiotics do not work [17]. The worst type of *Mtb* drug resistance is called totally drug resistant TB (TDR-TB) and is resistant to all known TB treatment options [4].

In order to address this problem, this study will look at ways to find novel drugs that will inhibit *Mtb* and combat this extremely difficult problem.

2.2. Mechanisms of Drug Resistance

The scope of the problem of drug resistance is due to the multiple mechanisms that *Mtb* can utilize to gain resistance. Mechanisms of drug resistance in bacteria include single nucleotide polymorphisms (SNPs), drug tolerance mediated by efflux pumps, and acquisition of drug resistance genes. Many of these alterations in nucleotide sequences are due to SNPs, where a single DNA nucleotide is different in a particular strain of *Mtb* in comparison to other types of *Mtb*. SNPs can be used to study drug resistance in *Mtb,* as SNPs are a major mechanism of drug resistance [19].

Unlike other bacteria, such as *E. coli*, *Mtb* doesn't use some other "common" mechanisms such as horizontal gene transfer (HGT) to acquire drug resistance. *Mtb* has been known to become resistant to these antibiotics through altering nucleotide sequences, rather than taking up new genetic material, which is common for other types of bacteria [20]. Ninety six percent of *Mtb* resistance to rifampin, one of the first-line drugs used to treat *Mtb*, is due to mutations that occur in the region that encodes for the beta subunit of RNA polymerase (*rpoB* gene) [20]. The beta subunit of RNA polymerase is responsible for initiation and elongation of DNA replication, in addition to rifampin resistance [21, 22]. Isoniazid resistance in *Mtb* is

characterized by mutations in the *katG* gene, which leads to nucleotide substitutions that occur in a gene regulatory region (*inhA*) [20, 23]. Due to these different mechanisms that lead to MDR-TB, it is extremely important that there is discovery of new drugs that target novel distinct mechanisms that are able to kill MDR-TB. In addition, it is important to look at the conditions that the *Mtb* were screened in. If the screening occurred under normal non-*in vivo* conditions, then the types of drugs that are "hits" may not represent drugs that would work in a real infection. Specific drug targets are important when figuring out methods for drug discovery.

2.3. Traditional Methods for Drug Discovery

Common strategies for drug discovery include targeting bacterial DNA synthesis, RNA synthesis, cell wall synthesis or protein synthesis [24]. However, these approaches have been thoroughly researched, and some of these aspects have been easily evaded by the bacteria they are intended to kill. For example, some antibiotics that target the cell wall of bacteria are only effective when the cells are actively undergoing cell division. When the cell is dormant and not dividing, as is the case of *Mtb* within granulomas, the bacteria are not affected by the drug. The bacteria are also able to turn on genes that produce proteins that can destroy the drug, such as beta-lactamases that are used to cleave beta-lactam drugs [25]. In these cases, targets of cell wall synthesis would be ineffective at treating the disease caused by the microbe.

In order to discover drugs that treat dormant *Mtb*, it is important to research conditionally essential processes required *in vivo* during persistent infections. This can aid in designing a screen that will find drugs that would be missed in *in vitro* screening. By looking at inhibitors of DosR:DosR, we will be looking for drugs that might have been missed in other drug screens that target *Mtb* during non-representative *in vitro* conditions. The importance of this specific PPI is

that it is predicted to be used as a target to screen for drugs that effectively kill *Mtb* in a persistent infection.

2.4. Protein-Protein Interactions (PPIs) as Potential Drug Targets

Studies on protein-protein interactions (PPIs) have been done in order to discover protein signaling and secretion pathways that are necessary for *Mtb* survival [26]. Proteomics, the study of expression levels of proteins and protein post-translational modifications, is a growing area of research, as it is crucial to understand the interaction pathways that can lead to pathogenesis in infectious bacteria [27, 28]. Exogenous chemicals and compounds can have a profound effect on PPIs, as these interactions can be sensitive to many environmental changes, such as temperature, pH, and other factors in addition to compounds that can enter the cell [28]. PPIs are a target for drug discovery. PPIs have been an underexploited target when identifying drugs that can inhibit different bacteria. Blocking essential PPIs can inhibit signaling and virulence factor regulation, blocking assembly of critical enzyme complexes, and the physiological and pathological processes of the bacteria can be halted [28].

For this project, the drug screen is looking for drugs that prevent essential PPIs for *Mtb*. Looking at PPIs can be important, as they directly relate to a pathogen's virulence. Since *Mtb* needs these PPIs in order to survive and cause disease, inhibiting these interactions could lead to weakening of the bacterial defense mechanisms, and enhance clearance of the infection by the immune system or it could outright kill *Mtb*. For this project, the PPI targeted will be one that is necessary for pathogenesis of *Mtb*. Targeting pathogenesis in bacteria, rather than specifically trying to kill the bacteria, is called anti-virulence. Inhibiting PPIs is one way to develop anti-virulence drugs.

2.5. M-PFC Basics

The mycobacterial protein fragment complementation (M-PFC) is a modified yeast two-hybrid method to monitor and measure PPI strength inside a bacterial cell. It has applications in dissecting pathways, understanding the function of virulence factors, determining the function of genes, and defining protein networks or complexes [29, 30].

M-PFC works by using the reporter protein called dihydrofolate reductase (DHFR). DHFR is a protein that is required in prokaryote and eukaryotic cells. It reduces folate cofactors that are necessary for metabolic functions, such as synthesis of some amino acids [31]. In the M-PFC assay, the DHFR regions are split up and attached to the two proteins of interest. The two interacting proteins are referred to as "bait" and "prey." When the DHFR protein is split into two components, the bait and the prey can be attached to each DHFR region, using a glycine linker region (Gly linker) to connect the two halves, as depicted in Figure 1. When the two DHFR regions come back together due to bait and prey interactions, a functional DHFR is reconstituted, conferring trimethoprim (TRIM) resistance to the bacteria [32]. Survival in the presence of TRIM therefore serves as an indicator of PPI between the bait and prey proteins. The M-PFC assay relies on Alamar Blue as the viability read-out dye [29]. Alamar Blue dye is reduced to Resorufin when a living organism is growing and actively respiring; the dye changes from blue to pink, with pink being the reduced form of the dye [33]. This color change is indicative of the presence of living bacteria that survived the addition of TRIM. Alamar Blue gives a quantitative measure of the relative strengths of interaction between binding partners, and can also function as a drug screen readout. One goal of this project is to re-engineer the M-PFC assay to include

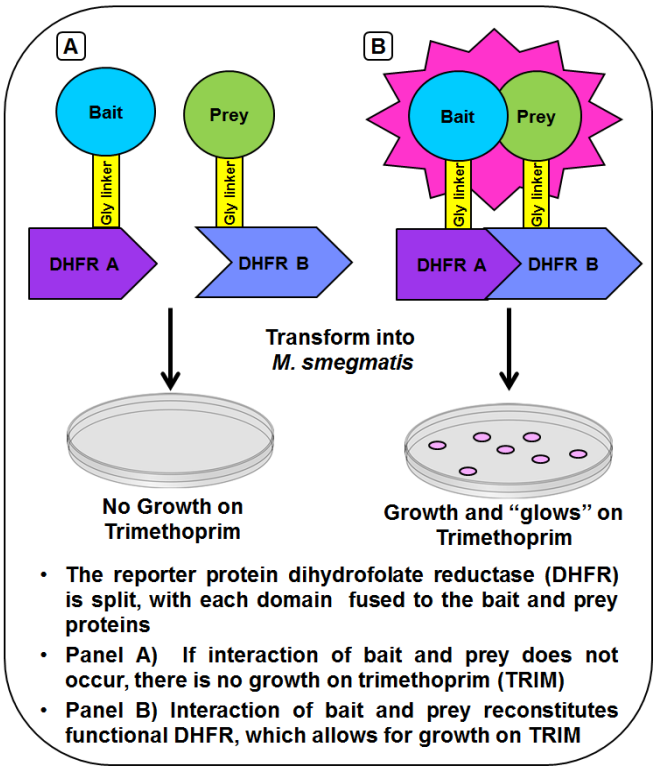


Figure 1: Basics of M-PFC

the red fluorescent protein mCherry reporter to maximize assay efficiency and sensitivity to make it amenable to high-throughput screening (HTS).

The M-PFC method has been validated in multiple studies looking at PPI in virulence pathways in *Mtb* [30, 34]. In the work done by Dr. Steyn's lab, M-PFC was used to validate known PPIs, such as homodimerization of yeast GCN4, interaction between KdpD, and KdpE *Mtb* proteins, and interaction between Esat-6 and Cfp-10 *Mtb* proteins [30]. After validation of the screening method, they were able to identify proteins that bind to Cfp-10 [30]. This is a validated, good method for studying PPI. For this project, we will be using M-PFC by adding a fluorescent component to the assay.

mCherry red fluorescent protein is one of the best red monomeric fluorescent proteins, due to its high photostability [35-37]. mCherry also has a low molecular weight, and does not interfere with protein function [35]. This is essential for the re-engineered M-PFC assay, as any

interference with the studied PPI would inhibit the reliability of the screening process. The fluorescent protein is also the fastest fluorescent protein to mature, taking only fifteen minutes at 37 degrees Celsius, the incubation temperature required by the M-PFC assay [36]. These characteristics of the mCherry fluorescent protein make it ideal to use in this study.

2.6. DosR: Initial Drug Target

Initial evaluation of the enhanced M-PFC assay will focus on homodimeric interactions between DosR monomers, the response regulator component of a well characterized two-component regulator of the hypoxia regulon [38]. DosR is a validated drug target because mutant strains lacking DosR are attenuated for growth under hypoxia and in animal infection models [39]. There are two sensor kinases, DosS and DosT, which sense cues and trigger DosR activation [32, 40]. DosT is a secondary sensor kinase that mediates the early *Mtb* response to hypoxia through phosphorylation of DosR [41]. Also, unlike DosS it is not encoded in operon with DosR [41]. In addition, DosS senses environmental cues near the end of an *Mtb* infection [41]. The two-component signaling cascade begins when environmental cues, such as extracellular concentrations of nitric oxide, carbon dioxide, and oxygen are increased. During hypoxic, or low oxygen, environments the heme-sensor in DosS is activated [42]. These outside factors allow DosS and DosT, the transmembrane proteins, to signal to DosR, the intracellular protein. These signals activate more than fifty genes that are used to help *Mtb* survive in a granuloma or macrophage [43]. Figure 2 depicts the intracellular signaling when DosR is activated [44].

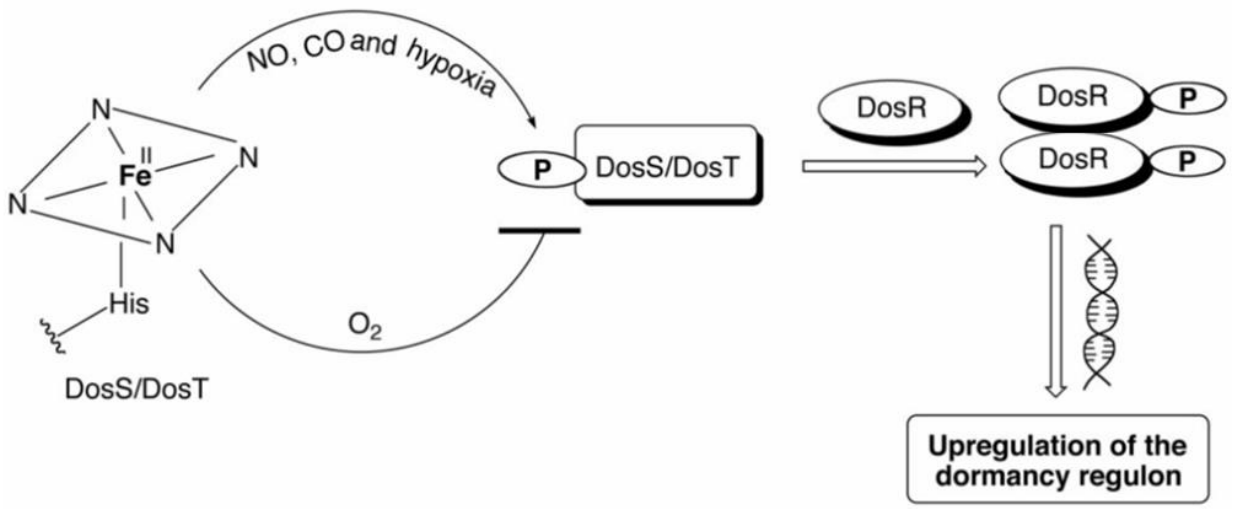


Figure 2: DosR Signaling in the Cell

Immune cell stresses, such as NO, CO, and hypoxia, are targeted at killing *Mtb*. Cues trigger autophosphorylation of sensor kinase DosS/DosT, subsequently leading to phosphorylation and dimerization of the response regulator, DosR. This triggers dimerization and promotor binding to DNA, which leads to the upregulation ~50 dormancy genes.

Inhibition of DosR:DosR homodimer is predicted to effectively kill dormant *Mtb* in granulomas, since it is necessary for survival of *Mtb in vivo* [45]. The drug screening platform will have translational applications, such as testing protein-protein interactions, structure-function analysis of protein complexes, pathogenic processes, and other physiological interactions.

The goals for the project are as follows:

Aim 1: Develop M-PFC drug target strains with interacting protein partners.

Different known and putative PPIs will be tested using the M-PFC method. For proof-of-principle, GCN4 homodimerization, a known PPI pair, will be tested as a positive control. GCN4 is a general control protein found in yeast, which is a zinc-finger protein that exhibits very strong homodimeric binding or assembly. [46]. However, for this project, we will focus on the DosR-DosR homodimeric interaction, a transcriptional regulator that allows *Mtb* to survive and

persistently infect the human host [38]. Finding drugs that can inhibit this mechanism will lead to *Mtb* death *in vivo*, as it cannot survive without this essential PPI.

Aim 2: Develop a fluorescent drug screening platform for inhibitors of protein-protein interactions.

A goal of this project is to engineer a derivative of the M-PFC assay with a red fluorescence protein mCherry read-out system. This can maximize efficiency and sensitivity of the assay, enhancing its capabilities of HTS. Utilizing a fluorescence based approach will reduce the amount of time and number of steps that are necessary when compared to using the M-PFC method with Alamar Blue dye.

Aim 3: Drug screening for PPI inhibitors of DosR-DosR homodimer.

After optimizing the fluorescent drug screening platform, a drug screen will be performed in order to find compounds that may kill or inhibit *Mtb* growth by blocking protein interactions. We will use position-scanning combinatorial synthetic libraries of compounds provided by a collaborator at the Torrey Pines Institute for Molecular Studies (TPIMS). In addition to this, the Sanford-Burnham Medical Research Institute will be performing a separate drug screen on the *Msm* strains using a 1,280 compound Library of Pharmacologically Active Compounds (LOPAC).

The final outcome of this project will be the fluorescent M-PFC and a validated new fluorescent drug screening platform for discovery of drugs that will inhibit DosR:DosR protein-protein interactions.

3. Methodology

3.1. General Overview of M-PFC

For this project, there are two pairs of plasmids that are used for M-PFC. They are called pUAB100, pUAB200, pUAB300, and pUAB400. pUAB200/400 integrate with the chromosomal DNA of *Msm*, and contain the "bait" protein genes. The pUAB100/300 plasmids continue to replicate as an episomal plasmid, and will contain the "prey" protein genes (as seen in Figure 3).

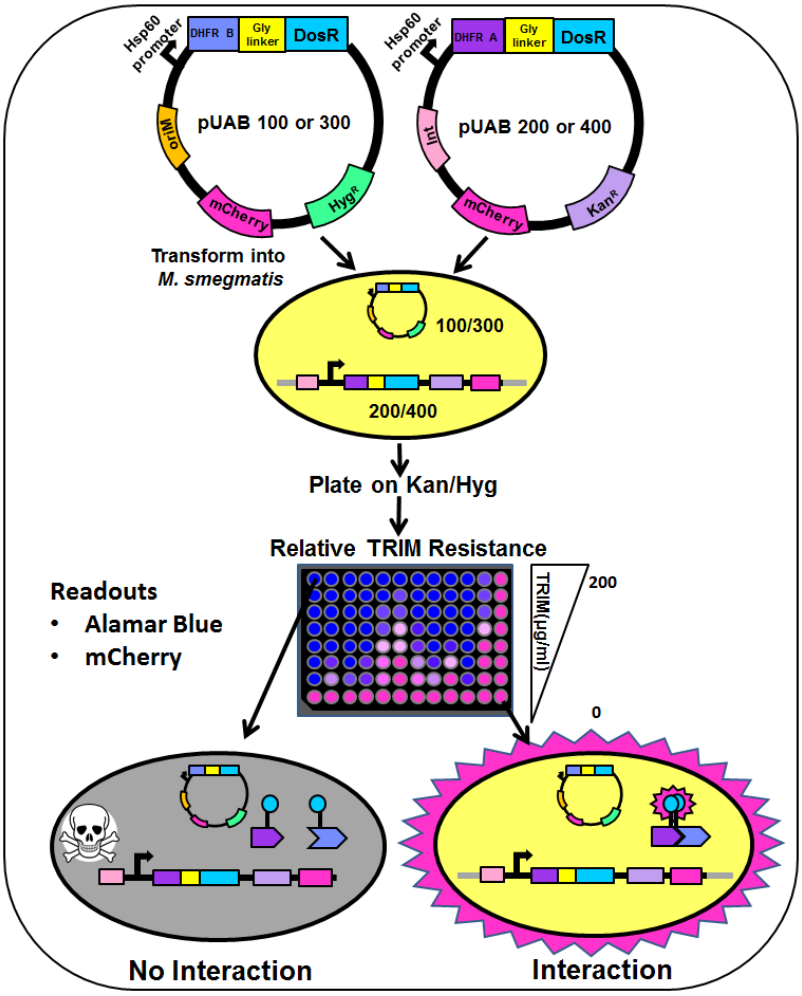


Figure 3: mCherry M-PFC Design
Overview of methods used for M-PFC. Two different plasmids contain genes encoding for the bait and prey proteins. After transformation, cultures can be tested using Alamar Blue or mCherry readout systems.

The pUAB200/400 plasmids contain a gene for kanamycin (Kan) resistance, and the pUAB100/300 plasmids contain a gene for hygromycin B (Hyg) resistance. These plasmids were genetically engineered and constructed before being transformed into *Msm*. Bacteria possessing the plasmids were selected via antibiotics. Once selected, the *Msm* were grown up for use in the M-PFC assay, where the level of PPI was measured. When there was no interaction, the bacteria in that column of the assay did not survive, and did not reduce Alamar Blue dye or express mCherry fluorescent protein. When the PPI was present, the bacteria survived and were able to reduce the Alamar Blue dye to a pink color or express mCherry. This general outline of the methods is explained in detail in the next sections of the methods.

3.2. Bacterial Cell Culture

NEB 10-Beta Competent *Escherichia coli* High Efficiency *(E. coli)* and *Mycobacterium smegmatis mc^{2}155 (Msm)* were used to conduct the experiments for this project [47]. *E. coli* was used as a host for recombinant DNA manipulations, to amplify the DNA for routine cloning, and as a source of plasmid DNA for transformation into *Msm*. *Msm* was used to express the DosR genes to make DosR proteins in a high G+C rich environment in non-pathogenic mycobacteria, and as expression host for M-PFC.

E. coli was grown at 37°C in lysogeny broth (LB) with shaking at 250 revolutions per minute (rpm). Depending on the plasmids used, different concentrations of antibiotics were used. When constructing plasmids using pUAB100 and pUAB300, hygromycin B (Hyg) was used at 250 μg/mL. To select for pUAB200 and pUAB400, kanamycin (Kan) was used at 50 μg/mL. Cultures were generally grown up in a 5 mL culture tube overnight.

Msm grows at 37°C in LB broth with 0.05% polysorbate 80, also called Tween 80 (LB Tw broth). The antibiotic concentrations used for *Msm* were lower than what were used for *E. coli*. For the selection of pUAB100 and pUAB300 plasmids, 50 μg/mL concentration of Hyg was used. To select for pUAB200 and pUAB400 plasmids, 25 μg/mL of Kan was used. 5mL cultures were incubated for three days, and shaken at 40 rpm.

3.3. Creating M-PFC strains to monitor DosR:DosR Interactions

The following genetic techniques were used in order to create the M-PFC strains used to monitor the DosR:DosR PPI.

3.3.a. Genetic Engineering

FastCloning is a method used to construct plasmids for the M-PFC assay. It is a ligation independent method that bypasses many of the steps involved in 'regular' cloning, such as gel purification, restriction digestion, and ligation [48]. The target plasmid and insert were amplified using Polymerase Chain Reaction (PCR), described in detail in the next paragraph. Complementary overhangs of base pairs that are located at the ends of the replicated DNA were annealed together. A restriction enzyme, called Dpn1, was used to digest and get rid of the methylated DNA, which were the original 'parent' strands used as PCR templates, while leaving the unmethylated amplified PCR products untouched. Once the plasmids were constructed, they were transformed into *E. coli*. Figure 4, below, is a representation of the steps involved in creating the PPI plasmids needed for M-PFC:

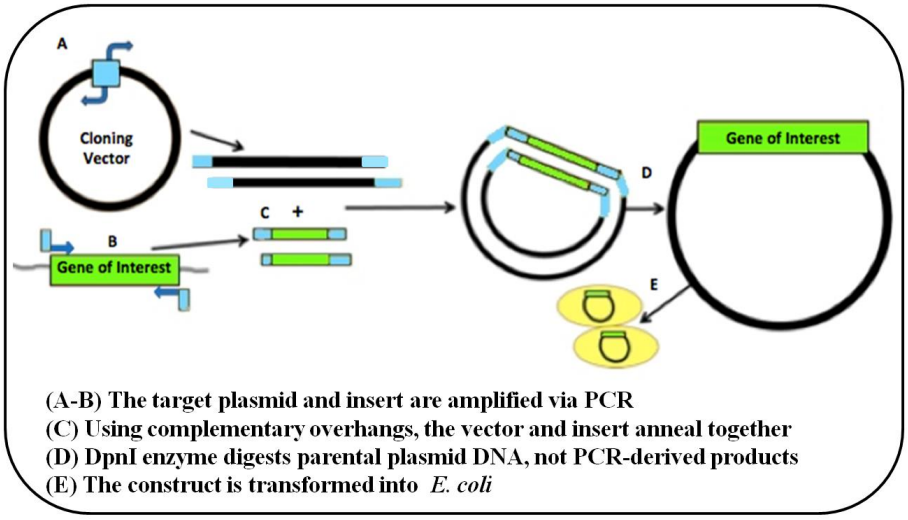


Figure 4: FastCloning

Ligation-free plasmid creation. Primers (designated by light blue rectangles) used to amplify vector and gene of interest with complementary overhangs that anneal together, allowing formation of the vector with the gene of insert.

This method enables the project to proceed at a faster rate, as non-essential steps and reagents are not being utilized. In addition, this method also allows for more flexibility when designing cloning experiments.

Polymerase Chain Reaction (PCR) is a method used to amplify both the plasmid and the insert DNA [49]. Each fragment of DNA can be logarithmically amplified to have many more copies of the original DNA. There are general steps that are followed in order to amplify DNA using this method. First, there is an initial denaturation of the DNA fragment, which allows the complementary strands to come apart, and often used to activate the enzyme. Then there is a secondary denaturation, which continues to denature the DNA fragments, allowing the removal of the hydrogen bonds between the DNA strands. This step is followed by an annealing temperature phase that adjusts the temperature for the DNA primer to allow it hybridize with the template DNA. Then there is an extension temperature that is specific to the DNA polymerase used, which in this case is Phusion® enzyme. During this step, the polymerase is replicating the

template strand of DNA. These three steps are repeated for multiple rounds of logarithmic amplification of the target DNA sequence. To conclude, there is a final extension time which allows for the newest DNA copies to be completed. The protocol that was followed is called "PCR Protocol for Phusion® High-Fidelity DNA Polymerase (M0530)," by New England BioLabs [50]. For a representative PCR reaction, 1uL of template DNA (~10-20 ng/µL), 1 µL of forward primer (100 ng/µL), 1 µL of reverse primer (100 ng/µL), 5 µL of 5x GC Buffer, 0.5 µL of 10mM deoxynucluotide (dNTP), 0.25 µL of Phusion® Enzyme, and 16.25 µL of DNAse free water were used, for a total volume of 25µL in a reaction tube.

Once the PCR reaction was set up, the reaction ran in a BioRad thermocycler to change the temperature of the reaction at preset times, which allowed the Phusion® enzyme to be activated at certain temperatures, and allowed for proper denaturation and annealing of the DNA fragments.

The typical thermocycler settings used for PCR are shown below:

Table 1: PCR Reaction Settings

Temperature (°C)	Time	PCR Step	
98	30 seconds	Initial denaturation	
98	10 seconds	Secondary denaturation	Repeat 20 times
55-70	30 seconds	Annealing. Variable depending on the primers' melting temperature.	
72	15 seconds/kb	Extension temperature	
72	3 minutes	Final extension	
12	∞	Hold time	

For the reactions, the annealing temperature was dependent on the melting temperature (Tm) of the primers that were used. The primers were designed to amplify the vector and the insert using software called "ApE – A plasmid Editor," and additional online Mycobacteria DNA

databases, such as “MycoDB – xBASE –” [51]. For each plasmid, primers were designed for each of the vectors used. Below is a table of the primers that were used, and their DNA sequences (Table 2).

Table 2: Primers for PCR Reactions

Primer	Sequence	Purpose
DosR_100_F	ACAATTGCGGATCCTTCGAACGTG GTAAAGGTCTTCTTGGTCGAT	Forward primer for DosR insert for pUAB100/300
DosR_100_R	CACCCGGTGATGGACCAATCGAT GGTGGCGGTGG	Reverse primer for DosR insert for pUAB100/300
pUAB100F	ATCGATGGTGGCGGTGG	Forward primer for vector amplification of pUAB100/300 to insert DosR
pUAB100R	ACAATTGCGGATCCTTCGAAC	Reverse primer for vector amplification of pUAB100/300 to insert DosR
mCh_FC_100F	CGCTCGCCGCAGCGGATCGTCGG CACCG	Forward primer for mCherry insert into pUAB100/300
mCh_FC_100R	CATGGACGAGCTGTACAAGTGAG AGCGCAACGCGTGAG	Reverse primer for mCherry insert into pUAB100/300
100_mCh_F	GAGCGCAACGCGTGAGC	Forward primer for vector amplification of pUAB100/300 to insert mCherry
100_mCh_F	GCTGCGGCGAGCGGTA	Reverse primer for vector amplification of pUAB100/300 to insert mCherry
DosR_200_F	TGGCCAAGACAATTGTTCGAGTG GTAAAGGTCTTCTTGGTCGAT	Forward primer for DosR insert for pUAB200/400
DosR_200_R	CACCCGGTGATGGACCAATCGAT GGTGGCGGTGG	Reverse primer for DosR insert for pUAB200/400
pUAB200F	ATCGATGGTGGCGGTGG	Forward primer for vector amplification of pUAB200/400 to insert DosR
pUAB200R	TGGCCAAGACAATTGTTCGA	Reverse primer for vector amplification of pUAB200/400 to insert DosR
mCh_FC_400F	CCTTGTCCCGGTCTATTCTCTTGG ATCGTCGGCACCG	Forward primer for mCherry insert into pUAB200/400
mCh_FC_400R	CATGGACGAGCTGTACAAGTGAA ATCTGGTGTGAATGCCCC	Reverse primer for mCherry insert into pUAB200/400
400_GFPmCh_F	AATCTGGTGTGAATGCCCC	Forward primer for vector amplification of pUAB200/400 to insert mCherry
400_GFPmCh_R	AAGAGAATAGACCGGGACAAGG	Reverse primer for vector amplification of pUAB200/400 to insert mCherry

Once the plasmids and inserts were amplified using Phusion® PCR, the PCR products were run on a 1% agarose gel, to verify that the DNA fragments were present, and were the

correct size band on the gel. In Section 4.1, Figure 8 depicts an example of an agarose gel used to visualize PCR products. Once verified, the vector and the insert were FastCloned together. 1.5 µL of Dpn1 enzyme was added to each tube with varying ratios of vector to insert DNA. Varying ratios were used to find the optimum concentration of vector:insert for efficient plasmid assembly. There were typically three tubes that had a 4:1, 1:1, and 1:4 ratio of insert to vector, with a total volume of 8 µL of DNA in each tube. For example, for the tube with a 4:1 ratio, there was 6.4 µL of the insert DNA (in this case either DosR or mCherry), 1.6 µL of vector DNA (typically pUAB100/200), and then 1.5 µL of Dpn1 enzyme. The reaction was incubated at 37°C for two hours in order to allow for the methylated (parental strands) of DNA to be properly digested.

Once the vector and the insert were FastCloned, the products of the reaction were transformed into *E. coli*. The "NEB 10-beta Competent *E. coli* (High Efficiency)" protocol was used as described by the manufacturer [47]. Transforming the DNA into *E. coli* was necessary for amplification of the circular DNA plasmids containing the gene of interest that were inserted into the vectors. There were modifications made to the protocol used above. After thawing an aliquot of 10-beta Competent (cc.) *E. coli*, the cells were split into thirds, for a total of 16.5 µL of 10-beta cc. *E.coli* in each tube. 2 µL of the FastCloned DNA was added into each tube (three tubes for three ratios of insert:vector). The cells then sat on ice for 30 minutes before heat shocking at 42°C for 30 seconds. Then, the cells were put on ice for 5 minutes before 200 µL of Super Optimal broth with Catabolite repression (SOC) was added to each tube. The cells were then incubated at 37°C and shaken at 250 rpm for an hour before 100 µL was plated onto LB agar plates that had antibiotics with varying concentrations depending on which plasmid was

being used. The plated cells were incubated at 37°C overnight. If there were colonies present on the plate, they were PCR screened using the primers for the insert to verify that the insert was present in the vector. Colony PCR screening involves lysing the bacteria and extracting DNA to use in a PCR reaction. To lyse the bacteria, an isolated colony on a plate was labeled, picked, and added to a tube containing 20 μL of diH_2O. The tube was boiled at 100°C for 5 minutes, and then spun down at 13,000 rpm for 2 minutes to pellet the bacterial debris. 2 μL of the supernatant was used for the template DNA in the PCR reaction. In Section 4.1, Figure 14 depicts an example of an image of an agarose gel used for PCR colony screening.

Once the colonies have been screened and verified, the DNA was confirmed via sequencing. *E. coli* colonies determined to contain the desired insert were picked and grown in a 5 mL overnight culture, in order to amplify the DNA. The DNA was then extracted from the *E. coli* cells using an alkaline lysis protocol using EconoSpin™ Spin Column for DNA, from Epoch Life Science Inc. The DNA concentration was measured using a Nanodrop spectrophotometer.

The M-PFC plasmids were constructed using the techniques mentioned above. The DosR genes were linked to the different DHFR regions using a glycine linker region, which was inserted into the M-PFC plasmids. Below, Figure 5 shows an outline of what the vectors look like and their different components.

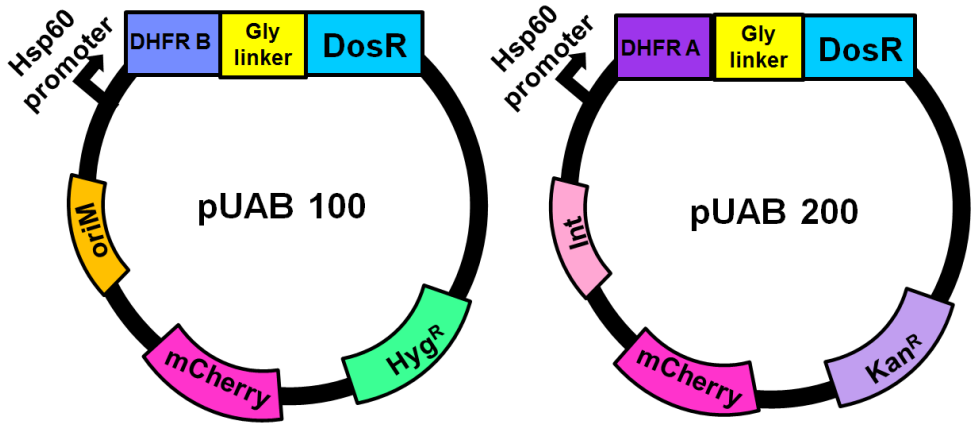


Figure 5: pUAB Plasmids
Episomal plasmid, pUAB100, used to amplify and express DosR glycine linked to DHFR B, and the chromosomal plasmid, pUAB200, used to amplify and express DosR glycine linked to DHFR A.

Both plasmids have a *hsp60* promoter region that allows for strong constitutive expression of the genes. In addition, the *mCherry* gene has its own promoter called the *smyc* promoter, which allows for transcription of the gene encoding the fluorescent protein.

pUAB100 has an origin of replication, called oriM, which enables the plasmid to replicate independently in the cytoplasm of the cell. A domain of the DHFR is linked to DosR using a glycine linker region. The plasmid has a *Hyg* resistance gene, which is used for selection of the plasmid when grown in bacteria.

pUAB200 has an integration segment, called Int, allowing the plasmid to integrate into the chromosomal DNA of the bacteria. The second half of DHFR is linked to DosR with a glycine linker region. A *Kan* resistance gene is present to allow for plasmid selection.

3.3.b. Transformation into *Msm*

Once the M-PFC plasmids were constructed, they were transformed into *Msm* before use in the optimized mCherry M-PFC assay. Transformation uses a process called electroporation,

which uses an electrical field to alter the permeability of the cell membrane [52]. This allows the cell to take in the engineered plasmids. These plasmids were transformed into *Msm* in order for the DNA to be introduced into the cell and to express the proteins of interest, in this case the DosR:DosR homodimer fused to the DHFR protein.

100 μL of electro-competent wild type *Msm* was thawed on ice for 10 minutes. Around 200-500 ng of DNA was added, for a maximum total of 4 μL of DNA added to the *Msm* cells. The cells were then transferred to an electroporation cuvette. The cuvette was placed into the BioRad Gene Pulse X machine, and 2,500 V (volts), 25 μF (capacitance), and 1,000 Ω (resistance) were applied to the cells in the cuvette. The cells were transferred to a 2 mL screw-cap conical tube containing 250 μL of LB Tw broth. The cells were then incubated at 37°C and shaken at 40 rpm for 4 hours before 100 μL was plated onto an LB agar plate with the appropriate antibiotic selection.

Once the plasmids were transformed into *Msm*, the incorporation of both plasmids was confirmed based on resistance to Kan or Hyg, conferred by antibiotic resistance genes on the pUAB plasmids.

3.4. Mycobacterial Protein Fragment Complementation (M-PFC) Assay

To assess potential PPI between bait and prey pairs, each M-PFC strain was diluted from a starting optical density of 0.5 to an optical density of 0.0005. The 135 μL of culture was then added to a 96 microwell plate with 15 μL of TRIM of varying dilutions from 200 μg/uL to 6.25 μg/uL. The gradient was used in order to determine the relative strengths of the PPI, specifically DosR. The TRIM dilutions showed the concentration of TRIM that was necessary to inhibit growth of the bacteria; growth at higher TRIM concentration means that stronger interactions are

taking place. After a forty-eight hour incubation with TRIM, 15 μL of Alamar Blue dye was added. For this project, Resazurin (1X) dye was used instead of commercial Alamar Blue dye. The current M-PFC assay uses Alamar Blue/Resazurin dye as an indicator [29]. Resazurin dye is reduced to Resorufin when a living organism is growing and actively respiring; the dye changes from blue to pink, with pink being the reduced form of the dye [33]. Once added, the cells were incubated for an additional four hours before the reduction of the dye can be detected by a fluorescent plate reader with an excitation wavelength of 530nm and an emission wavelength at 590nm. The control used for these readings were "blank" wells that only contained LB Tw broth with added Resazurin dye, and no bacteria. This control served to determine the levels of nonspecific signal (i.e. due to media interference), and also if there was any contamination in the plate in which the assay was performed.

One drawback of using Alamar Blue/Resazurin is that it requires additional handling steps and takes a large amount of extra time. The mCherry version of the assay addresses these issues by reducing the assay time, as less reagents and steps are necessary. Thus, the addition of mCherry makes the assay amenable for high-throughput screening (HTS).

3.5. Fluorescent M-PFC Assay

For this project, the M-PFC assay will serve as the basis for a novel fluorescent platform for TB drug discovery. Positional-scanning combinatorial synthetic libraries, in addition to other drug libraries, will be used to screen for drugs that can target the PPIs essential to *Mtb*.

A general outline of preparation and function of the mCherry M-PFC assay is depicted in Figure 3. The design shows that when the dihydrofolate reductase (DHFR) domains are reconstituted by the bait and prey coming together, the relative strength of the protein-protein

interaction can be measured. For example, in the 96 well plate graphic, the column with pink fluorescent mCherry shows a very strong interaction, as the bacteria are able to survive to the highest concentration of TRIM. In the sample to the left, there is little-to-no protein-protein interaction, seen as an inability of bacteria to survive even low amounts of TRIM. This shows that the bait and prey proteins did not interact and did not bring the two halves of the DHFR domain back together. The basis of this assay will be used in the drug screen, as the compounds that inhibit the protein-protein interaction will prevent DHFR assembly, causing death in the presence of trimethoprim and loss of mCherry fluorescence.

We developed a fluorescence-based M-PFC assay for monitoring PPIs, by FastCloning the red fluorescent mCherry reporter onto both pairs of pUAB plasmids. In addition, different mCherry configurations were tested by adding mCherry to the episomal, chromosomal, and both plasmids. The DosR inserts where cloned off of CDC 1551 *Mtb* chromosomal DNA. The mCherry insert was amplified off of the pVVR plasmid, which is a derivative from the pVV16 base plasmid [53]. The list of primers that were used can be found in Table 2 in Section 3.3.a.

3.5.a. Microwell Plate Optimization

The mCherry M-PFC assay was compared to the original M-PFC assay under the same conditions, such as OD of cultures, concentration of TRIM, total volume of microwells, and incubation temperature and time, in order to determine the efficiency of the mCherry version of the assay. Both assays were analyzed, looking for any changes in sensitivity and reproducibility. When these conditions were optimized, the assay was subjected to the drug-screening compounds in order to find “hits” that target the DosR-DosR protein interaction as a HTS platform.

The mCherry fluorescent assay was miniaturized in order to enhance efficiency and to reduce the amount of reagents used for each assay. The 96 microwell plate had a total volume of 150 µL per well. As mentioned previously, 135 µL of culture (0.0005 OD) and 15 µL of TRIM was added to each well. Optimization to a 384 microwell plate reduced the total volume of the well to 50 µL, using 45 µL of culture (0.0005 OD) and 5 µL of TRIM. In the data section, varying conditions were experimented with for the 384 microwell plate.

3.5.b. Drug Screen

The basis for the drug screen is to find inhibitors of DosR homodimerization. Figure 6 depicts the set up for the drug screen. Compounds that inhibit DosR: DosR PPI will not produce the mCherry fluorescence reporter protein, as the bacteria are not able to survive trimethoprim. Inactive compounds that do not disrupt DosR homodimerization will confer trimethoprim resistance, and will produce mCherry fluorescence.

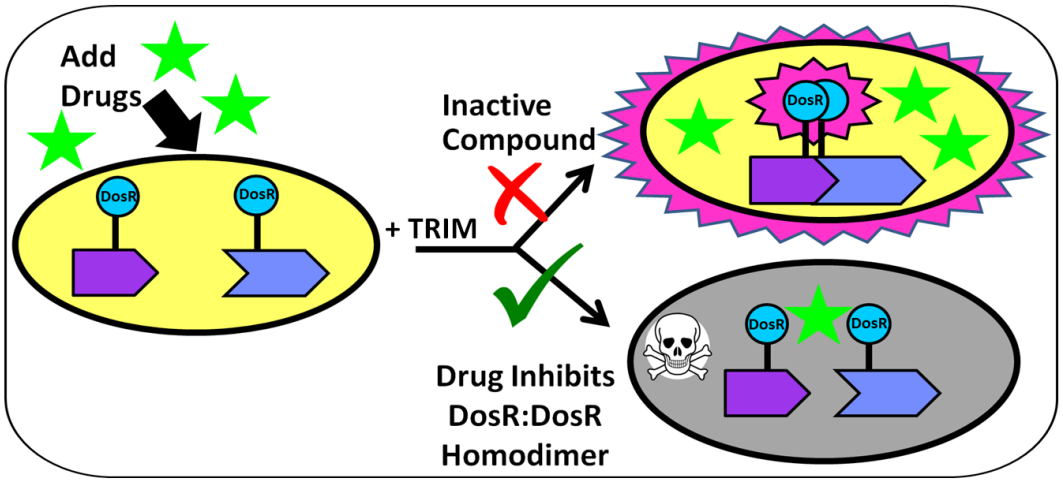


Figure 6: Fluorescent Inhibitor Drug Screen
Inactive compounds do not disrupt DosR:DosR interaction, which allows the bacteria to fluoresce and survive. Compounds that disrupt the DosR:DosR interaction lead to TRIM-dependent cell death due to disruption of DHFR and lack of fluorescence.

A pilot screen of 1,280 compounds has been tested at the Sanford-Burnham Medical Research Institute through the Florida Translational Research Program. In the lab, we are conducting a drug screen using positional-scanning combinatorial synthetic libraries of compounds from collaboration with the Torrey Pines Institute for Molecular Studies (TPIMS). The positional-scanning combinatorial library will allow us to cover a diverse range of compounds using a small scale approach. The library contains "scaffold" compounds that have varying side chains. Each well in the microwell Scaffold Ranking plate will contain a single scaffold with up to hundreds of thousands of modified forms from the variations to the backbone compound [54, 55]. If a well shows up as an inhibitor of DosR homodimerization, TPIMS will provide us with a sub-library containing each compound in its pure form for secondary screening. This method will allow us to determine structure activity relationship (SAR), as variants of the same backbone will be present in the library [54, 55].

For each drug screen, the mCherry M-PFC strains will be grown in a microwell plate with the compounds at 10μg/ml dissolved in dimethyl formamide (DMF) and TRIM at an optimized concentration. The final DMF concentration in each well will be 0.1%, which has been determined to have minimal deleterious effects on *Msm*. After sufficient incubation time to allow for growth (optimal time to be determined empirically), the plate will be read for fluorescence. The wells where the mCherry *Msm* did not fluoresce will show that the compound inhibited the DosR:DosR PPI. These "hits" will be tested further to verify their specificity in inhibiting the PPI. In addition to HTS applications, the enhanced M-PFC assay facilitates the study of protein-protein interactions relevant to *Mtb* pathogenesis.

Once hits have been found using this method, a counter-screen without TRIM will be conducted. If the compounds kill the bacteria in the absence of TRIM, then the compound does not inhibit the DosR:DosR dimerization, but has a different mode of action in killing the bacteria.

Chemical compounds that effectively block DosR:DosR dimerization would be good candidates for novel antimicrobials for the treatment of drug resistant MDR-TB. These drug screens will serve as the basis for additional drug screens targeting distinct PPIs critical for *Mtb* virulence.

3.6. Alternative Approaches

Potential problems using the fluorescent M-PFC assay include a low hit rate due to the specificity of the compound needed to be found. Alternative approaches that can be used to counter this include testing numerous and diverse compounds. The TPIMS compounds contain over thirty million different structures, due to positional-scanning combinatorial synthetically made compounds. This screen will increase the size and diversity of the compounds being tested, and will increase the chances of finding an inhibitor of the DosR:DosR homodimer.

There is also the possibility of false positives from compounds that kill the *Msm* cells without inhibiting the DosR homodimerization. Hits found in the primary screen of the compounds will be "cherry picked" for a secondary screen. In this case a viability screen (i.e. Alamar Blue M-PFC) could identify the hits that are bactericidal, that do not inhibit DosR homodimerization. Another way to filter out false "hits" is to use an additional PPI with a different fluorescent color to be tested at the same time. For example, if the GCN4/GCN4 PPI,

discussed in previous sections, was labeled with green fluorescent protein (GFP), that *Msm* strain could reduce the rate of false positives. When the culture loses both mCherry and GFP fluorescence, the compound did not inhibit a specific pathway and can be eliminated as a false positive. Inhibition of DHFR interaction or enzymatic activity could also lead to false positives. A multiple M-PFC screen, as mentioned above, would also eliminate false positives that inhibit the DHFR, and not the specific PPI being studied.

Additional ways to confirm "hits" is to use fluorescent promoter:reporter strains to determine if a compound inhibits DosR mediated transcriptional activation of known downstream genes (i.e. *hspX*). This could determine if the compound inhibits growth and viability under hypoxic conditions, which would biochemically validate that the compound binds to DosR and blocks DosR homodimerization.

Further ways to improve the assay would be to have a "real-time" readout of the assay, instead of viability assay readout (Alamar Blue and mCherry M-PFC). One way this can be achieved is by using a split mCherry approach; two halves of mCherry are used instead of two halves of dihydrofolate reductase (DHFR) [56]. The cells will only fluoresce when the bait and prey proteins interact. This could lead to a more sensitive and effective readout mechanism of the assay.

4. Results

The creation of the fluorescent mCherry assay included genetic engineering, transformation into bacteria, and measurement of interaction strength of PPI using M-PFC before addition of the mCherry fluorescent protein onto the plasmid pairs. The pUAB plasmids were given to our lab curtesy of Dr. Adrie Steyn at the University of Alabama. Proof-of-concept of the M-PFC assay has been tested in previous experiments [29, 30], and modified in this project to provide a fluorescent version of the assay.

4.1. Construction of Plasmid Pairs

In order to measure the PPI in DosR homodimerization, the two pUAB plasmids were genetically engineered to contain DosR fused to one domain of DHFR via a glycine linker and *smyc:*mCherry inserted at a separate location on the plasmid. Techniques described in the methods section were used. For each pUAB plasmid, primer design and PCR amplification were necessary before the vector and the insert could be FastCloned together. After confirming the amplified DNA by running the vector and insert on an agarose gel, the PCR products were FastCloned. After FastCloning, the DNA was transformed into *E. coli* in order to amplify the DNA. These steps were necessary to have amplified plasmids in order to transform into *Msm*. Once both plasmid pairs were transformed into *Msm*, the bacteria could be tested using M-PFC to determine PPI strength [29, 30]. Table 3 includes the names of the plasmids that were constructed using these methods.

Table 3: Table of Constructed Plasmid Pairs

Name	Plasmid Pairs	Vectors	Inserts
DosR/DosR	pUAB100-DosR + pUAB200-DosR	pUAB100 and pUAB200	DosR
DosR/Empty (Negative Control)	pUAB100-DosR + pUAB200	pUAB100 and pUAB200	DosR
GCN4/GCN4 (Positive Control)	pUAB100 + pUAB200	pUAB100 and pUAB200	None
DosR/DosR mCherry	pUAB100-DosR-mCh + pUAB200-DosR-mCh	pUAB100 and pUAB200	DosR and mCherry
DosR/Empty mCherry (Negative Control)	pUAB100-DosR-mCh + pUAB400-mCh	pUAB100 and pUAB400	DosR and mCherry

To verify the size of the PCR products on an agarose gel, Thermo Scientific 1 kilobase (kb) Gene Ruler was used [57]. This marker was loaded into the first lane next to the DNA samples before the gel was run. An example of a gel that was imaged, after being stained in Gel Red dye, is one that shows the vector (pUAB200-DosR), and the insert (*smyc*:mCherry) in Figure 7. In Figure 7, the mCherry insert is verified because it is around 1000 bp. The size of the mCherry insert is 1012 bp. In the Supplemental Figures (APPENDIX), the pUAB plasmids are labeled with the sizes of each insert, in addition to primers that were used. In the image, the size of the vector is roughly 5000 bp, which correlates with its actual size of 5,351 bp. In addition to imaging the DNA fragments before FastCloning, the DNA purified from *E. coli* was also sequenced, confirming that the insert was added to the correct part of the plasmid (data not shown).

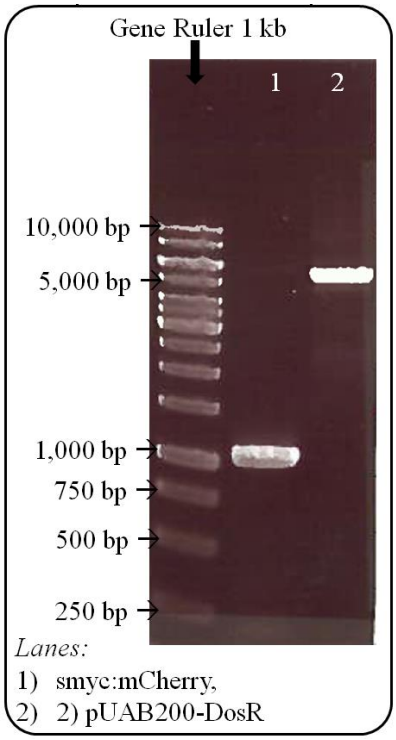


Figure 7: PCR Gel
Example of verification of PCR product DNA size on an agarose gel.

After amplification of the vector and insert were confirmed, the two pieces were FastCloned together and transformed into *E. coli*. The bacteria that incorporated the resulting plasmid successfully were selected for using appropriate antibiotics. The colonies that grew were tested to ensure that the correct plasmids were present. Figure 8 shows an example of a colony screening PCR gel. For this example, the presence of a DosR insert was being verified from a pUAB100-DosR-mCh plasmid. A "DosR Positive Control" was added to ensure that the PCR and gel were run correctly. In this example, all of the colonies tested (lanes 1-8) were positive for DosR (a 650 kb sized piece of DNA). In addition to visualization of the DNA on a gel, the plasmid was sequenced to ensure it had DosR in the correct place on the plasmid (data not shown). Once verified, the *Msm* strains were ready to be tested using the M-PFC method.

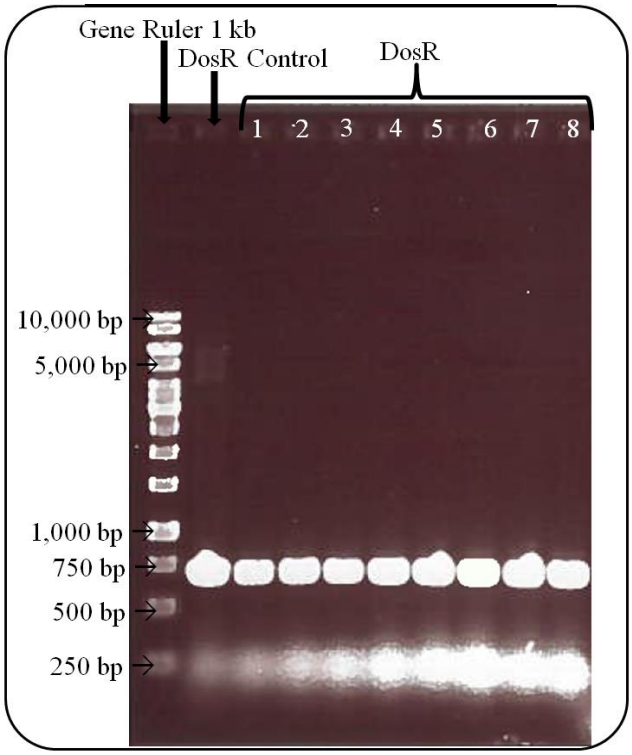


Figure 8: Colony Screen PCR for DosR
Agarose gel verification of PCR colony screen indicating all colonies contain the DosR insert in the vector.

4.2. Confirmation of DosR Homodimerization

In order to determine the reaction strength between DosR homodimerization, multiple M-PFC Alamar Blue assays were performed. Figure 9 shows a visualization of the color change in the Alamar Blue dye from blue to pink when there is PPI interaction and the two halves of DHFR come back together to confer trimethoprim resistance. In Figure 9, lane 1 is DosR/Empty, which is the negative control that does not have a "prey protein" for DosR to interact with (pUAB100-DosR+pUAB200). Lane 2 has DosR/DosR, which tests for interaction strength between DosR homodimerization (pUAB100-DosR + pUAB200-DosR). Lane 3 is

GCN4/GCN4, which is the positive control. It shows the interaction strength between GCN4 homodimerization, a known, strong PPI.

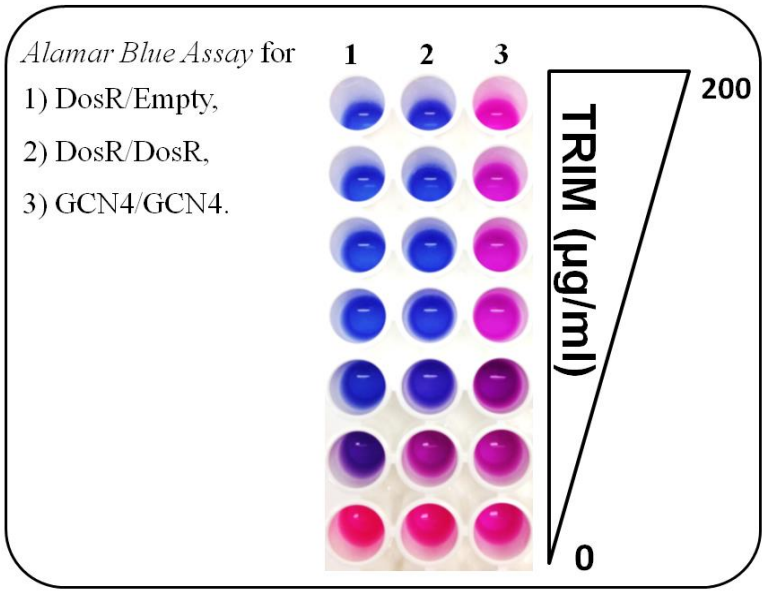


Figure 9: DosR-DosR PPI
Alamar Blue Assay indicating protein-protein interaction strength between negative control (DosR/Empty), DosR homodimerization (DosR/DosR), and the positive control (GCN4/GCN4).

A more quantitative measurement of the number of viable bacteria was generated using a plate reader by exciting at 530nm and measuring the fluorescence emission at 590nm. A heat map of the assay is depicted in Figure 10, with a 2-fold dilution of TRIM.

In Figures 9 and 10, the interaction strength of DosR/DosR is relatively lower than that of GCN4/GCN4. This could be due to multiple factors. When looking back at Figure 2, which shows the DosR dormancy pathway, the DosR homodimer is phosphorylated when it interacts. Due to a lack of a hypoxic environment, the DosR proteins may not be phosphorylated as frequently, which can lead to perceived lower interaction strength. Further research regarding

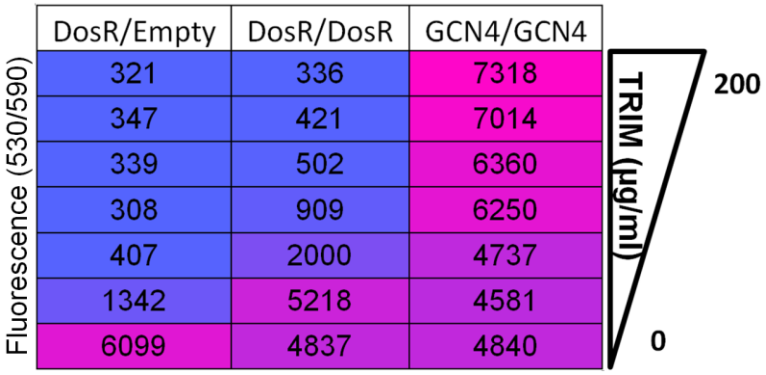


Figure 10: Alamar Blue Assay
Quantitative fluorescent read-out of the Alamar Blue M-PFC Assay indicating PPI strength between negative control (DosR/Empty), DosR homodimerization (DosR/DosR), and the positive control (GCN4/GCN4).

DosR homodimerization would need to be completed in order to better understand the binding strength.

After confirmation of PPI between DosR/DosR, the plasmids were genetically adjusted to produce mCherry fluorescent protein by addition of the mCherry gene in addition to its own promotor region (*smyc*:mCherry).

4.3. Conversion of Alamar Blue Assay to mCherry Fluorescent Assay

In order to convert the Alamar Blue assay to a mCherry fluorescent assay, we needed to optimize the amount of mCherry produced by the cells. The mCherry gene was added to a chromosomal plasmid (single copy in the cell), an episomal plasmid (~5 copies per cell), or both to test for the brightest fluorescence. Data from empty plasmids (not containing DosR:DosR) were used to test the optimal placement of mCherry. Figure 11 shows a graph of the fluorescent readout of mCherry on the chromosome, on the plasmid, and on both the chromosome and plasmid DNA constructs (data collected by Hodges, 2014). Preliminary data regarding optimization showed that the fluorescent reporter needed to be added to both the chromosomal and episomal plasmids in the assay for maximum sensitivity.

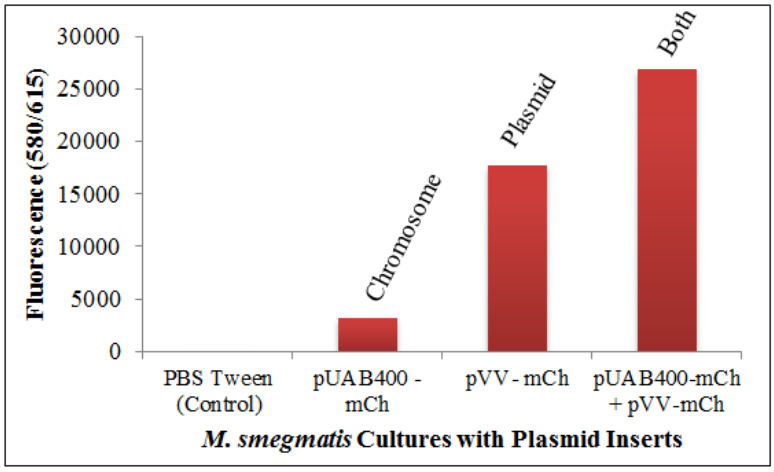


Figure 11: mCherry on Plasmid vs. Chromosome
Indication of fluorescence on the chromosome, plasmid, or both chromosome and plasmid. Fluorescent readout suggests that mCherry signaling is stronger when on both the chromosomal and episomal plasmids.

After genetically modifying the plasmids to include mCherry on both the chromosomal and episomal plasmids, we compared the results of the Alamar Blue and mCherry assays. Figure 12 shows the DosR/DosR interaction strength using an Alamar Blue M-PFC Assay to compare to a mCherry M-PFC Assay using the same strains of *Msm* and the same varying levels of TRIM. Both experiments were performed in a 96-well plate. The triplicate data has an additional column, called "Ratio of DosR/DosR : DosR/Empty," which is the ratio of the DosR/DosR to the negative control. Based on the data, the optimal range for drug screening would be at 12.5 µg/mL and 6.25 µg/mL of TRIM, as indicated by the red boxes in Figure 12. At these concentrations, the signal-to-background ratio is the highest. When looking at the data, the ratio is greater for the Alamar Blue Assay than the mCherry Assay. Additional experiments were performed in order to optimize this ratio, and to prepare the assay for high-throughput screening (HTS).

Alamar Blue Assay

Fluorescence (530/590)

TRIM (μg/mL)	DosR/DosR mCherry	DosR/Empty mCherry	Ratio of DosR/DosR: DosR/Empty
200	155	199	0.78
100	192	208	0.92
50	311	195	1.60
25	629	159	3.96
12.5	2402	176	13.67
6.25	3749	382	9.82
0	4423	3923	1.13

mCherry Assay

Fluorescence (580/615)

TRIM (μg/mL)	DosR/DosR mCherry	DosR/Empty mCherry	Ratio of DosR/DosR: DosR/Empty
200	140	137	1.02
100	271	206	1.31
50	325	189	1.72
25	536	203	2.64
12.5	1389	166	8.37
6.25	2251	346	6.51
0	3427	4987	0.69

Figure 12: Alamar Blue vs. mCherry Assay
Both assays show similar interaction strengths for both the fluorescent DosR homodimerization. The fourth column in each assay shows the signal-to-background ratio between the DosR/DosR mCherry and the negative control (DosR/Empty mCherry).

4.4. Optimization for High-Throughput Screening (HTS)

To optimize the mCherry Assay for high-throughput screening (HTS), different parameters of the assay were studied. The total volume of each well, the optical density of the bacterial cultures, the incubation time, and the concentrations of TRIM were varied. To begin optimization, the assay was miniaturized in order to reduce the total volume of the cultures used in the assay. For HTS, this reduces the volume of compounds from the chemical libraries needed for drug screening, allowing for repeat experiments and resource efficiency. The fluorescent strains were also tested in growth conditions with 0.1% DMF, which was the solvent that the drug screening compounds were dissolved in. This showed that the DMF had minimal damaging effects on *Msm*, and would not give a false negative (data not shown).

By testing these different parameters, we are looking for an optimum assay that consists of a high signal-to-background ratio, assay reproducibility, and a good drug screening selectivity range.

4.4.a. Miniaturization from 96- to 384-well plate format Plate Optimization

The total volume of each well was reduced by two thirds, from 150 µL to 50 µL. The concentrations of TRIM, optical density of the cultures, incubation time, and strains were kept constant. The data (Figure 13) showed a reduction of the level of mCherry fluorescence in the 384-well plate when compared to the 96-well plate, as indicated by the signal-to-background ratios indicated (Ratio of DosR/DosR : DosR/Empty). This could be due to a number of factors, such as less oxygen in the smaller wells, or evaporation of cultures during the incubation of the assay. In order to optimize this ratio, the incubation time and optical density (OD) of the cultures were varied to get the most optimum signal-to-background ratio for the assay.

96-well mCherry Assay

Fluorescence (580/615)

TRIM (µg/mL)	DosR/DosR mCherry	DosR/Empty mCherry	Ratio of DosR/DosR: DosR/Empty
200	203	188	1.08
100	312	200	1.56
50	597	201	2.97
25	1095	209	5.24
12.5	2717	253	10.74
6.25	4176	521	8.02
0	6005	9084	0.66

384-well mCherry Assay

Fluorescence (580/615)

TRIM (µg/mL)	DosR/DosR mCherry	DosR/Empty mCherry	Ratio of DosR/DosR: DosR/Empty
200	233	190	1.23
100	240	199	1.21
50	366	188	1.95
25	539	202	2.67
12.5	931	220	4.23
6.25	921	337	2.73
0	2129	1555	1.37

Figure 13: mCherry Assay Miniaturization
Comparison of mCherry Assay from a 96-well plate (150µL well volume) and a 384-well plate (50µL well volume). Signal-to-background ratios did drop in the miniaturized plate format; additional experiments were designed to optimize the miniaturized assay.

4.4.b. Optimization of Optical Density and Incubation Time

The optical density (OD) of the cultures and the incubation time of the 384-well mCherry assay were studied to determine the ideal time and concentration of bacteria for the assay. The OD of the cultures varied by a factor of 10; OD=0.05, 0.005, and 0.0005. The assays were read at

varying time points following inoculation; 48 hour (2 days), 72 hour (3 days), and 120 hour (5 days) incubation times.

For the 48 hour 384-well plate (Figure 14 below), averages of two replicate assays indicated that the OD=0.005 culture had the best signal-to-background ratio. However, the ratio of around 2.5 was significantly lower than the original assays discussed in previous sections. This could be due to the length of time that the culture had to grow. Perhaps the bacteria were not able to produce significant levels of mCherry during this time. The culture with an OD=0.05 suggests that there was still a high amount of mCherry fluorescent protein that was made by the negative control (DosR/Empty mCherry), as the signal-to-background ratio is very low. In addition, OD=0.05 has a broad range of TRIM concentrations with a high background signal. An optimum assay would have the signal-to-background ratio of the higher concentrations of TRIM at a 1:1 ratio. For this specific culture, the ratio is at 1.5, while the OD=0.0005 culture has a ratio of 1. In this case, although the 0.05 OD culture has a higher signal-to-background ratio initially, the 0.0005 OD has a better background ratio at the higher TRIM concentrations, which makes the 0.0005 OD culture more ideal.

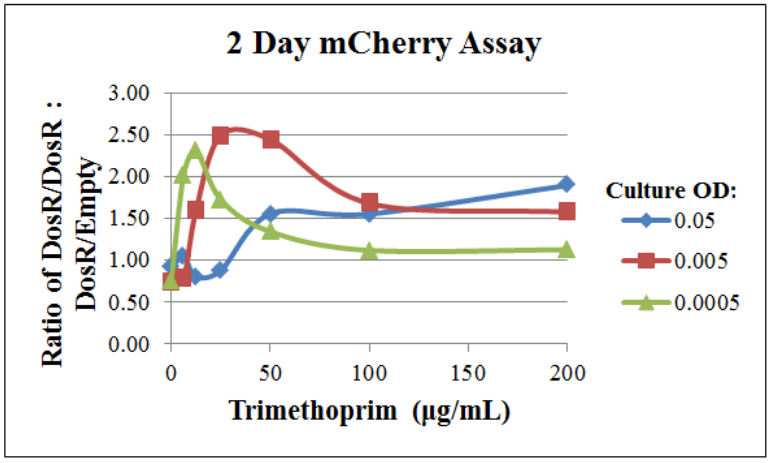


Figure 14: mCherry Variables (48 Hour)

Two-day mCherry Assay with three different starting optical densities of cultures; 0.05, 0.005, and 0.0005. Y-axis represents the signal to background ratio, and x-axis shows the increasing concentration of TRIM.

The 72 hour 384-well mCherry assay (Figure 15 below), shows an average of duplicate data from two separate assays. The data suggests that with additional incubation time, the signal-to-background ratio improves drastically, from a 2.5 to a 10 ratio of DosR/DosR : DosR/Empty for the OD=0.005 culture. From this data set, the 72 hour incubation time with an OD of 0.005 seems more optimal for HTS. However, the OD of 0.0005 culture has a better signal-to-background ratio at the higher TRIM concentrations.

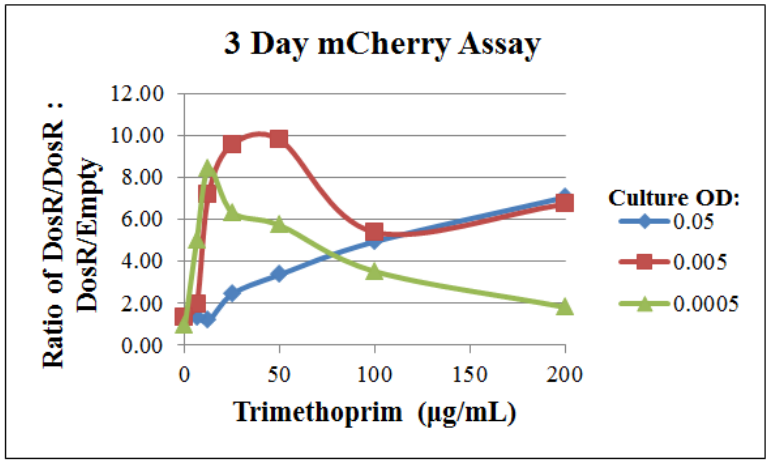


Figure 15: mCherry Variables (72 Hour)

Three-day mCherry Assay with three different starting optical densities of cultures; 0.05, 0.005, and 0.0005. Y-axis represents the signal to background ratio, and x-axis shows the increasing concentration of TRIM.

The signal-to-background ratio was increased significantly in the average of the triplicate data in the 120 hour mCherry assay (Figure 16 below). The ratio increased to ~45 for the ratio of DosR/DosR : DosR/Empty for the OD=0.0005 culture. This ratio is most optimal at the 12.5 µg/mL and 6.25 µg/mL concentrations of TRIM. The additional incubation time improves the signal-to-background ratio 20 times in comparison to the 2 day assay. The high signal-to-background ratio is important when conducting HTS, as it allows for easier detection of a "hit" (no signal), or of an inactive compound (high mCherry signal). Another important factor is that the overall signal-to-background ratio at the higher concentrations of TRIM is relatively lower than in previous assays. This is could be because there is more time for the mCherry protein to degrade from bacteria that are no longer living.

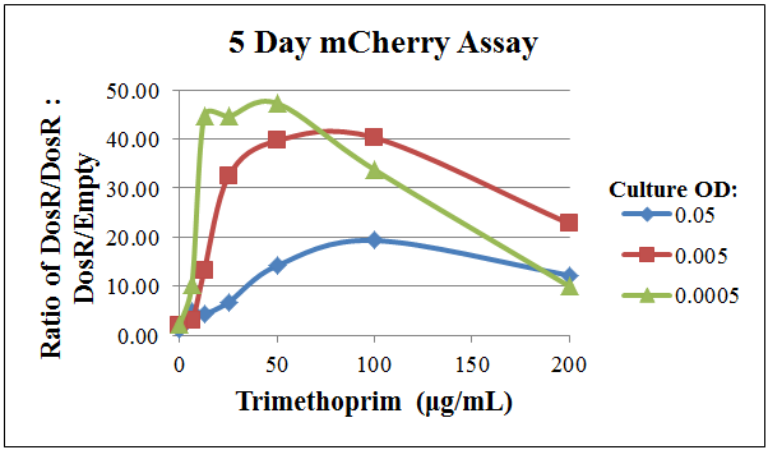


Figure 16: mCherry Variables (120 Hour)
Five-day mCherry Assay with three different starting optical densities of cultures; 0.05, 0.005, and 0.0005. Y-axis represents the signal to background ratio, and x-axis shows the increasing concentration of TRIM.

Each of the graphs above (Figures 14-16) have different y-axis scales. When changing the y-axis to a maximum height of 50 for all three assays, it is easier to compare the signal-to-background ratios for the two, three, and five day assays.

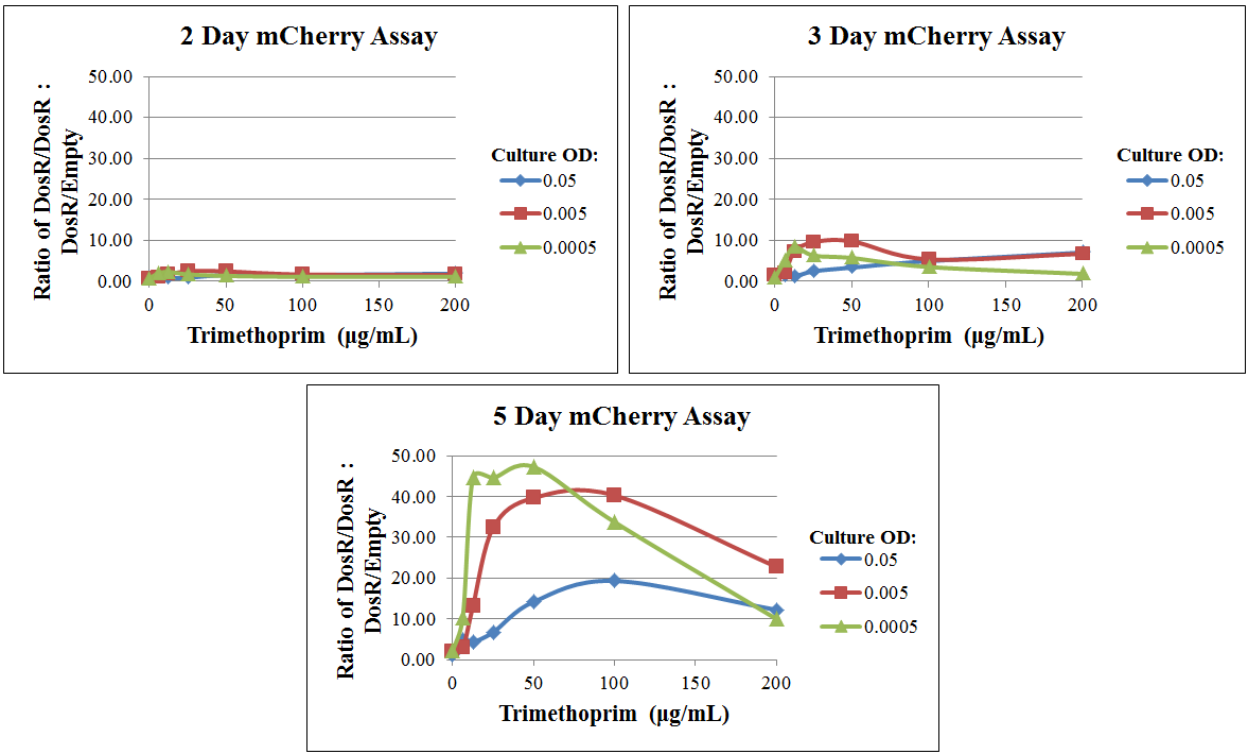


Figure 17: Comparison of mCherry Assays

Two, three, and five day mCherry Assay data, comparing the signal-to-background ration with the same scaling of the y-axis.

After finding the optimum conditions for drug screening, a 120 hour incubation time with a 0.0005 OD inoculum, another assay was set up with multiple columns of the mCherry strains with the two best concentrations of TRIM: 12.5 µg/mL and 6.25 µg/mL. The results from this assay were used to calculate a Z-factor for the fluorescent drug screening assay. A Z-factor (Z-prime or Z') is a statistical measurement of the quality of an HTS assay [58]. A Z-factor between 0.5 and 1.0 is an indicator of an excellent assay that is suitable for HTS. A score between 0 and 0.5 could potentially be used as a marginal or borderline assay, while a score below 0 shows that it would not be suitable for HTS [58]. The following equation was used to calculate Z', where SD is the standard deviation [58]:

$$Z = 1 - \frac{3SD \text{ of sample} + 3SD \text{ of control}}{|\text{mean of sample} - \text{mean of control}|}$$

Figure 18: Equation to Calculate Z'
Z-factor calculations are based on the standard deviation and mean of the sample (DosR/DosR mCherry) and the control (DosR/Empty mCherry).

Using the data from the additional mCherry assay with a starting OD of 0.0005 and a 120 hour incubation time, calculations for the TRIM concentration at 12.5 μg/mL, the Z-factor was 0.347. Calculations for the TRIM concentration at 6.25 μg/mL the Z-factor was at 0.683. This shows that the optimal concentration of TRIM that should be used in the fluorescent drug screening assay is 6.25 μg/mL. With a Z-factor of 0.683, the assay has a high statistical validity for its use in HTS for drug screening. Due to initial mCherry Assay data, in addition to the 5 day mCherry Assay, we calculated Z' for the 12.5 μg/mL and 6.25 μg/mL concentrations of TRIM. Future work will be to calculate the Z' for the 50μg/mL TRIM concentration, since it also had a good signal-to-background ratio.

4.4.c. Sanford-Burnham Drug Screen

Using the same *Msm* fluorescent M-PFC strains that were created, our collaborators at the Sanford-Burnham Medical Research Institute were able to further miniaturize the drug screening platform to a 1536-well plate format, with a Z' greater than 0.6. For preliminary testing, they used 1,280 compounds from a Library of Pharmacologically Active Compounds (LOPAC). Their pilot study initially gave 36 hits. After counter-screening of those hits, 11 of them were active. Dose-response curves were performed on these hits in order to test their effectiveness on the DosR PPI with different concentrations and exposure times. Their screen was successful with a total of 5 confirmed hits that inhibit DosR homodimerization.

5. Discussion

Due to the rise of MDR-TB cases worldwide, the need for new drugs to target novel pathways and treat *Mtb* has increased. In 2013, 9 million people became infected with TB, and thirty three percent of the world's population is currently living with TB [59]. The number of deaths per year from a TB infection is around 1.3 million people [1]. Due to the nature of transmission of this disease (breathing in aerosolized droplets from an infected patient), TB has the potential to spread to others without them realizing it. The issue of MDR-TB maximizes the negative consequences of TB, as drug-resistance limits the available drugs that can be used to treat the disease. There is also difficulty when trying to treat non-replicating, phenotypically drug tolerant *Mtb* in granulomas, which are present in patients that carry dormant or latent *Mtb*. In order to try to address this problem, this project was aimed to develop a fluorescent based drug screening platform to find inhibitors of essential PPI in *Mtb*.

The aims of this project were to develop target strains with PPI for M-PFC, develop a fluorescent drug screening platform for PPI, and to drug screen for inhibitors of DosR homodimerization. Through addition of the mCherry fluorescent protein onto M-PFC strains, the development of a fluorescent drug screening platform has been created and validated. The addition of mCherry eliminates the use of Alamar Blue dye, which removes additional use of reagents and incubation time which is advantageous when preparing strains for HTS drug screening. DosR homodimerization was used as the target for the fluorescent mCherry assay, as it is an essential PPI necessary for *Mtb* to survive *in vivo* [45]. Targeting PPI is a novel way to screen for drugs, and may prove beneficial as *Mtb* is becoming resistant to more common drug targets. The drug screen for inhibitors of DosR homodimerization comprises of two compound

libraries; a drug screen by the Sanford-Burnham Medical Research Institute, and a positional-scanning combinatorial synthetic library screen from the Torrey Pines Institute for Molecular Studies (TPIMS) that will be performed in the lab. Both of these drug screens will provide valuable data for "hits" that could potentially lead to treatment of *Mtb* infections.

During the process of optimization of the mCherry assay, different parameters of the assay were tested in order to enhance the proficiency of the assay. During the initial phases of the project, the assay was performed in 96-well plates. Through repetitions of the experiments, the assay was miniaturized to be performed in a 384-well plate. The total volume of the assay was reduced by two thirds; allowing the assay to be better suited for HTS, as fewer resources are needed for the assay. In addition to overall volume, the optical density of the cultures and incubation time of the assay were compared to find an optimal signal-to-background ratio for drug screening purposes. After multiple versions of the assay were set up, the 120 hour mCherry assay with an initial OD of 0.0005 seemed to provide the best level of mCherry fluorescence for the drug screen. The assay has been statistically analyzed to show that it is suitable for HTS, with a Z-factor of 0.683. The reproducibility of this data has been tested at Sanford-Burnham, resulting in 5 hits that actively inhibit DosR homodimerization.

Future work for this project includes conducting the positional-scanning combinatorial synthetic library drug screen from TPIMS, and to further analyze the hits from the Sanford-Burnham drug screen. Using the counter-screen by re-running the experiment without TRIM will determine if the compounds kill the bacteria regardless of DosR homodimerization. Drugs that will be further analyzed will be those that do not kill the bacteria without TRIM. Cytotoxicity

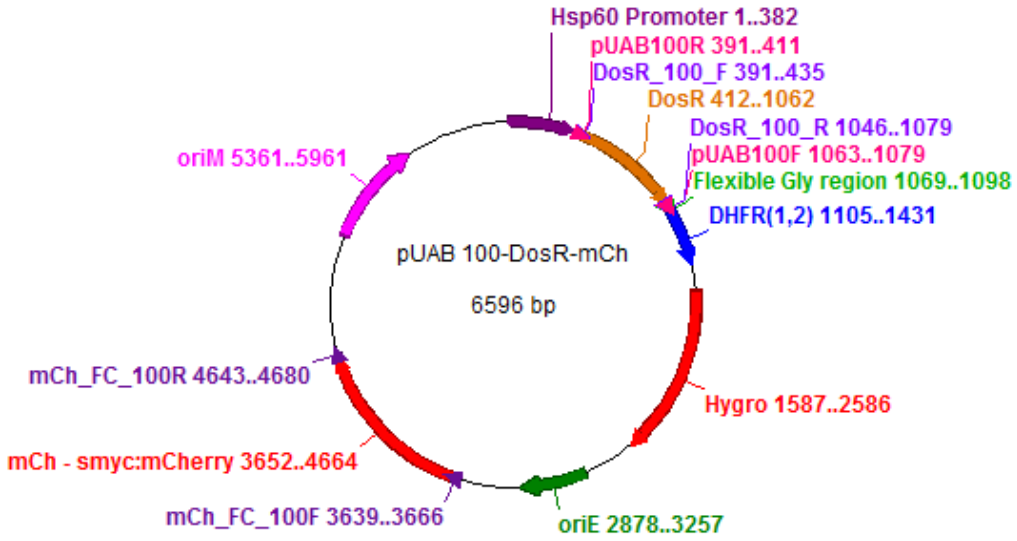
screening of the drugs will also ensure that the compounds do not also damage mammalian cells, as compounds that are toxic to humans would not be good drug candidates. Confirming the mode of action of the drug can be done using quantitative real time PCR (qRT-PCR). This method will quantitatively determine if the DosR dormancy genes are up-regulated in the presence and absence of hypoxic stress [60]. If the drug is effective at preventing these genes from upregulation in a hypoxic environment, then it can be inferred that the drug does effectively inhibit DosR homodimerization. The use of a multiple target M-PFC drug screen, with the addition of a green fluorescent strain with GCN4 homodimerization would also help to eliminate false positives during the screening process and eliminate the need for a counter-screen.

Once preliminary high-throughput screening of the positional-scanning combinatorial synthetic library has been performed using the mCherry M-PCF platform, sub-libraries of the "hits" will need to be tested further. Additional screening will be necessary to narrow the compounds down to the pure compound that inhibits DosR homodimerization.

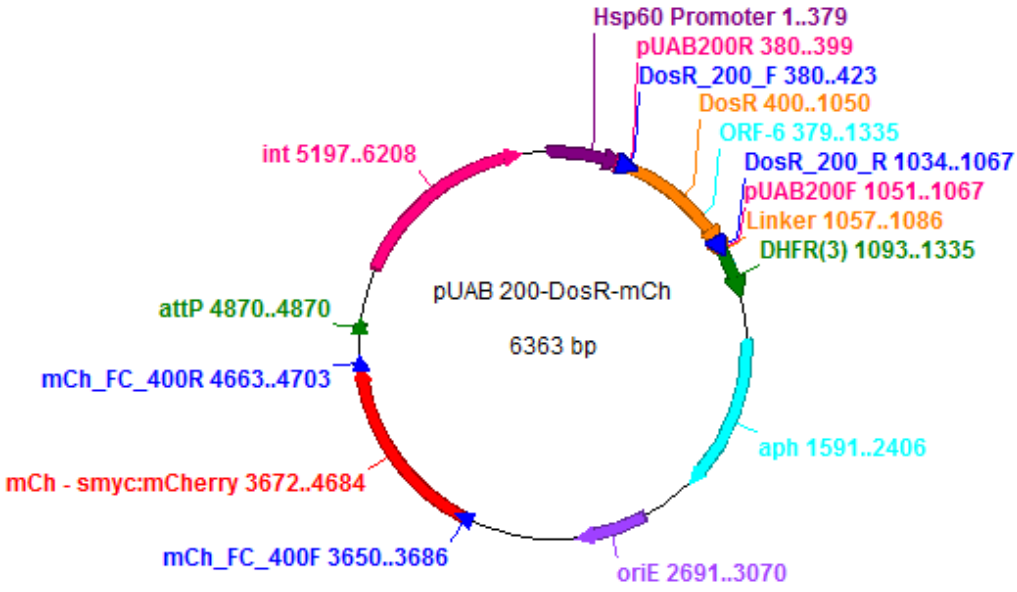
The final outcome of this project has led to a new drug screening platform, which is a novel approach to drug screening, and a method for studying protein-protein interactions. With the emergence of MDR-TB, this research project has the ability to screen for new drug targets and allow for new treatment options for those that suffer from TB worldwide. This project has the possibility to change the way that drugs against TB are screened, and can lead to the discovery of novel protein-protein interactions that can help us understand the mechanisms behind the resilience of this infectious pathogen.

Appendix: Supplemental Figures

Appendix: Supplemental Figures



Supplemental Figure 1: pUAB100-DosR-mCh Plasmid



Supplemental Figure 2: pUAB200-DosR-mCh Plasmid

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