Development of Novel Fluorescent Tools for Investigating Virulence Factors and Drug Susceptibility in Mycobacterium tuberculosis

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DEVELOPMENT OF NOVEL FLUORESCENT TOOLS FOR INVESTIGATING VIRULENCE FACTORS AND DRUG SUSCEPTIBILITY IN MYCOBACTERIUM TUBERCULOSIS

by

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ABSTRACT

*Mycobacterium tuberculosis* (Mtb) is the causative agent of Tuberculosis (TB), a life-threatening disease primarily affecting the lungs that infects about one third of the world’s population and causes 1.3 million deaths annually. It is estimated that TB has been infecting humans for around 70,000 years and has killed more people than any other infectious disease. The highly effective, persistent, and multifaceted virulence strategies that have allowed Mtb to continue to spread and thrive for so long are still poorly understood at the molecular level. This lack of knowledge contributes to ongoing challenges to curing TB. Although drugs capable of killing Mtb exist, even strains that are susceptible to these drugs remain so difficult to treat that stringent six- to nine-month courses of four-drug cocktails are required. Practical difficulties in administering full treatments and patient noncompliance have contributed to a rise in drug-resistant TB cases globally. To combat this increasing world health problem, new antibiotic treatments that kill Mtb and drug-resistant Mtb more effectively via new mechanisms of action are necessary. Discovering these antibiotics expediently requires that innovative Mtb-specific drug-screening assays are developed.

An ideal and innovative TB drug screening method would target validated protein-protein interactions (PPI) essential to Mtb’s pathogenesis and would be performed on whole Mtb cells under relevant *in vivo*-like conditions. This project focused on engineering several tools relevant to creating an ideal TB drug screen. A protein fragment complementation assay capable of studying PPI of the TB gyrase complex was created, and this assay was assessed for future HTS applications. To streamline the readout, this assay was re-engineered to include green fluorescent protein. Modifications to the red fluorescent protein mCherry, including the creation of a large Stokes shift mutant mCherry and an mCherry bimolecular fluorescence complementation assay, were also engineered and investigated.
DEDICATION

To my parents, who taught me that stubbornness, too,
can be a virtue when wisely applied.
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CHAPTER ONE: AN OVERVIEW OF TUBERCULOSIS

1.1 Tuberculosis: A Persistent World Health Problem

*Mycobacterium tuberculosis* (Mtb) is the bacterium that causes the respiratory disease Tuberculosis (TB) which, according to the World Health Organization (WHO), was responsible for the deaths of 1.3 million people in 2013 [1]. In the same year, 8.6 million people were newly infected with Mtb, contributing to the estimated 33% of the world’s population that is latently infected with TB [1, 2]. The most heavily affected regions are in Asia and Africa, especially in very densely populated countries like India [3]. TB continues to spread and thrive because it features highly effective, persistent, and multifaceted virulence strategies that are still poorly understood, as well as a pattern of infecting populations that are prominently afflicted with human immunodeficiency virus (HIV) like those in Sub-Saharan Africa [3].

The toll on human life and health caused by Mtb is not a new problem. In fact, it is thought that more people have died from TB throughout history than from any other infectious disease [4]. Humans have been afflicted with TB for about 70,000 years, and it is this remarkable “timelessness” in the face of human lifestyle changes and advances in modern medicine over millennia that makes the puzzle of curing TB both interesting to study and challenging to unravel [4]. Despite global tuberculosis reports by the WHO that imply TB is gradually declining, other evidence suggests that TB still ought to be treated as a growing world health problem [5]. For example, more people are currently afflicted with the contagious form of TB than at any other time in history, and global control of the disease is dually threatened by the emergence and spread of drug-resistant TB [4, 5]. Considering these challenges alongside the ongoing lag in production of improved antibiotics to
combat them, it is important that we advance our knowledge of the pathogenesis of Mtb. Hopefully we can exploit this knowledge to generate more effective treatments for TB in the future, mitigating TB’s global detrimental impact.

1.2 Basic Pathogenesis of Mtb

Critical to the spread, survival, and evolution of Mtb is its ability to cause infection and disease in a human host. The primary site of infection is the alveolar space. Depending on the interactions between the host and the bacteria following the initial infection, two distinct disease states are observed: latent or active TB. In 90% of cases, infection with Mtb does not result in an active disease state, at least at first, because the immune system contains the pathogen inside walled off, aggregated clusters of immune cells called granulomas in the lungs [3]. This containment in many cases may sterilize the infection, particularly if the rate at which Mtb is attacked by the adaptive immune response is relatively high [6]. However, Mtb is often able to survive the host’s defenses, remaining dormant in the body inside these granulomas. This is termed a latent tuberculosis infection. From inside this niche, Mtb is able to continue its life cycle, using specialized virulence factors to take advantage of the latent infection and persist until the host’s immune defenses break down. At this point, Mtb escapes its replicative niche and becomes transmissible via aerosolized droplets, which is termed active TB.

Unlike other highly successful bacterial diseases—cholera, typhoid, plague, and diphtheria, to name a few—the causative agent of TB does not feature many of the virulence factors that are classic to these other pathogens [4]. For example, it is notable that Mtb features no capsule for evading phagocytosis, no pili or adhesins for attaching to host tissue, no flagella for motility, and few enzymes or toxins with which to damage host cells [4]. Rather, Mtb’s pathogenic lifecycle has been
honored so that it does not need these virulence factors to establish disease; Mtb sidesteps innate immune system barriers and hijacks host macrophages in order to reach deep sterile sites and cause infection [4]. Normally a pathogen is engulfed by macrophages via phagocytosis and a phagolysosome forms containing degradative enzymes and toxic molecules to destroy the pathogen, but Mtb is able to survive inside the host macrophages. Mtb does this by manipulating phagosome maturation to avoid acidification and fusion with the lysosome. From this point, Mtb is able to continue evading, modifying, and exploiting both the adaptive and innate immune system to further disease in its host.

Infection with Mtb is initiated via an aerosolized form of the bacteria that is produced when individuals who are in the contagious, or “active” stage, of TB cough in the vicinity of others. Upon inhalation, small droplets containing one to three bacteria enable Mtb to access lower lung tissue and to begin establishing disease by engaging with alveolar macrophages [4]. Mtb’s surface is awash in pathogen-activated molecular patterns (PAMPs) that would typically activate Toll-like receptor (TLR)-mediated signaling to recruit microbicidal macrophages, which are primed to destroy invading mucosal pathogens. However, pathogenic mycobacteria express a surface lipid that masks their PAMPs and reduces the activation of TLRs to save them from destruction by these innate immune cells [4]. Additionally, Mtb is able to express a related surface lipid that triggers recruitment of growth-permissive macrophages that allow Mtb to invade them [4]. This strategy is aided by the small infectious droplets delivering Mtb directly to the alveolar space in the lower lung, where few commensal bacteria exist to stimulate TLR-signaling and ruin Mtb’s disguise [4]. The fact that alveolar macrophages are programmed not to induce strong inflammatory responses upon engulfing pathogens that enter the alveolar space is also conducive to the initial establishment of the infection [7].
The growth-permissive macrophages first recruited by Mtb to its entry site become infected with the bacteria, and the bacteria are subsequently transported across the epithelium of the lung to deeper tissues [4]. Migratory immune cells of the host respond to the early infection by transporting some of the bacteria to nearby lymph nodes [6]. Here, the process of generating T cells that are primed to act against Mtb begins; although, about two weeks elapse between Mtb entering the alveoli and the beginning of this adaptive immune response [6]. In this time, the early stage infection gives way to expansion as new groups of growth-permissive macrophages are recruited by Mtb and subsequently infected [4]. The initially infected macrophages die via apoptosis once the number of bacteria inside them is sufficiently high [4]. Still containing viable bacteria, the dead macrophage contents are engulfed by the newly recruited macrophages to expand the infection [4]. By the time antigen-specific CD4+ T cells proliferate in the lymph node and are distributed to the infection site, their ability to reach infected macrophages and aide in resolving the disease is limited by excessive accumulation of recruited macrophages around the infection site [6]. As the host’s immune response advances, the bacteria eventually cause granuloma formation in the lungs through an intricate process of manipulation and subsequent aggregation of macrophages and other immune cells into these tightly-linked, epithelioid clusters [4]. In this way, the granuloma exists alongside the expansion of Mtb’s niche in the host, as Mtb continues to transcriptionally induce new genes it needs to adjust to the ever more hostile environment of the growing granuloma. An adaptive immune response to Mtb’s expansion of the granuloma may wipe out the tubercle bacteria, but in many cases the mycobacteria employ further strategies to persist against the adaptive immune system. For example, it is believed that by delaying the priming, arrival, and activation of T cells while continuing to delay responsiveness of macrophages, Mtb is able to hold on to its replicative niche long-term [4]. From the host’s perspective, combatting Mtb becomes a delicate balance. On one hand, the immune
response must contain the bacteria to prevent it from disseminating outside the granuloma and infecting other organs [6]. On the other hand, the immune response must be tempered to prevent excessive inflammation that hinders the efficacy of the adaptive response and causes collateral tissue damage [6].

After forming a mature granuloma, the final step in Mtb’s life cycle is to escape from the granuloma so it can exit its host and spread to a new one, beginning the infection cycle again. Indeed, transmission occurs most efficiently from hosts with cavitating mature granulomas [4]. How the shift between a granuloma supported by the apoptosis of macrophages and a cavitary granuloma occurs is not fully clear; however, it is clear that the necrosis of macrophages infected with Mtb is responsible for releasing intact bacteria into the tissue of the bronchial tree [4]. From within this medium, the mycobacteria can multiply and enter the airway to be aerosolized in cough droplets and spread readily to new hosts through proximal air [4]. This is called an active TB infection. Some people’s immune defenses are overcome by Mtb later than others, which is why the bacteria may remain latent for years before an active disease state manifests. For individuals with latent TB there is a 5-10% risk each year that the latent state will develop into an active infection, and in HIV-TB coinfected individuals there is approximately a 50% lifetime risk of reactivation [3]. When drug treatment is not effectively applied or the Mtb causing the infection is resistant to treatment, TB remains contagious and also causes life-threatening complications like cavities in the lungs that bleed and can be infected with different bacteria, holes in the airways of the lungs, or blocked airways that strain breathing [3]. At this stage, active TB is simultaneously a serious danger to the infected individual’s life and a threat to their community.
1.3 Challenges To Curing Tuberculosis

Once an infection has begun, Mtb is very difficult to eliminate because of its persistent and diverse virulence factors, which continuously challenge the immune system’s ability to clear the infection. In this way, Mtb creates niches in the body where it can persist in evading the immune system as it acquires nutrients and continues to grow. These Mtb-friendly niches combined with unique features of Mtb also give the bacteria an advantage when drug treatments are administered to clear the infection. For example, the signature feature of Mtb’s cell wall is its high mycolic acid content, which provides intrinsic resistance to antibiotics [8]. The location of Mtb inside macrophages and within granulomas further reduces the accessibility of the bacteria to antibiotics. Other features of Mtb, like its efflux pumps or its ability to persist in a dormant state and develop phenotypic drug resistance, also challenge administered drug treatments.

Although TB has been considered a treatable and curable disease because drugs capable of killing Mtb exist, even strains that are susceptible to these drugs remain so difficult to treat that stringent six- to nine-month courses of four-drug cocktails are required [5]. The small variety of drug treatment regimens available for TB, both active and latent, are limited in efficacy and difficult for the patient to maintain. Typical treatment recommendations for drug susceptible strains advise an initial two-month intensive regimen of isoniazid (INH), rifampicin (RIF), pyrazinamide, and ethambutol [5]. Afterwards, a four-month continuation phase with just INH and RIF is common for up to six-months for active infections, or a four to nine months for latent TB [9]. Every dose during these phases must be taken daily without fail to cure the patient, so doctors are frequently required to monitor patients directly to ensure compliance. Also, since these drugs are aggressive and prescribed for extended periods of time, treatment causes side effects that further discourage patients from completing the regimen. The high financial burden of completing treatment is an
additional barrier to curing Mtb infection even when it is susceptible to these first-line drug cocktails.

### 1.3a An Emerging Threat: Drug Resistant Mtb

The poor efficacy of current antibiotics and the noncompliance associated with their regimens are contributing to the advancement of drug resistant Mtb strains. Records indicate a significant increase in Mtb strains worldwide that are resistant to the front-line TB drugs. The WHO estimated in 2014 that 3.5% of newly acquired TB and 20.5% of previously treated TB cases are resistant to first-line drug treatments [5]. Classified by their level of resistance, Mtb causes Multidrug resistant TB (MDR-TB), eXtensively drug resistant TB (XDR-TB), and Totally drug-resistant TB (TDR-TB). MDR-TB is caused by Mtb strains resistant to the powerful activities of RIF and INH, and currently has a cure rate of only around 48% globally [5]. Approximately 480,000 people developed MDR-TB in 2013 [1]. Treatments for MDR-TB rely on second-line drugs, like fluoroquinolones and aminoglycosides, and last upwards of two years in many cases [5]. These second-line drugs are remarkably more toxic to the patient, less effective, and more expensive [5]. XDR-TB results from strains of Mtb that are additionally resistant to second-line drug treatments, and the strains that cause TDR-TB are entirely resistant to all available treatment options [10]. Patients infected with XDR- and TDR-TB face a bleak prognosis, and some postulate that the spread of these strains would result in a “return to a pre-antibiotic era” [11]. Weak adherence to treatment courses and drug regimens that are incorrectly chosen or poor in quality are the central contributing factors to the emergence of drug resistant TB in individual patients [5].

Mtb generally develops drug resistance in two ways, either genetic or phenotypic [5]. Genetic drug resistance arises when fixed genetic mutations occur in a particular strain of Mtb that disrupt
the ability of a drug compound to interfere with its target. For instance, spontaneous mutations may arise that modify the target and block drug binding, the target may become overexpressed, or a series of genetic changes may occur in concert within the bacteria to mediate drug resistance [5]. Phenotypic drug resistance to an antibiotic arises when Mtb in the host becomes transiently resistant without fixed genetic mutations occurring [5]. For example, Mtb replicating within macrophages are able to turn on efflux pumps that allow them to tolerate antibiotics by actively pumping the foreign compounds out of their cells [5]. Understandably, this contributes significantly to the long treatment periods required to cure patients of TB, which in turn contributes to an increase in opportunities for drug resistant strains to take hold.

Considering the significant threat posed by TB and drug-resistant TB worldwide and the limited number of drug treatments available, it is important to discover new antibiotics that treat TB more effectively by acting against shrewdly selected targets. Current drugs inhibit general targets, like the synthesis of cell wall components or RNA and protein synthesis. By contrast, novel specific drug targets, like under-studied essential protein-protein interactions occurring within Mtb, have the potential to reveal antibiotic compounds that are both new and more effective at combatting drug-resistant and dormant TB.
CHAPTER TWO: DRUG DISCOVERY EFFORTS

The outlook on TB drug discovery is bolstered by extraordinary potential for the future, but currently the establishment of new TB treatments is still marked by slow progress and limited success. In fact, in 2012 bedaquiline became the first TB drug approved by the Food and Drug Administration in over forty years that kills Mtb through a novel mechanism of action [12]. Arguably the most significant aide to TB drug discovery has been knowledge of Mtb’s entire genome sequence and the advances in understanding the molecular biology of Mtb. Considering that the majority of the genes encoded in Mtb still have unknown functions, there remain significant gains to be made both in unraveling the biology of Mtb and in exploring novel drug targets. This potential combined with the current gap in new TB drug candidates and the desperate need for novel TB drugs motivates us to engineer innovative drug discovery platforms that improve upon current TB drug discovery methods.

2.1 Existing TB Drug Discovery Strategies and Their Problems

TB drug discovery and development today is a costly and time-consuming endeavor that requires investigators to take an interdisciplinary approach at each step of the process. The drug development pipeline can be broken down into two parts: early pharmaceutical research and late pharmaceutical research and development. Projects focused on the early stage of drug development aim to identify and validate targets and to identify and optimize lead compounds based on “hits” identified for these targets during drug screens. Within this stage, two main strategies are commonly employed: target-based biochemical screening and whole-cell based screening [13]. Target-based screens are hypothesis-driven, analyzing the effects of a library of compounds on a purified target protein that is mechanistically relevant to pathogenesis. Meanwhile, whole-cell screens analyze the
ability of drug compounds to kill or inhibit a pathogenic phenotype of bacterial cells without focusing on a particular pathway. Target-based screens have traditionally proven problematic and unsuccessful because of their tendency to identify hits that are potent against the target of interest but that ultimately are not capable of eliminating bacterial growth [13]. This most commonly occurs because the compound cannot actually penetrate the bacterial cell, either \textit{in vitro} or \textit{in vivo}, or because the bacteria are capable of inducing efflux of the compound from effectively inhibiting its target inside the cell or from crossing the cell wall to begin with [12]. Whole-cell screening, by comparison, overcomes issues of cell-penetration and cell-efflux and has therefore been relatively successful as a strategy for TB drug discovery, making it the go-to method for identifying hits [12]. Through whole-cell screening, investigators may identify hits for sensitive and accessible pathways that might not have been predictable targets, but this strategy still leaves room for improvement. Mainly, the efficiency of whole-cell screening is compromised because it requires that the target of the hit be identified retroactively in order to optimize the potency of the hit compound [12]. If identification of the hit’s target takes a long time or is completely unsuccessful, this optimization process can be significantly delayed. Recent reviews also point out some informative patterns that have emerged as a result of extensive use of whole-cell screening over the past 5-10 years, which provide reason to think that moving beyond simple whole-cell screening has become necessary [12]. For instance, it has been observed that whole-cell screens performed by various groups exhibit redundancy, identifying hits that are structurally identical or nearly identical repeatedly [12]. This is likely due to the lack in diversity of the screening methods utilized and the compound libraries [12]. Additionally, whole-cell screening results exhibit redundancy in the mechanistic targets identified for various hits, most of which are proteins involved in the cell surface or bound to the cell membrane [12]. On the one hand, this is a positive aspect of simple whole-cell screening—the most accessible targets are the
first to be identified. However, to combat a disease as prone to drug resistance as TB, it is not enough to recycle the same targets and the hits for these targets redundantly. It would be remiss not to use knowledge recently gained about the molecular biology of Mtb to investigate the potential of other novel, sensitive targets by rethinking traditional screening methods. Specifically, methods that are capable of combining target-specificity and whole-cell screening to eliminate the main drawbacks of each of these methods alone are the ideal next step.

2.1a Characteristics of an Ideal TB Drug Discovery Approach

An ideal TB drug screening method would focus on validated targets and would be performed on whole Mtb cells, with the option to perform the screen under relevant in vivo-like conditions. Target-specific whole-cell screens of this sort are more likely to succeed in identifying hits that will translate to drug development because they ensure that the hit can penetrate the cells while also demonstrating that a given hit interferes with the desired target. This allows the efficacy of the lead compound to be optimized expediently and also more quickly provides information that may assist investigators to identify patterns that make particular target/hit combinations successful. Targets selected for these screens ought to be novel and known to be essential for Mtb’s pathogenesis. Novelty becomes an increasingly important factor as treatments are sought to combat the spread of drug-resistant strains of Mtb. Re-examining old targets that are already at the crux of drug-resistant strains is not likely to produce new therapies that would be clinically effective long-term, as these targets have already proven they can be readily mutated to the bacteria’s advantage. An ideal drug discovery platform would also be well-adapted to high-throughput screening (HTS), with emphasis on optimizing the time, costs, and quality of the screen for lead discovery [13]. A high quality screen features few false positives and few false negatives, a chemically diverse and novel
screening library, high signal/noise ratio, and a Z’-factor between 0.5 and 1 [13]. The Z’-factor is a coefficient that defines the optimal window within which a particular assay’s readout signal is statistically significant [14]. The Z’-factor is used as an indicator of the quality of an assay, and it reflects the level of confidence with which the assay will be able to identify hits [14]. Other factors of an ideal assay include a relatively inexpensive and simple set up, an easily quantifiable, highly specific readout, and great versatility in the targets to which the screen can be applied. A screen meeting these standards would represent a promising step toward discovering novel drug therapies to treat TB.

2.2 Protein-Protein Interaction-Based Drug Discovery: An Innovative Strategy

The significance and complexity of protein-protein interactions (PPIs) that are vital for regulating most of the biological processes in both prokaryotic and eukaryotic cells cannot be overstated. The ability of Mtb to evade immune defenses and to cause disease is intrinsically dependent on its unique network of protein-protein interactions. A variety of novel methods for studying PPIs have been applied across many species of bacteria to elucidate the mechanisms underlying vital processes like DNA replication, metabolism, and formation of enzyme complexes as well as virulence factors that interact with host cells. Knowledge of these mechanisms has driven the design of improved and novel treatments for diseases that result directly from specific cellular processes. However, the PPIs that are critical for Mtb pathogenesis have not been explored in depth and are still very poorly understood. This gap in knowledge signifies vast potential because increased examination of essential Mtb PPIs would reveal important aspects of Mtb pathogenesis that could be exploited as specific drug targets. Inhibiting the formation and activity of critical protein complexes by targeting the interfaces between interacting proteins would represent a
promising yet underexploited avenue to address the urgent need for improved treatment options for TB. This approach is also highly compatible with the ideal target-specific whole-cell screen already described.

Discovering small molecules capable of interfering with PPIs was once viewed as far too challenging and was initially marked by very little success [15]. However, it has become increasingly evident that successfully targeting PPIs with small-molecules is a promising path to drug discovery. Over 40 PPIs relevant to various diseases have been successfully targeted with synthetic molecules, and several of the lead compounds identified have progressed to clinical trials [16]. The advances that have been made in specifically designing peptides and small molecules to interfere with certain PPIs will only continue to accelerate the efficacy of this approach. The progress made in targeting PPIs primarily came from the identification of “hotspots” critical to the interactions of protein-protein interfaces [16]. These hotspots are small subsets of the residues involved in the total, large protein-protein interfaces that contribute the most to the free energy of binding between the proteins [15]. The existence of hotspot regions refuted the concern that PPI interfaces could not be targeted by small molecules, when it was initially thought that large, flat contact surfaces between proteins were necessarily rigid and would not offer spots for small molecule binding [15]. Recent successes have demonstrated consistently that contact surfaces do provide conformationally-flexible cavities available to small molecules for tight binding and interference of PPI [15]. Concerns over the sheer lack of success of HTS applied to protein-protein interfaces have also been raised. But because empirical evidence has shown that HTS can successfully lead to valid hits against PPIs, it is important to consider that HTS’s lack of success has likely been due to the libraries of compounds that are traditionally most readily available and most utilized for screening [15]. These libraries were often optimized for screens against traditional drug targets like G-protein-coupled receptors and
enzymes, while it has become clear that each PPI is distinct and the scaffolds that worked to unlock these traditional targets are unlikely to do the same for the unique interfaces that are critical for distinct PPIs [15]. A third common objection to PPI-based screening was the notion that small molecules are not capable of displacing protein partners effectively because native protein complexes have a higher affinity for one another. However, it has been demonstrated that under equilibrium conditions small molecule inhibitors can often be found to have an advantage over the natural protein partner, binding deep within the contact hotspot with high, drug-like efficiency [15]. Given the mounting evidence that PPIs can be successfully exploited for drug discovery, the incredible potential exploiting novel PPIs in Mtb holds, and the compatibility of PPI-based screening with the ideal TB drug discovery approach, pursuing the development of a drug screening platform that targets PPI is an innovative and favorable endeavor.

2.2a Mycobacterial-Protein Fragment Complementation

Protein-fragment complementation (PFC) assays have been remarkably useful for studying PPI in a variety of biological systems. This utility led to the development of a method called Mycobacterial-Protein Fragment Complementation (M-PFC). The M-PFC assay uses a viability readout to assess whether two proteins interact in *Mycobacterium smegmatis* (Msm) which is utilized as a surrogate host in place of Mtb. This works by fusing the “bait” protein to one domain of the split dihydrofolate reductase (DHFR-A) reporter and fusing the “prey” protein to the other domain of the split DHFR reporter (DHFR-B) [17]. Each of the two plasmids containing the bait/DHFR A and prey/DHFR B elements are also resistant to kanamycin or hygromycin so Msm cells containing both plasmid inserts can be selected following transformation [17]. The two halves of DHFR protein are inactive when they are apart, but if the bait and prey proteins interact in Msm, the
DHFR halves are reconstituted into an active DHFR protein [17]. DHFR confers resistance to trimethoprim in Msm. Therefore, if the bait and the prey are positive for interaction, the bacteria survive when grown in the presence of trimethoprim (see Figure 1).

To acquire a quantitative measure of PPI binding, cultures of the M-PFC Msm strains are incubated in the presence of TRIM. Afterwards, Alamar blue dye (resazurin) is used as a readout to reflect the viability of the bacteria in response to their exposure to trimethoprim. When bacterial growth occurs, the blue resazurin is reduced by the bacteria to a fluorescent pink color, the intensity of which can be readily quantified using a plate reader [17].

The M-PFC has also been translated to screen a variety of targets for compounds that are effective at preventing the targeted PPI from occurring in Mtb, killing the bacteria’s virulence [17, 18]. Effective compounds prevent the protein partners from interacting, so DHFR-A and DHFR-B are not reconstituted. Therefore, the bacteria cannot survive when grown in increasing
concentrations of trimethoprim, which is displayed by a lack of fluorescence upon incubation with resazurin (See Figure 2). Prior to the development of M-PFC, it was not possible to set up screens for inhibitors of PPI in Mtb because tools capable of reporting the interruption of PPI inside mycobacteria were not available [17]. Now the M-PFC assay is a highly versatile approach for screening for compounds that are active against specific PPI targets in a whole-cell context in mycobacteria. Thus, it is an ideal starting-point for developing an innovative TB drug screening platform based on PPI inhibition. Establishment of strains showing PPI of novel targets combined with fluorescent improvements to the M-PFC are critical to improving the quality and practicality of this platform.

![Figure 2: The Relation Between PPI, TRIM Resistance, and Resazurin Reduction in M-PFC Strains of Msm.](image)

The stronger the interaction between the protein partners of the M-PFC strain, the greater the reduction is of blue Resazurin to pink, fluorescent Resorufin at increasing concentrations of TRIM.
CHAPTER THREE: TB GYRASE AS A NOVEL TARGET OF THE M-PFC DRUG DISCOVERY SYSTEM

3.1 The Role of TB Gyrase

TB gyrase is a well-studied and clinically validated target for TB drug discovery that plays a vital role in the bacterial cell. Like the topoisomerase enzymes found in other bacteria, TB gyrase is necessary to maintain the functionally required topology of the DNA molecules in Mtb [19]. During the processes of replication and transcription topoisomerase enzymes act to moderate the winding of DNA about itself, regulating the level of tension the DNA molecule experiences as a result of its double-stranded helical structure and closed circular domain in bacteria [19]. The type I topoisomerase family is able to cut one strand of the DNA at a time to reduce stress on the helix by passing it through a single-stranded break and reannealing it [20]. But type II topoisomerases act on both strands of DNA, cutting two strands to produce a double-stranded gap through which another portion of the duplex is passed and then reannealed [20]. Bacterial DNA gyrase is a type II topoisomerase that is present in all bacteria [20]. In Mtb, just two topoisomerase genes are present: DNA gyrase is the only Type II topoisomerase, and a homolog of the E. coli topoisomerase I gene is also encoded [20].

As the only available type II topoisomerase in Mtb, DNA gyrase introduces negative supercoils in the DNA, relaxes positive supercoiling, and moderates decatenation of replicated DNA [20]. The structure of the complete TB gyrase tetramer is not yet available; however, the structures and arrangements of components of the tetramer have been solved independently [21, 22]. Plus, the gyrase gene in E. coli and other bacteria and the gene in Mtb are sufficiently conserved so that it is reasonable to draw missing information about the structure and function of TB gyrase from facts
known about gyrase in other bacteria [23]. To form a series of gates through which DNA can be threaded, the enzyme forms a tetramer consisting of two A subunits and two B subunits, encoded by *gyrA* and *gyrB* respectively [20]. The opening and closing of these gates to break the DNA, pass another DNA segment through the break, and rejoin the break is coupled to ATP hydrolysis [24]. The breakage and reunion steps are catalyzed by the A subunit, while the B subunit contains the ATPase domain [20].

The A protein’s (*GyrA*) core features two dimer interfaces: the primary dimer interface and the head dimer interface [24]. It is believed that the N-terminal interface binds the DNA that will form the gate for strand passage, while the C-terminal domain of *GyrA* (*GyrA-CTD*) is thought to mediate changes in supercoiling by binding closely spaced segments of DNA and encouraging them to wrap around the protein in the required direction before reannealing takes place [20]. Studies of the full length *GyrA* reveal that the dimeric 59kDa core is flanked by the two pear-shaped *GyrA-CTD* monomers [20]. Importantly, *GyrA* is capable of dimerizing and binding DNA in the absence of the B subunit, but for all of the gyrase reactions to take place both subunits must be present [19].

The B protein (*GyrB*) features an ATPase site N-terminal domain, which dimerizes when bound to ATP [20]. The C-terminal domain of *GyrB* is responsible for interaction with the *GyrA* protein and its bound DNA [20]. Protein-protein interactions, recycling of the enzyme, and interaction of the transfer segment of the DNA with gyrase is dependent on the ATPase activity of *GyrB* [20].

### 3.2 TB Gyrase A: A Worthwhile M-PFC Target

While investigating novel targets for the development of new classes of antibiotics is a valid and promising strategy in the search for new TB drugs, exploring targets like TB gyrase that are
already relatively well-understood and clinically validated also has its merits. This approach benefits greatly from the knowledge that any chemical inhibitors discovered against the target will successfully inhibit the growth of Mtb. Additionally, more strategic selection of the compounds that will be screened against the target can be performed. The specific sites of the target that have been most successfully targeted by old antibiotic compounds can be examined and used as a reference for generating new compounds that will interfere with the target site more effectively. A caveat to this method concerns antibiotic resistance. Generating new drugs by studying a target site that has previously been extensively exploited is not effective if Mtb has already developed drug-resistance mutations at that site. This can happen when Mtb has been exposed to previous classes of antibiotics that target the same site redundantly. Therefore, investigating a validated target site that also contains low levels of drug-resistance mutations in clinical isolates is advantageous.

The fluoroquinolones are a class of powerful antibiotics that bind to the DNA-gyrase complex and interfere with the enzyme’s normal function, leading to the accumulation of double-stranded fragments of DNA and the death of the treated bacteria [20]. Fluoroquinolones are capable of killing mycobacteria and have shown promise in reducing the length of TB treatment regimens [20]. These abilities have been strongly correlated with their inhibition of DNA gyrase [20]. Inhibiting DNA gyrase is an effective way to kill non-replicating, persistent mycobacteria, and older fluoroquinolones have been effective as part of treatment regimens for MDR-TB [20]. Additionally, levels of resistance to fluoroquinolones remain low in clinical isolates of Mtb, suggesting that Mtb’s resistance to novel compounds that would act on DNA gyrase is desirably low [20]. Thus, TB gyrase is a valid and promising target for discovering new drugs to treat TB. Because fluoroquinolones are known to act on GyrA specifically, developing a drug screen capable of identifying compounds that disrupt the GyrA dimer is a worthwhile endeavor. While fluoroquinolones have been the most
effective existing compounds to target DNA gyrase thus far, the potential for exploring improvements to the spectrum, potency, and specificity of these drugs is becoming exhausted [23]. On the other hand, it remains clear that GyrA as a target holds significant potential for the development and discovery of new antibacterial compounds. Therefore, an M-PFC assay targeting GyrA, which could efficiently screen for novel compounds that disrupt the function of the GyrA dimer, would be highly useful in the search for new TB treatments.
CHAPTER FOUR: NOVEL FLUORESCENT IMPROVEMENTS TO THE CURRENT M-PFC DRUG SCREENING PLATFORM

4.1 Fluorescent M-PFC

M-PFC has become a compelling method for pioneering the validation of new PPI in Mtb. Furthermore, this has led to the development of an M-PFC based drug screen for molecular inhibitors of these intriguing PPI. However, a drawback of the original M-PFC is its readout because it requires the addition of resazurin followed by an extended incubation before results can be collected. Necessarily adding exogenous reagents increases the time and cost of completing the M-PFC assay, increases the likelihood of human error, and decreases the number of screens that can be completed per month. Ideally this additional step would be eliminated to make the M-PFC more amenable to HTS by increasing its efficiency and improving the reliability of the readout.

To eliminate the need for resazurin, an alternative fluorescent readout has already been tested for the M-PFC assay using the red-fluorescent protein mCherry. In this mCherry M-PFC system, an mCherry gene driven by a strong constitutive promoter was introduced on both the episomal and chromosomal plasmids that are used to construct the M-PFC assay. This changes the assay’s readout so that rather than measuring the reduction of resazurin by bacteria surviving in increasing concentrations of TRIM, the fluorescence due to the constitutive production of mCherry by surviving bacteria is measured instead. As before, growth of the bacteria in higher concentrations of TRIM indicates the presence of the protein-protein interaction of interest, but the bacterial growth is detectable due to the innate fluorescence of the mCherry protein. This fluorescent signal can be conveniently read on a plate reader without the addition of any exogenous reagents. The
mCherry M-PFC represents a more streamlined approach for studying novel PPI and screening for inhibitors of these interactions in Mtb.

4.2 Modifications to the Red-Fluorescent Protein mCherry

The study of bacterial pathogenesis benefits greatly from genetically encoded fluorescent proteins (FPs), which have served as the basis for versatile techniques for examining proteins and cells of interest, and have also been instrumental in examining these in the context of model hosts [25]. For example, fluorescence has been used for visualizing processes within live cells, reporting transcriptional and translational activity, and comparing characteristics of mutant cell types. Enhanced red-shifted fluorescent proteins (RFPs) have several advantages over FPs of other colors. Specifically, RFPs feature reduced autofluorescence, decreased phototoxicity, and reduced light-scattering which make them superior for experiments where low background noise and high viability of cells is important [25]. Perhaps the greatest additional benefit of RFPs is that they are complementary to the use of other FPs—blue, cyan, green, or yellow, for instance—when multicolor applications are desired [26]. The most popular general-purpose RFP is mCherry because it possesses high photostability and a low maturation time, performs well when fused to other proteins, provides a high signal-to-noise ratio, and is monomeric and thus generally nontoxic to the cells expressing it [26]. Although it is not as bright as mStrawberry or tdTomato, two other FPs in its class, the other features of mCherry combined make it superior to brighter RFP variants [26].

In our work with mycobacteria, mCherry has remained a reliable and useful fluorescent tool. Previous experiments have used mCherry as a reporter gene for assessing the results of reporter-fusion constructs in Msm and Mtb. The use of mCherry to monitor the activity of mycobacterial promoters is ideal because it provides a direct fluorescent readout that reflects cues and timing
associated with gene regulation. Other projects have utilized mCherry for simple phenotypic drug screening, in which Msm and Mtb bacteria have mCherry integrated into their genome, these strains are grown, and the cells are treated with drugs. It was demonstrated that bacteria surviving a given compound grow and produce fluorescence, but those that are killed by the compound lack fluorescence. As discussed above, recently mCherry has also been successfully integrated into the assessment of protein-protein interactions as the reporter for an improved M-PFC assay. Therefore, advancements in the uses of mCherry that result from optimizing its fluorescent and spectral properties have the potential to improve fluorescent tools in a wide sense, and to impact the study of pathogenesis in mycobacteria significantly, too.

4.2a Large Stokes Shift mCherry

A subclass of improved FPs are those that have been modified to feature a large Stokes shift (LSS). The Stokes shift of a given FP is defined as the difference in wavelength between the respective maximums of the excitation and emission spectra, as shown in Figure 3. An LSS FP distinctively exhibits a difference between these maxima of larger than 100nm [25]. Typically LSS RFPs have been utilized to increase the sensitivity and decrease the background of multicolor imaging applications like Förster resonance energy transfer (FRET), but the improved separation of the absorbance and emission maxima is potentially advantageous even for single-color imaging applications [25].

By characterizing the molecular networks that generate LSS fluorescence in LSSmKate, an RFP, Piatkevich et. al. successfully engineered a range of new LSS FPs, including LSSmCherry. Two point mutations were strategically introduced to the wild type mCherry gene, resulting in a serine at amino acid 158 and a glutamic acid at 160 [27]. These modifications were made to introduce support
for an excited-state proton transfer (ESPT) pathway analogous to those found in LSSmKate1 and LSSmKate2, shown to be responsible for the large Stokes shift of these proteins as well as several other LSS FPs [27]. Wild type mCherry exhibits an excitation maximum at 587nm and an emission maximum at 610nm, while LSSmCherry demonstrated a significantly shifted excitation maximum at 456nm when produced in *E. coli* and purified [27]. Similar success occurred when this method was applied to generate LSS variants of a handful of other commonly used fluorescent proteins including mNeptune, mStrawberry, and mOrange using *E. coli* [27]. LSSmCherry has not yet been used for applications in mycobacteria, and generating this mutant holds the potential to increase the sensitivity of the assays that currently utilize mCherry readouts. This may prove particularly impactful for the mCherry M-PFC since increasing the sensitivity of this assay would decrease the likelihood that weakly interacting protein-protein pairs are overlooked. Additionally, when the mCherry M-PFC is applied to drug-screening, increasing the signal-to-noise ratio by upgrading the sensitivity of the readout is desirable.

**Figure 3: Definition of the Stokes Shift of a Fluorescent Protein.** The difference between the peak of the excitation spectrum, shown in blue, and the peak of the emission spectrum, shown in red, defines the Stokes shift of a fluorescent protein. When this difference is larger than 100nm, the fluorescent protein is considered to have a large Stokes shift.
4.2b Bimolecular Protein Fragment Complementation and Split mCherry

While PFC assays utilizing split reporter enzymes like DHFR are widely accepted and reliable, the readouts of these assays have been uniquely improved by a subsequently developed method called bimolecular fluorescence complementation (BiFC). Rather than measuring the effect of a truncated enzyme’s reconstitution (i.e. TRIM resistance conferred by DHFR), a BiFC assay relies on the reconstitution of a split fluorescent protein as its reporter [28]. The protein partners of interest are each fused to a non-fluorescent fragment of the FP, typically by a flexible glycine linker region [29]. When brought together by the interacting pair of proteins in vitro the FP reforms and generates a functional fluorophore [29]. Thus, BiFC assays provide a real-time fluorescent signal indicative of PPI in living cells. Not only does this feature allow visualization of the PPI, but it also creates a highly sensitive assay with minimum background because fluorescence is not recovered without interaction of the two proteins of interest [29]. Thus, BiFC is often advantageous compared to other fluorescence-based visualization techniques, particularly FRET. While FRET is more desirable than BiFC for capturing dynamic, instantaneous interactions of protein partners, it is less sensitive than BiFC because it only reflects the small number of partners that happen to interact with each other at a given moment [29]. This often means the protein partners of interest ought to be overexpressed to increase the sensitivity of a FRET assay, which is importantly not required for BiFC [30]. Additionally, the construction of the assay and gathering of data for FRET is more involved than for BiFC [29]. FRET also requires that the interacting partners be located closer together than BiFC, increasing the likelihood FRET will miss some PPI occurring in macromolecular complexes that BiFC would not [29]. Even when visualization of the protein-protein interaction is not the primary goal, BiFC remains advantageous over enzyme-based PFC readouts because it removes the necessity of treating cells with exogenous reagents, like resazurin in
the M-PFC. Removing this step is more efficient in terms of cost, time, and error-reduction while increasing assay sensitivity.

Although the recently developed mCherry M-PFC does reflect PPI with a fluorescent readout, this fluorescence is importantly not a direct product of the proteins of interest fusing together. It is instead an indirect measure of PPI that reports the viability of the bacteria when exposed to various concentrations of TRIM. This report is then used to infer that the PPI of interest is present because, for the bacteria to survive in TRIM, the PPI must have facilitated reconstitution and function of the DHFR enzyme. As a result, the fluorescent M-PFC is less sensitive and less specific than a mycobacteria-compatible BiFC assay would likely be and it does not leave potential for visualization of the PPI itself. BiFC also holds more promise for studying multiple PPI at once than the fluorescent M-PFC does. Not only is it simpler to construct a multicolor screen to study multiple PPI occurring simultaneously within a single cell using BiFC, the information available about each partnership from such a screen would be more detailed and accurate since the readout is direct. These advantages become increasingly important when a given PPI assay is translated to HTS efforts, where easily quantified BiFC signals increase the efficacy of cell-based HTS.

For over a decade, BiFC has been successfully applied to detect PPI in a variety of species of living cells using a variety of fluorescent colors. Large-scale BiFC screens for specific PPIs in yeast, plant, mammalian cells, and even for interactions occurring between proteins from different species have been achieved using split yellow, cyan, green, and red FPs [29]. The development of split RFP variants was of particular interest after yellow and cyan variants were introduced since RFPs extended the range of available wavelengths for BiFC assays, opening up more potential for multicolor assay construction [28]. Multicolor BiFC assays are performed to visualize multiple PPI simultaneously in the same cell. They are constructed by fusing multiple split fluorescent proteins,
which must feature sufficiently different spectral properties, to different pairs of protein partners [31]. The results of a multicolor BiFC assay allow useful analysis of the relative distribution of multiple PPI in the same cell [31].

Split mCherry has been the most reliable of the RFP variants applied to BiFC, as it was demonstrated that an mCherry-based BiFC could be used to visualize both dimerization of EGFP and interaction of human p53 with SV40 large T antigen in Vero cells [29, 32]. Collectively, these BiFC successes have supplied knowledge about novel protein interactions and have stimulated drug development efforts. Specifically, HTS for small molecule inhibitors of PPIs using cell-based BiFC assays have also been validated in studies of HIV, cancer, and influenza A [29]. Interest in BiFC assays like these has increased because they combine uncovering deeper knowledge about the mechanisms of action of any identified inhibitors with the advantages of whole cell screening. Constructing a BiFC assay for use in Mtb with a novel PPI target would undoubtedly be an excellent tool not only for studying novel protein-protein interactions but also for discovering new compounds that effectively inhibit them.
CHAPTER FIVE: SPECIFIC AIMS

This project’s focus is to engineer and evaluate three plausible improvements to the tools we currently use when evaluating PPI in \textit{Mtb}. If these aims are successful, we will have a new PPI assay for HTS and improved fluorescent tools to enhance our PPI screen in \textit{Mtb} and to bolster our drug screening capabilities.

5.1 Aim 1: TB Gyrase A and the Fluorescent M-PFC

The TB gyrase complex is a validated and promising target for drug discovery in \textit{Mtb}. Because of its links to fluoroquinolone activity, the GyrA dimer component of the complex is of particular interest. Thus, the GyrA dimer was selected as a target of the M-PFC and subsequent improvements to the M-PFC. The specific goals of this portion of the project are as follows:

\textit{Aim 1.1: Create GyrA/GyrA M-PFC strains and validate the interaction of the GyrA/GyrA protein partners.}

An M-PFC assay that can detect the interaction of the GyrA dimer will be created using genetic engineering techniques. Then, this GyrA/GyrA M-PFC will be used to confirm that GyrA partners with itself independently of the GyrB dimer, as supported by the literature. Following this, the relative strength of the GyrA dimer interaction will be characterized using the M-PFC.

\textit{Aim 1.2: Enhance the GyrA /GyrA M-PFC by generating a GFP-based fluorescent GyrA /GyrA M-PFC.}

After confirming that the GyrA/GyrA M-PFC demonstrates significant interaction between the GyrA partners, the assay will be simplified and improved by replacing the current dye-based readout with a GFP-based fluorescent readout. This will not only increase the quality of the assay, decrease the time required to complete the assay, and decrease the costs associated with running the assay, it will also improve the assay’s applicability to drug screening in the future.
5.2 Aim 2: LSS mCherry

The unique spectral properties of LSS FPs can be used to increase the sensitivity of FP-based assays. Substituting certain amino acids in wild type mCherry results in a large Stokes shifted mutant of mCherry. Because mCherry is the basis of the readout of many of our assays, including one version of the fluorescent M-PFC, generating an LSS mCherry is desirable.

Aim 2.1: Generate an LSS mCherry strain of Msm.

Two amino acid substitutions will be made within the wild type mCherry gene to generate LSS mCherry. The LSS mCherry will be expressed constitutively in wild type Msm on an episomal plasmid.

Aim 2.2: Investigate the spectral properties of LSS mCherry expressed in Msm.

The characteristics of wild type mCherry will be compared to those expected of LSS mCherry. It is expected that the peak excitation wavelength will shift from 595nm in WT mCherry to 456nm in LSS mCherry.

5.3 Aim 3 Focus: The Split mCherry BiFC

While the M-PFC and fluorescent M-PFC assays work reliably for detecting PPI, their main weakness is that they feature an indirect readout. This decreases the sensitivity and specificity of the assay while also limiting its range of potential applications. It has been shown that mCherry is compatible with the construction of a BiFC assay in Vero cells, but this tool has not yet been demonstrated in mycobacteria. Because BiFC has the potential to significantly enhance our ability to study specific PPI in a direct and specific manner, we will work to engineer a functional BiFC in Msm.
**Aim 3.1: Generate split mCherry BiFC strains with protein partners known to interact strongly in Msm.**

To demonstrate proof-of-concept of the mCherry BiFC in *Msm*, the protein partners will be selected based on the strength of their interaction and their compatibility with the known requirements for constructing an mCherry-based BiFC. Multiple mCherry BiFC strains of *Msm* featuring different partners will be created to determine whether the reconstitution of mCherry can occur in *Msm*.

**Aim 3.2: Characterize the properties of the mCherry BiFC strains and optimize the BiFC assay for studying PPI.**

The mCherry BiFC strains will be assayed under various conditions mimicking those that have been optimal in other BiFC studies. An optimized procedure for performing the mCherry BiFC assay will be established so that this assay can be applied to studying other PPI or extended to drug screening in the future.
CHAPTER SIX: METHODS

6.1 Bacterial Cell Culture

In the following experiments, NEB 10-Beta Competent *Escherichia coli* High Efficiency (*E. coli*) and *Mycobacterium smegmatis* mc2155 (*Msm*) were used [33]. *E. coli* were needed to host recombinant DNA constructs, to amplify DNA for standard cloning procedures, and to produce plasmid DNA for transformations into *Msm*. *Msm* was selected as a surrogate host in place of *Mtb* for these experiments because it is non-pathogenic and can be handled safely in BSL-2 conditions while still exhibiting most of the relevant qualities of *Mtb*, it grows robustly after only two days of incubation, and it readily expresses *Mtb*-exclusive genes that are G+C rich. *Msm* was used as a host to express M-PFC constructs, the LSS mCherry protein, and BiFC constructs.

*S. coli* cultures consisted of lysogeny broth (LB) and different concentrations of selective antibiotics, depending on the plasmids that were transformed into the bacteria. When constructs were built from plasmids pUAB100 or pUAB300, hygromycin B (Hyg) was used at 250 μg/mL. For constructs derived from plasmids pUAB200 or pUAB400, kanamycin (Kan) was used at 50 μg/mL. These cultures were routinely grown at 37°C in a culture tube overnight at a volume of 5mL while shaking at 250 revolutions per minute (rpm).

*Msm* cultures consisted of LB broth plus 0.05% polysorbate 80, referred to as Tween 80 (LB Tw broth) and lower concentrations of the selective antibiotics than those used for *E. coli* cultures. For pUAB100 and pUAB300 constructs, the Hyg concentration was reduced to 50 μg/mL. When selection for pUAB200 and pUAB400 was needed, 25 μg/mL of Kan was used. Standard *Msm* cultures were incubated at 37°C for three days while shaking at 40 rpm.
6.2 Aim 1 Methodology

6.2a M-PFC Overview

Four types of base plasmids were used in this project to construct the M-PFC assay: pUAB100, pUAB300, pUAB200, and pUAB400. The Hsp60 promoter region included on both plasmids is a constitutive promoter region that drives strong expression of the genes that follow it. The oriM region of pUAB 300 is an origin of replication that is included so that the plasmid can replicate in the cytoplasm of Msm. On pUAB 400, the Int region denotes an integration segment that causes the plasmid to integrate into the chromosomal DNA of Msm upon transformation. The Hyg and Kan resistance genes are included on pUAB 300 and pUAB 400 respectively so that antibiotic selection for the presence of these plasmids can occur. See Figure 4 for details. When transformed into Msm, the pUAB100/300 plasmids are retained and replicated as episomal plasmids, containing genes for the Protein Partner 2 (PP2) and DHFR B proteins. By contrast, the pUAB200/400 plasmids integrate within the chromosomal DNA of Msm upon transformation, containing genes for the Protein Partner 1 (PP1) and DHFR A proteins. pUAB200/400 are suicide plasmids that lack a mycobacterial origin of replication. In order to retain them following transformation, they must integrate within the chromosome of Msm via recombination at the phage-derived Integrase (Int) site (See figure 4). One episomal plasmid (pUAB100 or pUAB300) can be transformed with one chromosomal plasmid (pUAB200 or pUAB400) into Msm at a time to generate a complete M-PFC strain. To confer selectivity during the genetic engineering process, these sets of plasmids also contain antibiotic resistance genes. Plasmids pUAB100/300 contain a gene for Hyg resistance and pUAB200/400 contain a gene for Kan resistance (See Figure 4). First
genetic engineering was used to insert the GyrA gene into base plasmids 300 and 400, in the position of PP1 and PP2. The details of the genetic engineering process can be found below.

![Diagram of M-PFC Base Plasmids](image)

**Figure 4: M-PFC Base Plasmids.** Completed M-PFC strains of Msm contain one episomal plasmid (pUAB 100 or pUAB300) and one plasmid that integrates into the chromosome (pUAB 200 or pUAB 400). Before they can be transformed into Msm, these plasmids must be genetically engineered to contain the target protein partners and then they must be amplified in *E. coli*.

The resulting constructs were transformed into *E. coli* and grown on LB plates containing their respective antibiotics to select for the bacteria containing the desired plasmid construct. Once it was confirmed that a certain colony of *E. coli* contained the target construct, these *E. coli* were used to produce copies of the plasmid for transformation into Msm. Antibiotic resistance was used to confirm the transformation of both the episomal and the chromosomal M-PFC plasmid into Msm, and then cultures of the complete M-PFC Msm strains, along with the necessary control strains, were grown for use in the M-PFC assay. The M-PFC assay measured the presence of PPI through a viability readout that reflects the reconstitution of DHFR due to positive PPI. When the bacteria in
a given column were positive for PPI, the bacteria survived in the presence of increasing concentrations of TRIM and were able to reduce Alamar Blue dye to a pink fluorescent color. When a lack of PPI was present in the bacteria, they were unable to survive in TRIM due to a non-functional DHFR enzyme, and as a result the Alamar Blue dye remained blue (See Figure 2). Further details about these methods are explained in the following sections, and an overview of the entire method is shown in Figure 5 below.

![Figure 5: Overview of the M-PFC Assay.](image)

Genetic engineering is used to construct a pair of plasmids, each containing a protein partner connected to a DHFR fragment via a glycine linker. These plasmids are transformed together into Msm and the resulting M-PFC strain is grown on selective media. Cultures of the M-PFC strain and the necessary control strains are grown. The M-PFC assay is set up by adding the Msm strains plus the dilutions of TRIM to the plate, incubating them together for at least 48 hours, and then adding Alamar Blue dye to the wells. After a four hour incubation with the dye, the plate is read in a fluorescent plate reader, and quantitative data about the relative strength of the PPI is gathered based on how pink the dye becomes.
6.2b Creation of M-PFC Strains to Monitor GyrA/GyrA Interaction

Genetic engineering followed by transformation into Msm was used to create the M-PFC strains. These strains were later applied to the M-PFC assay to study the TB GyrA/GyrA PPI. To generate these strains, first the plasmids for the M-PFC assay were constructed using PCR followed by a method called FastCloning. Compared to traditional cloning methods, FastCloning is advantageous because it does not require gel purification, restriction digestion, or ligation [34, 35]. Because of this, the efficiency of cloning experiments was accelerated and the design of possible plasmid constructs was more flexible.

**PCR and FastCloning**

To insert a new gene into a base plasmid, the original plasmid (the ‘vector’) and the target gene fragment (the ‘insert’) were amplified using Polymerase Chain Reaction (PCR). PCR is a technique used to logarithmically amplify a targeted segment of DNA, which in the case of FastCloning must include both the vector and the insert DNA. Specific forward and reverse primers are designed to flank the targeted DNA, causing many copies to be made of the desired DNA, compared to the original DNA. Due to the primer design, the ends of both the replicated vector and the insert DNA contained complementary overhangs of ~10-15 base pairs. The complementary overhangs allowed the vector and insert DNA to anneal together, fusing the new gene to its insertion site on the vector during the FastCloning reaction that followed. To ensure that the parent strands of DNA were degraded after they served their function as templates in the PCR reaction, a restriction enzyme called DpnI was added to digest methylated DNA while leaving the unmethylated vector and insert products of PCR unaffected. Products of the PCR reaction were analyzed by agarose gel electrophoresis on a 1% gel alongside a GeneRuler 1 kb DNA ladder to separate the DNA products based on their size and thereby verify the presence of the desired vector.
and insert products. Figure 6 depicts an example of an agarose gel used for this purpose. Specific details of the PCR reaction used to amplify the vector and inserts are covered in the next paragraph.

**Figure 6: An Agarose Gel Confirming the Presence of PCR Products of Different Sizes.** Lanes 1 and 2 contain fragments of ~2,500 base pairs amplified via PCR from template DNA. Lanes 3 and 4 contain different fragments of ~2,000 base pairs also amplified by PCR. The presence of these distinct fragments can clearly be distinguished by running PCR products alongside the 1kb ladder on the agarose gel.

During a PCR reaction, several temperature-dependent steps are followed to utilize the primers needed to amplify the DNA. First, the template DNA gets denatured so that the complementary strands of DNA begin to separate and the added polymerase enzyme is activated. A secondary denaturing step follows, in which denaturation of the DNA segment in continues so that the hydrogen bonds holding base pairs together are compromised in the regions where the primers will anneal. Then the temperature is shifted to begin an annealing phase in which the primers hybridize with the parent DNA. The ideal annealing temperature depends on the particular primers
being used. Next, the extension phase begins with another shift in temperature that favors the function of the DNA polymerase enzyme that is selected for the reaction. In this case, the Phusion® enzyme was used. The polymerase enzyme is responsible for assembling the replicated strands of DNA using the parent strand as a template. The secondary denaturation, annealing, and extension steps are repeated twenty times to cause logarithmic amplification of the target segment, resulting in many more copies of the segment DNA than the template DNA. To complete the PCR reaction, a final extension step is included to allow any incomplete DNA products to finish the reaction. To generate these temperature phases and to ensure correct denaturation, annealing, and extension times, PCR reactions were run in a BioRad thermocycler following a set Phusion®-specific protocol. See Table 1 for an example of a typical PCR reaction cycle. The annealing temperature was determined based on the melting temperature of the primers used in a given reaction. The primers themselves were designed using a program called “ApE – A plasmid Editor,” combined with sequences gathered from online Mycobacterial DNA databases, like “MycoDB – xBASE –” [36]. Table 2 indicates the names of the primers used to construct the GyrA/GyrA M-PFC assay and their sequences. To set up a PCR reaction the protocol “PCR Protocol for Phusion® High-Fidelity DNA Polymerase (M0530),” by New England BioLabs was followed [37]. A standard 25μL PCR reaction contains 1μL of template DNA (~10-20 ng/μL), 1μL of forward primer (100 ng/μL), 1μL of reverse primer (100 ng/μL), 5μL of 5x GC Buffer, 0.5μL of 10mM deoxynucluotide (dNTP), 0.25μL of Phusion® Enzyme, and 16.25μL of DNAse free water.
Upon affirming the presence of the desired PCR products, the vector and insert were FastCloned with one another. First, varying ratios of insert and vector PCR products were mixed together. Typically three ratios were used to find the concentration of insert:vector that was most efficient for a given fusion of insert with vector. Usually, three insert to vector ratios (4:1, 1:1, and 1:4) were added to three tubes containing a total volume of 8 μL of PCR product in each tube. To these tubes 1.5μL DpnI enzyme was added and then the reaction was incubated at 37°C for two hours to catalyze the complete digestion of the methylated, parental DNA. If the correct ratio of insert to vector DNA was present, the result of FastCloning was a fully assembled vector and insert.

### Table 1: A Typical PCR Reaction Cycle

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time</th>
<th>PCR Step</th>
</tr>
</thead>
<tbody>
<tr>
<td>98</td>
<td>30 seconds</td>
<td>Initial denaturation</td>
</tr>
<tr>
<td>98</td>
<td>10 seconds</td>
<td>Secondary denaturation</td>
</tr>
<tr>
<td>55-70</td>
<td>30 seconds</td>
<td>Annealing. Varies depending on primers’ melting temperatures.</td>
</tr>
<tr>
<td>72</td>
<td>15 seconds/kb</td>
<td>Extension temperature</td>
</tr>
<tr>
<td>72</td>
<td>3 minutes</td>
<td>Final extension</td>
</tr>
<tr>
<td>12</td>
<td>∞</td>
<td>Hold time</td>
</tr>
</tbody>
</table>

### Table 2: Primers Used to Construct the GyrA/GyrA M-PFC Assay

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUAB300_F</td>
<td>AAGCTTATCGATGTCGACGT</td>
</tr>
<tr>
<td>pUAB300_R</td>
<td>CTGCAAGCTGGATCCGG</td>
</tr>
<tr>
<td>GyrA_pUAB300_F2</td>
<td>CCGGATCCAGCTGCAAGATGCAGACACAGACGTGTCG</td>
</tr>
<tr>
<td>GyrA_pUAB300_R2</td>
<td>ACGTCGACATCGATAAGCTTTTAAATTGCGGTCGTTGCTG</td>
</tr>
<tr>
<td>pUAB400_R</td>
<td>CAATTTGAGGCCCAACCC</td>
</tr>
<tr>
<td>GyrA_pUAB400_F</td>
<td>GTGGTGGGTCCAAATTGATGACAGACACAGACGTGTCG</td>
</tr>
<tr>
<td>GyrA_pUAB400_R2</td>
<td>ACGTCGACATCGATAAGCTTTTAAATTGCGGTCGTTGCTG</td>
</tr>
</tbody>
</table>
combination. If the correct vector and insert were not assembled, the transformation into *E. coli* would not result in colony growth on the selective media. Following the FastCloning reaction, the resulting plasmids were transformed into *E. coli* so antibiotic selection, colony screening, and sequencing could be used to confirm the desired plasmids had been constructed. Figure 7 details the step-by-step procedure of FastCloning.

**Figure 7: The FastCloning Process.** A) Vector amplification via PCR with vector-specific primers. B) Fragment amplification via PCR with fragment-specific primers containing overhangs that are complementary to the amplified vector. C) Vector and fragment products are mixed in various ratios and exposed to DpnI to digest parental DNA. The remaining products begin to fuse at their complementary regions. D) The gene of interest is completely fused with the vector by the end of the incubation. E) The FastCloning product is transformed into *E. coli*.

**Transformation into E. coli, Colony Screening, and Sequencing**

To amplify the plasmids produced by the FastCloning reaction, the reaction products were transformed into *E. coli* according to the “NEB 10-beta Competent E. coli (High Efficiency)” protocol, distributed by the manufacturer [33]. Some modifications were made to this protocol. A 50μL aliquot of 10-beta Competent (cc.) *E. coli* was thawed, and the aliquot was split into thirds so that a total of 16.5μL of 10-beta cc. *E. coli* were present in each tube. Three tubes were needed for the three ratios of insert:vector. Usually 2μL of the FastCloning product (between 1pg and 100ng
DNA) was added per tube of *E. coli* and the tubes of *E. coli* were lightly flicked to distribute the DNA among them. The *E. coli* were incubated on ice for 30 minutes, and then they were heat shocked at 42°C for 30 seconds. Following heat shocking the cells were iced for 5 minutes, after which 200μL of Super Optimal broth with Catabolite repression (SOC) was added to each tube. The cells were placed in a 37°C incubator and shaken at 250rpm for an hour to recover. Then, 100μL of the SOC/*E. coli* suspension from each tube was plated on LB agar plates containing selective antibiotics (either Kan at a concentration of 50μg/mL or Hyg at a concentration of 150μg/mL) for the plasmid being transformed. The plated cells were incubated overnight at 37°C overnight, and colonies present on the plates were observed the next day. If colonies were present, then a colony PCR screening protocol was used to verify that the vector in fact contained the insert. To screen the colonies, the bacteria were lysed to extract their DNA, which was used in a PCR reaction with primers for the insert region to verify the insert’s presence. These were the same primers that were used to amplify the insert from the parental DNA. Lysis of the bacteria was achieved by picking an isolated colony on a plate, adding the colony to a tube containing 20μL of deionized water, and then boiling the tube at 100°C for 5 minutes. To pellet the bacterial debris and leave the DNA in the supernatant, the tube was centrifuged at 13,000 rpm for 2 minutes. From the supernatant, 2μL was used as the DNA template in the typical PCR reaction previously described. When multiple colonies were screened at one time, the colonies were labeled on the plate before they were picked so they could be identified for making cultures. A 1% agarose gel electrophoresis process was used to verify that the size of the colony PCR product matched the size expected for the insert. Figure 8 demonstrates an example of an agarose gel used for colony PCR screening.
Figure 8: An Agarose Gel Used During Colony PCR Screening. FastCloning products are transformed into *E. coli* and colonies are grown on selective media. DNA is isolated from these colonies and PCR is run with primers that amplify the fragment that was inserted into the vector. If a colony contains the target plasmid, then the PCR product will be the size of the intended fragment on an agarose gel. Lanes 2-5 on this gel are positive for the target plasmid, while lanes 1 and 6-8 are negative.

In preparation for sequencing, *E. coli* colonies that were identified as positive through the colony screening process were picked and grown in 5mL overnight cultures to amplify the plasmid containing the desired insert. An alkaline lysis protocol, along with the EconoSpin™ Spin Column for DNA from Epoch Life Science Inc., was used to extract the amplified DNA from the *E. coli* cells. Then a Nanodrop spectrophotometer was used to measure the concentration of the prepared DNA. To make certain that the DNA contained the target vector and insert, the prepared DNA was sequenced by Genewiz Inc. preceding transformation into Msm. The raw sequence data was analyzed using APE and *in silico* sequence models of the target plasmid constructs. Sequence data was aligned with the model sequences. DNA from a particular colony was positive when its sequence data matched the model sequence exactly.
**Transformation into Msm**

Using the genetic engineering techniques described above, the necessary pair of Gyra M-PFC plasmids were constructed. Figure 9 displays the specific arrangements of the Gyra, DHFR, and glycine linker regions relative to one another on the plasmids that were constructed for this experiment. The final step needed to generate the Gyra/Gyra M-PFC strains was transformation of the constructed plasmids into Msm. The cell membrane of competent Msm cells was altered by electroporation, which exposes the cells to an electric field that increases the permeability of the membrane, facilitating the intake of the constructed plasmids. The plasmids were transformed into *Msm* individually to generate M-PFC control strains, and also together to generate M-PFC test strains. To perform an electroporation, a 100μL aliquot of wild type electro-competent *Msm* was thawed for ten minutes on ice. Without exceeding a volume of 4μL, between 200 and 500ng of the prepared DNA was added to the *Msm* cells and the cells were transferred into an electroporation cuvette. In the BioRad Gene Pulse X machine, the cuvette was exposed to 2,500 V (volts), 25 μF (capacitance), and 1,000 Ω (resistance). The cells were combined with 250μL LB Tw broth and transferred to a 2mL screw cap conical tube. For four hours the cells were incubated at 37°C and shaken at 40 rpm. Following this recovery period, 20μL and 100μL of the cells were each plated on LB agar plates containing appropriate antibiotics for selection. To determine that both plasmids were successfully transformed into Msm together, the cells were plated on double selection (Kan and Hyg) LB agar plates. Confirmed M-PFC strains were resistant to both Kan and Hyg, conferred by the antibiotic resistance genes of the two incorporated pUAB plasmids. Once transformed into *Msm*, the Gyra fused to each half of DHFR were expressed in the cells and the resulting Gyra/Gyra homodimer could be observed through the M-PFC assay.
6.2c M-PFC Assay

For this project the M-PFC assay was performed to study the PPI between the GyrA homodimer using the GyrA/GyrA M-PFC Msm strain, which contained both GyrA_pUAB300 and GyrA_pUAB400. Negative and positive control strains were also used alongside the M-PFC strain. The controls included strains that contained GyrA_pUAB300 alone, GyrA_pUAB400 alone, and DosR_pUAB100+DosR_pUAB200. The GyrA_300 and GyrA_400 strains contain only one half of the DHFR protein linked to GyrA and serve as negative controls because no reconstitution of DHFR A and DHFR B can occur in either of these. The DosR_100+DosR_200 strain serves as a positive control because in previous M-PFC experiments it demonstrated moderately strong PPI [38]. To set up an M-PFC assay, cultures of each strain were diluted to a starting optical density of 0.5. Then serial tenfold dilutions were performed to reduce the optical density of the cultures to 0.0005. Next, 135μL of the cultures were added to their assigned positions in a 96 microwell plate. A row of wells containing LB Tw and no bacteria was included as a negative control. Then 15μL of two fold dilutions of TRIM from 200μg/uL to 1.56μg/uL were added to their assigned wells and
mixed with the cultures in those wells respectively. Using a gradient of TRIM reveals the relative strength of the PPI of interest. Initially, the gradient used ranges from 200μg/uL to 6.25μg/uL, but sometimes it is necessary to decrease the spread of the gradient to 50μg/uL-1.56μg/uL so that the relative strength of weaker PPI can be more accurately determined. The higher the TRIM concentration needed to inhibit the growth of the bacteria is, the stronger the PPI. Wells containing 15μL of water in place of TRIM (0μg/uL TRIM) were included as controls. In a typical Alamar Blue assay, after the plate was set up it was incubated at 37°C for 48 hours. However, based on optimization experiments previously performed in our lab, this incubation was extended to five days for the standard GyrA/GyrA M-PFC. Following the incubation of the strains with TRIM, 15μL of Alamar Blue dye was added to every well. During this project, Resazurin (1X) dye was substituted for commercial Alamar Blue dye to reduce the cost of the assay. As previously described, the Alamar Blue/Resazurin dye is used as an indicator of bacterial survival and growth in the presence of TRIM in the M-PFC assay [17]. Growing bacteria actively respire and reduce the blue Resazurin dye to Resorufin, which is pink and fluorescent [39]. It takes four hours of incubation at 37°C after the Resazurin is added for it to be adequately reduced by the bacteria. At this point the distinction between the Resazurin wells, which contain dead bacteria, and the Resorufin wells, which contain growing bacteria, can be detected using a fluorescence plate reader, set for an excitation wavelength of 530nm and absorbing an emission wavelength at 590nm. The “blank” wells used in this experiment contained only LB Tw broth without bacteria, water in place of TRIM, and Resazurin dye. These wells served as a control to determine the level of nonspecific signal generated by media interference, and the signals acquired from the rest of the plate were scaled to reflect this interference. The blank wells also served as indicators of contamination on the plate, in which case the assay was repeated.
The additional time, cost, and handling associated with the necessary addition of Resazurin in the traditional M-PFC assay is the biggest weakness of this method. Therefore, a fluorescent readout was designed and implemented to improve the quality of the M-PFC assay. With this improvement, the assay could be readily applied to drug screening, specifically HTS.

**6.2d Fluorescent M-PFC Assay**

A fluorescent M-PFC was developed to eliminate the need to add dye during the M-PFC assay while retaining the ability to detect relative TRIM resistance, and therefore relative strength of PPI in M-PFC strains of Msm. The fluorescent M-PFC assay was created by FastCloning the mycobacteria codon-optimized green fluorescent protein (GFP) gene onto both of the M-PFC plasmids. GFP attached to the Hsp60 promoter was PCR amplified off of pvvRG (a plasmid containing mCherry and the Hsp60 promoter:GFP cassette) for insertion into pUAB 300_GyrA, and off of pUAB400_GFP for insertion into pUAB 400_GyrA. The primers used for these FastCloning reactions can be found in Table 3. Before the fluorescent M-PFC is run, levels of fluorescence of the possible GFP strains were compared by transforming the fluorescent chromosomal M-PFC plasmid alone, the fluorescent episomal M-PFC plasmid alone, and both the fluorescent chromosomal and episomal M-PFC plasmids together into Msm. Once a configuration producing sufficient green fluorescence is acquired, the GFP GyrA/GyrA M-PFC assay can be run.
Table 3: Primers Used to Construct the GFP GyrA/GyrA M-PFC Plasmids

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>300_GFPmCh_F</td>
<td>GAGCGCAACGCACGTGAG</td>
</tr>
<tr>
<td>300_GFPmCh_R</td>
<td>GCTGCGGCGAGCG</td>
</tr>
<tr>
<td>GFP_FC_300F</td>
<td>CGCTCGCCGCGACGCGGTGACCACAACGACGC</td>
</tr>
<tr>
<td>GFP_FC_300R</td>
<td>CTACACCGGGTTCGCTCTAAATGATCATCCATGCCCCATGGTAA</td>
</tr>
<tr>
<td>400_GFPmCh_F</td>
<td>AATCTGGTTGTAATGCCCC</td>
</tr>
<tr>
<td>400_GFPmCh_R</td>
<td>AAGAGAAATAGACCGGGAAGG</td>
</tr>
<tr>
<td>GFP_FC_400F</td>
<td>CCTTGTCCCCGGTCTATTTCCTGTTGACCACAACGACGC</td>
</tr>
<tr>
<td>GFP_FC_400R</td>
<td>GGGGCATTCAACCAGATCTATAGATCATCCATGCCCATGGTAA</td>
</tr>
</tbody>
</table>

The GFP M-PFC assay should be compared to the original M-PFC assay using the Resazurin readout to determine whether the fluorescent M-PFC demonstrates comparable or better quality. Using cultures of the same OD, consistent concentrations of TRIM, the same total volume in the microwells, and identical incubation time and temperature, the assays would be run side by side and compared for sensitivity and reproducibility.

6.3 Aim 2 Methodology

6.3a Making LSS Mutations to Wild Type mCherry

Two mutations to the WT mCherry gene were previously identified to generate the LSS mCherry protein in *E. coli* [27]. To reproduce this modification and to examine its use in Msm, the same alterations were made to the mycobacterial-optimized mCherry gene. The effects of these alterations were then analyzed in Msm to determine whether a functional LSS mCherry protein had been generated.
A method called ‘Round the Horn Site-Directed Mutagenesis (RTHM) was used to make the following alterations to the WT mCherry gene from pvvRG: at base pair 497 of mCherry a T was replaced with a G and at base pair 502 a C was replaced with a G. These specific point mutations were selected to replace amino acid 158E with 158S and amino acid 160G with 160E according to codons optimal in mycobacteria. These specific amino acid replacements were selected to generate a certain excited-state proton transfer (ESPT) pathway that is believed to be the cause of the unique properties of related LSS FPs [27].

RTHM is a PCR-based method in which one or both of the forward and reverse primers can contain mutation(s). Primers are designed so that the mutation is present near the 5’ end, while the rest of the primer through the 3’ end matches the parent plasmid to form a readily-annealing portion. During initial PCR cycles, the forward and reverse primers anneal to the parent plasmid and the DNA template is copied to form a linear version of the template with the mutation(s) introduced at the primer sites. For the rest of the PCR cycles, the linear DNA containing the mutation(s) is copied. Compared to other site-directed mutagenesis procedures, RTHM causes exponential amplification of the mutated plasmid to increase the efficiency of transformation by increasing the yield of the reaction. Before the primers are added to the PCR reaction, they are phosphorylated so that the PCR product can later be ligated to form a closed circle. After ligation is complete, the circularized DNA can be transformed into bacterial cells. Figure 10 illustrates how RTHM operates.
**Figure 10: The Process of ‘Round the Horn Mutagenesis.** A) Phosphorylated primers containing the targeted point mutations are used to amplify the parent vector at the site of the gene of interest via PCR. B) The parent plasmid DNA is degraded using DpnI. The remaining product contains two phosphorylated ends where the primers annealed along the target gene, including the desired mutations. C) The phosphorylated ends are ligated to form the complete plasmid, with the target gene in tact and containing the point mutations.

The primer phosphorylation reaction was the first step in the standard RTHM protocol. The primer stocks were at 100μM in water. Then the following was mixed together for each primer, for a total volume of 50μL: 37μL of H2O, 5μL of 10 Kinase reaction buffer, 1μL 50mM MgSO4, 5μL of 100μM primer, 1μL 100mM ATP, and 1μL T4 polynucleotide kinase (PNK). This mixture was incubated at 37°C for 60 minutes, and then heated at 95°C for 5 minutes to kill the PNK enzyme. After primer phosphorylation was completed, the PCR reaction was performed. The PCR reaction for RTHM was modified slightly from the standard PCR reaction previously described. A RTHM PCR reaction contained 35μL DNAse free H2O, 10μL 5x GC Buffer, 1.5μL of the phosphorylated forward primer, 1.5μL of the phosphorylated reverse primer, .5μL of of 10mM dNTP, 1μL of the parent plasmid DNA, and .5μL Phusion® Enzyme. The specific PCR reaction, primers, and thermocycler settings used to create the LSS mCherry mutant are shown in Table 4.
The PCR product was run on a 1% agarose gel using gel electrophoresis to confirm the presence of the amplified plasmid, and then 1.5μL of Dpn1 was added to the PCR product. After incubation with Dpn1 for 2 hours, the PCR product was ligated. The ligation reaction required that 1.5μL of Fast-Link™ ligation buffer, .75μL 10mM ATP, 1μL of Fast-Link™ DNA ligase, and 8.75μL H₂O were added to 3μL of the post-Dpn1 PCR product. This mixture was incubated at room temperature for 30 minutes, and then the DNA ligase was heat inactivated by incubating the reaction tube at 70°C for 15 minutes. Following the ligation reaction, the DNA product was transformed into E. coli and plated on selective LB agar containing 250 μg/mL Hyg. In this experiment, the ligated RTHM PCR product was transformed into E. coli to amplify the circularized DNA containing the mutant mCherry gene. Because colony PCR screening could not be performed on the resulting colonies, several colonies were picked at random and cultured overnight. DNA was isolated from the E. coli cultures and sent for sequencing to confirm that only the desired mutations were made to mCherry.

Table 4: LSS mCherry Primers and PCR Cycle Settings

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSSmCherry_F_RTH</td>
<td>AGGAGCGGCTGAAGCTGAAGG</td>
</tr>
<tr>
<td>LSSmCherry_R_RTH</td>
<td>TGCTCTGCCCCTTCAGCGG</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time</th>
</tr>
</thead>
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<td>98</td>
<td>1 minute</td>
</tr>
<tr>
<td>98</td>
<td>30 seconds</td>
</tr>
<tr>
<td>63</td>
<td>30 seconds</td>
</tr>
<tr>
<td>72</td>
<td>2.5 minutes</td>
</tr>
<tr>
<td>25</td>
<td>∞</td>
</tr>
</tbody>
</table>

The PCR product was run on a 1% agarose gel using gel electrophoresis to confirm the presence of the amplified plasmid, and then 1.5μL of Dpn1 was added to the PCR product. After incubation with Dpn1 for 2 hours, the PCR product was ligated. The ligation reaction required that 1.5μL of Fast-Link™ ligation buffer, .75μL 10mM ATP, 1μL of Fast-Link™ DNA ligase, and 8.75μL H₂O were added to 3μL of the post-Dpn1 PCR product. This mixture was incubated at room temperature for 30 minutes, and then the DNA ligase was heat inactivated by incubating the reaction tube at 70°C for 15 minutes. Following the ligation reaction, the DNA product was transformed into E. coli and plated on selective LB agar containing 250 μg/mL Hyg. In this experiment, the ligated RTHM PCR product was transformed into E. coli to amplify the circularized DNA containing the mutant mCherry gene. Because colony PCR screening could not be performed on the resulting colonies, several colonies were picked at random and cultured overnight. DNA was isolated from the E. coli cultures and sent for sequencing to confirm that only the desired mutations were made to mCherry.
6.3b Creation of an LSS mCherry Strain of Msm

The DNA isolated from the colony containing the correctly mutated mCherry was transformed into WT Msm. The fluorescent properties of the LSS mCherry strain of Msm were analyzed using a fluorescent plate reader and compared to those of a WT mCherry strain of Msm and to the properties established in the literature. An excitation wavelength of 585 nm and an emission wavelength of 615 nm was used during this analysis. Additionally, an excitation scan from 400nm-585nm was completed for each sample using an emission wavelength of 615 nm. Before analysis, the cultures were pelleted and resuspended in phosphate-buffered saline (PBS) to eliminate any background fluorescence from the LB Tw media in which Msm was grown.

6.4 Aim 3 Methodology

6.4a BiFC Overview

A BiFC assay provides a direct fluorescent readout of the interaction between two protein partners. To do this, a fluorescent protein is first split at a specific point. This point must be selected so that the two halves of the fluorescent protein are not fluorescent when they are on their own, but are capable of rejoining with one another to reform the functional fluorescent protein. Using genetic engineering, each of the halves is fused to one of the protein partners via a flexible linker. When the BiFC plasmid constructs are transformed into cells the split FP, linker, protein partner complexes are produced. When the protein partners interact, the halves of the FP are brought into proximity with each other and reconstitution of the FP occurs. The restored FP is functional and emits a fluorescent signal, which indicates directly that the protein partners are interacting. Fluorescence can be detected either by using a fluorescent microscope or with a fluorescent plate reader. For this
experiment, mCherry was selected to be the FP based on previous work that demonstrated split mCherry can form the basis of a functional mCherry BiFC in Vero cells [32].

6.4b Creation of Split mCherry BiFC Strains

FastCloning was used to construct the mCherry BiFC plasmid constructs from the parent plasmids pUAB 100 and pUAB 400. The layout of the general mCherry BiFC constructs is detailed in Figure 11. It is important to note that the arrangement of the mCherry halves in these constructs leaves the split site interface of mCherry unattached to the glycine linker or to any other proteins; the split site of each half is left free to allow reconstitution to occur readily (See Figure 12). This feature was maintained within all of the mCherry BiFC constructs to mimic the constructs that were shown to be functional in Vero cells [32]. Two pairs of protein partners were selected as proof-of-principle PPIs for the mCherry BiFC constructs based on their high level of interaction according to previously collected M-PFC data. The M-PFC is the most similar assay to BiFC currently available in mycobacteria, which is why M-PFC data was used to make predictions about the BiFC constructs.

Figure 11: General Designs for the mCherry BiFC Plasmids. The arrangements of the mCherry fragments and the protein partners in these designs are consistent with those that were shown to create a functional mCherry BiFC in Vero cells.
The first interaction pair selected was the General Control Protein (GCN4) homodimer, which has demonstrated the strongest PPI in the M-PFC thus far and is regularly used as a strongly positive control in the M-PFC assay. GCN4 is not a mycobacterial protein, rather it is natively found in the yeast *Saccharomyces cerevisiae* where it operates as a transcription factor [40]. The two-stranded, parallel coiled coil structure of the GCN4 homodimer bound to DNA is shown in Figure 13 [40, 41].
The second pair selected for testing the mCherry BiFC were two protein partners that make up the Acid and Phagosome Regulated (apr) gene locus that is unique to \textit{Mtb}. Three genes are contained within the locus: \textit{aprA}, \textit{aprB}, and \textit{aprC}. These genes are upregulated in \textit{Mtb} growing in acidic conditions both \textit{in vitro} and in macrophages and deleting these genes inhibits \textit{Mtb}'s ability to grow, thus they are believed to be important to \textit{Mtb}'s ability to survive and persist through the hostile conditions it encounters inside the host during infection [42]. Predictions indicated that \textit{aprA} and \textit{aprB} both encode small proteins, but little else has been concluded about their structures [42]. When \textit{aprA} and \textit{aprB} were tested in a M-PFC assay, it was found that they exhibited strong levels of interaction, particularly when the N-terminus of \textit{aprA} was exposed to the C-terminus of \textit{aprB} [43].

\textbf{Figure 14: A Model of the Final mCherry BiFC Constructs.} Following transformation of the mCherry BiFC plasmid pairs into Msm, these are the protein constructs predicted to be expressed inside the cells. When interaction between the protein partners occurs, the mCherry fragments are brought into proximity with one another and they reconstitute to form a functional mCherry protein. This makes the Msm cell fluorescent. Before reconstitution, Msm will not appear fluorescent.
The configuration of *aprA* and *aprB* in the M-PFC constructs that produced this strong interaction is consistent with the constraints imposed by arrangement of the mCherry halves in the mCherry BiFC constructs. Because *aprA/aprB* is the strongest Mtb-exclusive PPI we have shown that fits this configuration and the *aprA/aprB* PPI is of interest as a novel drug target, this pair was also selected for the mCherry BiFC. The *aprA/aprB* mCherry BiFC constructs were also made via FastCloning, and diagrams of the resulting protein constructs are detailed in Figure 14. The primers used for these FastCloning reactions are displayed in Table 5.

**Table 5: Primers Used to Construct the mCherry BiFC Plasmids**

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>200 Split mCh R</td>
<td>TCCGGAGGACCCACC</td>
</tr>
<tr>
<td>pUAB100 F split</td>
<td>TAATCTGAACTAGTGGAAGTCTGATGTCG</td>
</tr>
<tr>
<td>100_RN_R</td>
<td>CCGGGGAATCCACTAGTTCTAGATTAGTCCCCTCCGGGTACATCGG</td>
</tr>
<tr>
<td>100_Rn_F</td>
<td>GTTGGGTCTCTGCGAATGGTCAGCAAGGGCGA</td>
</tr>
<tr>
<td>GCN4_pUAB400_F</td>
<td>GTTGGTGCTCTGCGCAATTGTAACACTGAAGCCGCA</td>
</tr>
<tr>
<td>GCN4_pUAB400_R</td>
<td>ACGTCGACATCCGAATAGCTTTCAGCTCGCCAACTAATTCTTAAATCT</td>
</tr>
<tr>
<td>pUAB500_F</td>
<td>AAGCTTATCGGTAGTCGACGT</td>
</tr>
<tr>
<td>pUAB400_R</td>
<td>CAAATTGGGACCCACC</td>
</tr>
<tr>
<td>splitR_GC4_400_F</td>
<td>CGGAGGAAATCCACTTGCGCAATGGGGCGGCTGAGG</td>
</tr>
<tr>
<td>splitR_GC4_400_R</td>
<td>ACCGGCCACCCGCCACCTCTGTACAGCTCGTCCATGCC</td>
</tr>
<tr>
<td>splitR_AprA_100RN_F</td>
<td>ACAATTGCGGATCCCTTGCAACTTGACATGAACAGCAGATGT</td>
</tr>
<tr>
<td>splitR_AprA_100RN_R</td>
<td>CCACCCGCCACCATGAGACTCTCGTCCATGCC</td>
</tr>
<tr>
<td>splitR_AprB_GC4_400_F</td>
<td>GTTGGTGCTCTGCGAATTGTCAGCTCGCCAGGG</td>
</tr>
<tr>
<td>splitR_AprB_GC4_400_R</td>
<td>ACGTCGACATCCGAATAGCTTTCAGCTCGCC</td>
</tr>
<tr>
<td>pUAB100_F</td>
<td>ATCGATGGTGCGCGGCGG</td>
</tr>
<tr>
<td>pUAB100_R</td>
<td>GTTGGTGCTCTGCGAATTGT</td>
</tr>
</tbody>
</table>

Once the GCN4/GCN4 and *aprA/aprB* BiFC plasmids were FastCloned, they were transformed into *E. coli* and colony PCR screening was performed as described in section 6.2b using
the insert primers. After growing the positive *E. coli* colonies overnight and isolating the DNA, sequencing was performed to confirm that the constructs had been made successfully. Then the completed BiFC constructs were transformed into Msm, both individually and as pairs.

6.4c BiFC Analysis

According to the literature, mCherry does not reconstitute well at 37°C, so following mCherry BiFC culture growth, cells must be incubated at room temperature (~25°C) overnight or at 4°C for 1-2 hours before reconstitution occurs [32]. The Msm BiFC cultures were grown at room temperature or at 37°C for three days. After diluting the cultures to equal ODs, they were distributed into 500μL aliquots and either left at room temperature or placed in a 4°C refrigerator for intervals between 1 hour and overnight. At each interval, both the room temperature and 4°C BiFC samples were examined for fluorescence in the fluorescent plate reader, using an excitation wavelength of 585 nm and an emission wavelength of 615 nm.
CHAPTER SEVEN: RESULTS

In an effort to improve our ability to study Mtb-specific PPI and to screen for new TB drugs, three new fluorescence-based tools were investigated in Msm. First an M-PFC assay was created to analyze the PPI of the GyrA homodimer in the TB gyrase complex. Then, the potential for translating this assay into a green fluorescent M-PFC assay was examined, in an effort to streamline the assay. The other two tools were focused on applications of the red fluorescent protein mCherry. To improve the sensitivity of assays that rely on mCherry-based readouts, altering mCherry’s spectral properties to generate a large Stokes shifted mutant was desirable. Mutations were made to the mCherry gene, replicating alterations that generate LSS mCherry in E. coli [27]. Spectral characteristics of our mCherry mutant in Msm were subsequently analyzed. To create a more diverse assay that is capable of studying mycobacterial PPI with a direct fluorescent readout, an mCherry BiFC system was created based on one previously constructed in mammalian cells [32]. Proof-of-concept of this assay was then tested using two distinct PPI pairs. Overall, these fluorescent tools were designed to improve the quality of the assays used to study PPI in Mtb so that enhanced drug screening platforms can be developed to discover antibiotics that combat TB better than those currently available.

7.1 Aim 1: TB Gyrase A and Fluorescent M-PFC

A GyrA/GyrA M-PFC assay was created to determine whether GyrA dimerization is detectable via M-PFC and to measure the strength of the interaction between the GyrA homodimer in the TB gyrase complex. Genetic engineering, transformation of the M-PFC constructs into Msm, and measurement of the relative interaction strength of the GyrA/GyrA pair were used as described above. The pUAB plasmids used to construct this assay were provided to our lab by Dr. Adrie Steyn
from the University of Alabama. Proof-of-concept of the M-PFC assay was demonstrated in previous experiments [17, 18] and the assay has since been modified and used successfully to characterize the strengths of a variety of protein partners in our lab [43].

To construct the GyrA/GyrA M-PFC strains, several steps were required. First, genetic engineering was used to generate the pair of target plasmids by modifying pUAB300 and pUAB400. Models of the target M-PFC plasmids shown in Figure 9 (GyrA_pUAB300 and GyrA_pUAB400) were constructed. On each target plasmid, the GyrA gene was fused via a flexible glycine linker to one domain of the DHFR reporter protein. Based on these models, primers were designed to amplify the vectors and GyrA fragments needed for FastCloning. The vectors and fragments were amplified via PCR and their identities were confirmed on an agarose gel based on their respective sizes, in base pairs. The GyrA fragments were 2517 base pairs in length, the pUAB300 vector was 4895 base pairs, and the pUAB400 vector was 4703 base pairs. These sizes could be clearly distinguished using the GeneRuler 1 kb DNA ladder on the agarose gel, as previously described. Next, the two vector and fragment pairs were fused together using FastCloning. The FastCloning products were transformed into E. coli to amplify the GyrA_300 and GyrA_400 plasmids. Following isolation of the plasmid DNA and positive confirmation by sequencing (data not shown) the plasmids were transformed into Msm individually and together as a pair. Once the three Msm M-PFC strains were created, the M-PFC was set up to measure the strength of the GyrA/GyrA interaction. Table 6 provides a description of the strains used in this assay. The two negative control strains, consisting of only one half of the PPI pair on its own in Msm, were included to ensure that neither construct alone was responsible for results indicating interaction in the GyrA_300+GyrA_400 strain. The positive DosR/DosR control was included because it is another M-PFC strain in which PPI have previously been confirmed using the same assay. DosR/DosR was
selected specifically because it exhibits moderate-strength PPI, similar to the strength expected of the GyrA/GyrA PPI.

Table 6: Strains Utilized During the GyrA/GyrA M-PFC Assay

<table>
<thead>
<tr>
<th>Strain Name</th>
<th>Plasmid Pairs</th>
<th>Vectors</th>
<th>Inserts</th>
</tr>
</thead>
<tbody>
<tr>
<td>GyrA_300 (Negative Control)</td>
<td>GyrA_pUAB300 Alone</td>
<td>pUAB300</td>
<td>GyrA</td>
</tr>
<tr>
<td>GyrA_400 (Negative Control)</td>
<td>GyrA_pUAB400 Alone</td>
<td>pUAB400</td>
<td>GyrA</td>
</tr>
<tr>
<td>GyrA_300+GyrA_400</td>
<td>GyrA_pUAB300+GyrA_pUAB400</td>
<td>pUAB300 and pUAB400</td>
<td>GyrA</td>
</tr>
<tr>
<td>DosR/DosR (Positive Control)</td>
<td>mCh_pUAB100-DosR + mCh_pUAB200-DosR</td>
<td>pUAB100 and pUAB200</td>
<td>DosR</td>
</tr>
</tbody>
</table>

The GyrA/GyrA M-PFC assay was run several times until conditions were optimized, minimizing variation between runs. It became evident that a critical factor in attaining consistency between repeats of the assay was creation of fresh TRIM stocks for each run. A qualitative measurement of the strength of the GyrA/GyrA PPI can be observed by observing the color change from blue to pink in each well visually. An example can be seen in Figure 15, which is a representative example of a GyrA/GyrA M-PFC assay result. Compared to negative control strains, the GyrA_300+GyrA_400 strains survive (appear pink or purple) at a higher concentration of TRIM, indicating that they are positive for PPI between GyrA.
During each assay, the GyrA_300+GyrA_400 strain exposed to the full range of TRIM concentrations was run twice to increase the accuracy of the results. Two additional columns were included in each assay: GyrA_300+GyrA_400 exposed to the maximum concentration of TRIM (50μg/mL) and GyrA_300+GyrA_400 exposed to no TRIM. The column exposed to the maximum amount of TRIM was used to calculate the background of each plate during quantitative analysis of the assay (described later). The column exposed to no TRIM was included to measure the maximum signal so that the experimental wells could be calculated as a percentage of the maximum signal for each plate.

To perform quantitative analysis of the GyrA/GyrA M-PFC assay, each plate was read in a fluorescent plate reader to quantify the fluorescent signal due to reduced Resazurin in the wells for
each column relative to the LB Tween control wells. A quantitative map is generated from this for each plate, displaying the fluorescent signal of the wells in Relative Fluorescence Units (RFUs). All plates were read using the same settings on the plate reader to ensure the RFU readings were scaled consistently. An example is shown in Figure 16. The map, like the visual result shown above, reflects the strength of the GyrA/GyrA PPI; however, it provides much more specific data about the presence of PPI in the strains. Quantitative maps were collected for three representative runs of the assay, and then used to assess the quality of the assay and the strength of the GyrA/GyrA PPI.

### Quantitative Map of GyrA_300/GyrA_400 M-PFC

<table>
<thead>
<tr>
<th>TRIM (ug/mL)</th>
<th>GyrA_300</th>
<th>GyrA_400</th>
<th>GyrA_300+GyrA_400</th>
<th>GyrA_300+GyrA_400</th>
<th>DosR/DosR</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>165</td>
<td>162</td>
<td>160</td>
<td>178</td>
<td>1638</td>
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<tr>
<td>12.5</td>
<td>163</td>
<td>198</td>
<td>892</td>
<td>936</td>
<td>1939</td>
</tr>
<tr>
<td>6.25</td>
<td>156</td>
<td>378</td>
<td>3799</td>
<td>3339</td>
<td>2593</td>
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<td>6982</td>
<td>6092</td>
<td>6727</td>
<td>2856</td>
</tr>
<tr>
<td>0</td>
<td>6688</td>
<td>6453</td>
<td>6251</td>
<td>6243</td>
<td>2851</td>
</tr>
<tr>
<td>LB Tween Control</td>
<td>171</td>
<td>157</td>
<td>175</td>
<td>185</td>
<td>195</td>
</tr>
</tbody>
</table>

**Figure 16: The Quantitative M-PFC Map.** This figure is an example of the quantitative map generated by reading an M-PFC plate in a fluorescent plate reader. This data is necessary to analyze the quality of the assay.

In all three maps, as in Figure 14, 6.25ug/mL is the concentration of TRIM at which a GyrA_300+GyrA_400 can be distinguished from the negative controls. Therefore, 6.25ug/mL TRIM was selected as the optimal screening point of this assay, were it to be used to screen for compounds that inhibit the GyrA/GyrA partnership. With this goal in mind, calculations assessing the quality of the assay were performed using data collected at the screening point. First, the signal-to-background ratio was calculated by averaging the fluorescence values at 6.25ug/mL TRIM for GyrA_300, GyrA_400, GyrA_300+GyrA_400, and the DosR/DosR control and dividing these averages by the background signal. The background was calculated by averaging the values of the
wells in the 50ug/mL TRIM columns. For each plate one outlier was excluded because they were likely generated by slight contamination from a neighboring well and were not representative of the background signal. The Z’-factor of the assay was also calculated at the screening point. The Z’-factor is a coefficient used to determine the range within which a signal is significant for an assay, which also reflects the quality of the assay. It is calculated from the standard deviations and the averages of the positive and negative controls of a single run of the assay. The Z’-factors of multiple plates are then averaged to generate a representative Z’-factor. The result is a number between 0 and 1; an assay with a result above 0.5 is judged to be well-suited for HTS applications. The Z’-factor formula is shown and explained in Figure 17 [14].

\[
Z' = 1 - \frac{3\sigma_{+c} + 3\sigma_{-c}}{|\mu_{-c} - \mu_{+c}|}
\]

**Figure 17: The Z’-factor formula.** The standard deviations are represented by \(\sigma\) and the averages are represented by \(\mu\). The subscripts refer to these values for the positive (+c) and negative (-c) controls.

The signal-to-background results and the Z’-factor are shown in Figure 18. The signal of the GyrA_300+GyrA_400 strain at the screening point is nearly 21 times greater than the background (the signal generated when the bacteria are completely killed by 50ug/mL TRIM). This is 23 times greater than the signal-to-background of the GyrA_300 negative control and 7 times greater than the GyrA_400 negative control. This indicates that at the screening point, the presence of the GyrA/GyrA PPI can be very clearly detected, and is easily distinguished from the assay’s negative controls. It is unusual that the signal of the GyrA_400 control is closer to the GyrA_300+GyrA_400 signal than the GyrA_300 control. However, because there is no way DHFR could be more active in the GyrA_400 control, this observation would likely diminish as more repetitions of the screen are
performed. Calculated from the same three representative plates, the Z’-factor of this assay is .582 which indicates that the assay is high enough quality to be used in a HTS application in the future but is not of exceptional quality. It is likely that the Z’-factor could be improved substantially if further measures were taken to optimize the screening conditions, including the number of days the bacteria are grown in TRIM, the starting OD of the bacteria, and the precision of the volumes added to each well by using automated liquid dispensers. This assay may also be improved by inserting a stronger promoter in place of Hsp60 to increase the production of the M-PFC constructs in the bacteria.

Figure 18: The Signal-to-Background and Z’-factor Results of the GyrA/GyrA M-PFC Assay.
Following the success of the GyrA/GyrA M-PFC, designs to convert the assay to a GFP-based fluorescent M-PFC were constructed. Models of the GyrA_pUAB300 and GyrA_pUAB400 plasmids were altered to include the GFP gene, inserted following a constitutive promoter (Hsp60) on both plasmids. The GyrA_GFP plasmids are shown in Figure 19. Primers were designed for amplifying the vectors and inserts via PCR according to the GyrA_GFP models. The vectors for constructing the fluorescent M-PFC plasmids were amplifying the GyrA_pUAB300 and GyrA_pUAB400 plasmids, and the GFP fragments were amplified from the pvvRG and

![Figure 19: Designs for the GFP GyrA/GyrA M-PFC Plasmids](image)

pUAB400_GFP plasmids. The presence of the vectors and fragments was confirmed on an agarose gel based on size. Then the GyrA_pUAB300 vector was FastCloned with the GFP fragment from pvvRG, and the GyrA_pUAB400 vector was FastCloned with the GFP fragment from pUAB400_GFP. Recently, the FastCloning products were transformed into *E. coli*, and plated on selective media. Colony PCR screening was performed, and thus far only colonies from the GFP_GyrA_pUAB300 transformation were positive for GFP. Further attempts at acquiring GFP positive GFP_GyrA_pUAB400 *E. coli* are ongoing. In the meantime, the GFP_GyrA_pUAB300
positive colonies were grown overnight to amplify the DNA. The DNA was isolated from the cultures and transformed into Msm already containing GyrA_400, which was plated on selective media. The colonies on the resulting plates, unusually, were both yellow and white. GFP-positive Msm may appear yellow when plated as a lawn, but isolated colonies do not typically produce high enough levels of fluorescence to appear yellow. Therefore, three colonies were selected and grown in cultures: a bright yellow colony, a weakly yellow colony, and a white colony. A negative control culture, GyrA_300+GyrA_400, was also grown. These cultures were examined for green fluorescence on a fluorescent plate reader after three days of growth. The ODs of the cultures were normalized, and then 500uL of each culture was pelleted and resuspended in 100uL of PBS Tween to eliminate background signal from LB Tween. A PBS control was also included, and the fluorescent signals of the cultures were scaled to this negative control. A positive GFP control was not examined during this initial screen, but under similar conditions and at a similar OD, an Msm strain containing the pvvRG plasmid expresses GFP signal that is ~25 times brighter than the background signal from PBS Tween. Although all of the GFP_GyrA_300+GyrA_400 colonies did exhibit higher fluorescence than the GyrA_300 negative control, even the highest signal (Colony 1) was only 2.27 times brighter than negative control. The signal-to-background of the colonies is shown in Figure 20.
**Figure 20: Green Fluorescence of the GFP_GyrA_300+GyrA_400 Colonies.** These colonies were analyzed to determine whether the insertion of GFP on the GyrA_pUAB 300 plasmid produces a green fluorescent signal that is sufficient for strains containing this plasmid to be used in a fluorescent GyrA/GyrA M-PFC assay.

Because it is typically expected that a fluorescent protein inserted onto a plasmid vector will have a signal-to-background value many times higher than this and because the plate contained multiple morphologies, all three colonies were PCR screened for GFP to confirm that they contained GFP_GyrA_300+GyrA_400 in Msm and were not merely contaminants or false positives. Using the same colony screening protocol used on *E. coli*, it was found that only Colony 3 contained the GFP gene. Therefore, from this preliminary experiment, it appears that the GFP_GyrA_300+GyrA_400 Msm strain does not emit a high enough fluorescent signal to be applied to a fluorescent M-PFC. Due to time constraints, sequencing of this plasmid was not performed, so it is possible that a mutation in the GFP gene or the inserted promoter is causing the absence of fluorescence. Sequencing should be performed to rule this out, after which alternative approaches could be taken to improve the fluorescent signal. Creating a double GFP (GFP-GyrA_300+GFP_GyrA_400) Msm strain, with GFP on both the chromosomal and episomal plasmids, may improve the quality of the signal. This is likely since GFP would be expressed on both the chromosomal and the episomal plasmids, instead of just on the episomal. Another potential
approach would be to substitute the Hsp60 promoter on both constructs for a stronger promoter, like the smyc promoter used to increase the production of mCherry in the mCherry fluorescent M-PFC. Selecting a fluorescent protein that does not have an excitation wavelength in the GFP range may also improve the readout of the assay because Msm and the LB Tween media it is grown in does exhibit higher than ideal background signal in this range.

7.2 Aim 2: LSS mCherry

Based on knowledge of other LSS FPs, two amino acids within the mCherry gene were strategically mutated to generate LSS mCherry, which was expressed in *E. coli* \[27\]. In *E. coli*, LSS mCherry exhibited levels of fluorescence comparable to those of wild type mCherry, but its maximum excitation wavelength shifted from 587nm to 456nm (see Figure 21) \[27\]. RTHM primers were designed to replicate these mutations, generating a serine at amino acid 158 and a glutamate at amino acid 160 by in an mCherry gene that is codon optimized for expression in mycobacteria. To exchange these amino acids, a T was replaced with a G at base pair 497 of mCherry and a C was replaced with a G at base pair 502 \[27\]. To make these two point mutations, RTHM was performed
using pvvRG as a vector, which contains the codon optimized mCherry gene. The RTHM product was transformed into *E. coli* to amplify the DNA, and then the DNA was sequenced to confirm that only the target point mutations had been made to the mCherry gene (See Figure 22). Sequencing was also performed to ensure no mutations to the promoter region were made and the stop codon of the

**Figure 21: The Spectral Properties of Large Stokes Shift mCherry Due to Two Amino Acid Mutations**

mCherry mutant was intact. Then, the LSS mCherry DNA was transformed into Msm and cultures of the LSS mCherry strain were grown from three colonies of the single transformation, along with a
culture containing a strain positive for wild type mCherry. These cultures were then pelleted, resuspended in PBS Tween, and screened for their excitation spectra at an emission of 610nm using a fluorescent plate reader. The excitation spectra of the three colonies were averaged and a graph comparing the LSS mCherry mutant in Msm to a control containing PBS alone and the positive mCherry control is found in Figure 23. As the figure shows, the LSS mCherry contains neither a significant excitation peak at 456nm as predicted, nor an excitation peak at 587nm as wild type mCherry does. Rather, no significant excitation peak was observed in the LSS mCherry mutant, while an excitation peak is clearly observed in the wild type mCherry control at ~587nm.

![Mutant mCherry Excitation Scan](image)

**Figure 23: An Excitation Scan Comparing the LSS mCherry Mutant to Wild Type mCherry.** This excitation scan was performed from 400nm through 585nm for an emission wavelength of 615nm to examine the spectral properties of the mutant mCherry to those of the wild type.
These observations can be explained in several ways. For example, it is possible that the mutations to the mCherry gene cause the protein to misfold or to be degraded, due to differences in the Msm cell environment compared to the environment inside E. coli. To determine whether this hypothesis is true, a Western Blot could be done to test for the presence of mCherry. This step was not performed during this project because, while it might have helped explain the observations, it would not have altered the fact that this LSS mCherry mutant is not useful to us in Msm. It also was not performed because we do not currently have either an antibody or a tag on mCherry. Another approach to improve the outcome of this aim is to make different point mutations to the mCherry gene. A different set of amino acid substitutions generated another LSS mCherry mutant in E. coli and this alternate mutant may be expressed more successfully in Msm than the first mutant [27].

7.3 Aim 3: The Split mCherry BiFC

Two sets of mCherry BiFC plasmids were designed to attempt proof-of-concept of the mCherry BiFC with two different PPIs: GCN4/GCN4 and AprA/AprB. These two PPIs have previously been identified for having strong interactions using the M-PFC assay [43]. First, from the plasmids pUAB 100 and pUAB 400, the split mCherry plasmids pUAB100_mCh and pUAB400_mCh were designed. Primers were made to amplify the mCherry gene in two fragments, split between amino acids 159 and 160 [32]. N-terminal mCherry (amino acids 1-159) was inserted into the pUAB 400 vector, and C-terminal mCherry (amino acids 160-236) was inserted into the PCR amplified pUAB 100 vector through FastCloning. The pUAB 400 and pUAB 100 vectors used already contained GCN4 in the PP1 and PP2 positions. Also, the N-terminal and C-terminal mCherry fragments took the place of the DHFR A and DHFR B fragments in these plasmids. These new constructs were transformed into E. coli, amplified, isolated, and sequenced. Following sequence
confirmation, the process of creating the target GCN4 BiFC constructs (GCN4_pUAB 100_mCh and GCN4_pUAB 400_mCh was complete). For each of the final AprA and AprB constructs, primers were designed to amplify the fragments (AprB and AprB) and vectors (GCN4_pUAB 100_mCh and GCN4_pUAB 400_mCh) via PCR. Then the presence of the fragments and vectors were confirmed on an agarose gel by size and FastCloned together to create the target plasmids. The FastCloning products were transformed into *E. coli* to amplify the DNA, and sequencing was performed on the two new constructs to ensure they matched those modeled in Figure 24.

![Completed mCherry BiFC Plasmids](image)

**Figure 24: Completed mCherry BiFC Plasmids**

The plasmids were then transformed into Msm in pairs: GCN4_pUAB 100_mCh with GCN4_pUAB 400_mCh, and AprB_pUA B400_mCh with AprA_pUA 100_mCh. Individual control strains were also generated, although better controls for the BiFC technique exist and will be discussed later. The BiFC Msm strains used for this experiment are detailed in Table 7.
Table 7: The Msm Strains Examined for Proof-of-Principle of the mCherry BiFC

<table>
<thead>
<tr>
<th>Strain Name</th>
<th>Plasmid Pairs</th>
<th>Vectors</th>
<th>Inserts</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCN4_100_mCh (Negative Control)</td>
<td>GCN4_pUAB 100_mCh</td>
<td>pUAB100_mCh</td>
<td>GCN4</td>
</tr>
<tr>
<td>GCN4_400_mCh (Negative Control)</td>
<td>GCN4_pUAB 400_mCh</td>
<td>pUAB400_mCh</td>
<td>GCN4</td>
</tr>
<tr>
<td>GCN4_100_mCh + GCN4_400_mCh</td>
<td>GCN4_pUAB 100_mCh + GCN4_pUAB 400_mCh</td>
<td>pUAB100_mCh and pUAB400_mCh</td>
<td>GCN4</td>
</tr>
<tr>
<td>AprA_100_mCh (Negative Control)</td>
<td>AprA_pUAB100_mCh</td>
<td>pUAB100_mCh</td>
<td>AprA</td>
</tr>
<tr>
<td>AprB_400_mCh (Negative Control)</td>
<td>AprB_pUAB 400_mCh</td>
<td>pUAB400_mCh</td>
<td>AprB</td>
</tr>
<tr>
<td>AprA_100_mCh + AprB_400_mCh</td>
<td>AprA_pUAB 100_mCh + AprB_pUAB 400_mCh</td>
<td>pUAB 100_mCh and pUAB 400_mCh</td>
<td>AprA and AprB</td>
</tr>
<tr>
<td>mCh_DosR_100 + mCh_DosR_200</td>
<td>mCh_DosR_pUAB100 + mCh_DosR_pUAB200</td>
<td>DosR_pUAB100 and DosR_pUAB200</td>
<td>mCh</td>
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The first mCherry BiFC strains designed and completed were the GCN4/GCN4 strains because GCN4/GCN4 is a stronger PPI than AprA/AprB according to previous M-PFC data [43]. More is also known about the structure of GCN4, which is necessary when creating the ideal negative controls for a BiFC assay. Ideal negative controls for a BiFC assay are strains that contain BiFC constructs in which the interaction interface between PP1 and PP2 has been strategically mutated to interrupt their interaction, but to generate these controls the structure of the partners should be well understood [28]. These ideal negative controls are only truly necessary to rule out false positive signals from a BiFC assay because they confirm that a positive signal was not due to non-specific interactions generated from unanticipated associations with the BiFC protein constructs [28]. Rather than expending considerable resources to generate these ideal negative controls at the outset of the project, alternative negative control strains were constructed instead. If a positive signal
were generated from the GCN4/GCN4 BiFC strains being examined for proof-of-principle, then the ideal negative controls would have been engineered to ensure the validity of the signal. The alternative negative control strains examined in this experiment were GCN4_100_mCh and GCN4_400_mCh, generated by transforming either the episomal or the chromosomal plasmid alone into Msm. Each of these strains contains only one half of the split mCherry protein fused to one of the protein partners. Thus, these strains are negative controls in that they reflect the signal produced when only one of these constructs is present at a time and in that they should produce no signal at all. A strain containing intact mCherry on both the episomal and chromosomal plasmids, producing mCherry constitutively, was also used during this experiment as a positive control for the signal produced by functional mCherry (what a positive BiFC signal should look like).

Assessment of the GCN4/GCN4 BiFC strain, along with the negative and positive control strains, was performed as described previously, and the level of fluorescence of the strains relative to the background over time is shown in Figure 25. As already mentioned, split mCherry does not reconstitute well at 37°C. Rather, cells must be incubated at room temperature (~25°C) overnight or at 4°C for 1-2 hours before reconstitution is observed. Therefore, fluorescence was assessed at both room temperature and 4°C, and periodically over a span of 1-24 hours to ensure any reasonably expected signal was not overlooked. At both temperatures and across all time points, no signal from the GCN4/GCN4 BiFC stain sufficiently similar to the signal of the positive control was observed. Although the GCN4/GCN4 homodimer is a strong PPI, its leucine zipper structure may explain the negative BiFC results. The mCherry fragments must be arranged on the BiFC constructs so that the end of the N-terminal fragment is exposed to the beginning of the C-terminal fragment. This necessitates that the mCherry fragments are attached at opposite ends of the GCN4 protein.
When these constructs are expressed and the GCN4 portions dimerize, the mCherry N-terminal and C-terminal fragments are separated across the leucine zipper from one another (See Figure 26 for illustration). It is possible that this arrangement does not allow adequate proximity of the mCherry
fragments, so reconstitution does not occur, even though this length was sufficient for DHFR A and B reconstitution. Increasing the length of the glycine linker may solve this problem by increasing the distance across which the fragments are free to move, increasing the likelihood that the non-fluorescent fragments will meet and reconstitute mCherry. Because alternative plasmids with many different lengths of glycine linkers would need to be constructed to examine this hypothesis, this route was not pursued due to time constraints. Instead, the focus of the project shifted to testing the BiFC on a pair of much smaller protein partners (AprA and AprB), which were already of interest and seemed unlikely to pose the same hypothesized problems as the GCN4/GCN4 BiFC.

The AprA/AprB BiFC strain was designed based on previous M-PFC data that demonstrated significant interaction between the protein partners, as long as the C-terminus of AprB remained free (See Figure 27) [43]. In accordance with this requirement, AprA was inserted in GCN4’s place on the GCN4_pUAB 100_mCh plasmid and AprB was inserted in GCN4’s place on the GCN4_pUAB 400_mCh plasmid. These plasmids were used to construct the AprA/AprB BiFC strain. Negative controls analogous to those selected for the GCN4/GCN4 BiFC experiment were also made, so that AprA_pUAB 100_mCh and AprB_pUAB 400_mCh were each transformed alone into Msm. The same positive control was used as in the GCN4/GCN4 BiFC experiment.

Figure 26: Hypothetical Illustration of Structural Issues Preventing Success of the GCN4/GCN4 BiFC
After the *AprA/AprB* BiFC strain, the negative control strains, and the positive control were grown, the same protocol used to analyze the signal over time for the GCN4/GCN4 BiFC was used to assess the *AprA/AprB* BiFC. The fluorescence of the strains relative to the background over time for both room temperature and 4°C are shown in Figure 28. The AprA_100_mCh strain did not grow sufficiently, so it could not be included in this analysis.

Like with the GCN4/GCN4 BiFC, at both temperatures and across all time points, no signal from the *AprA/AprB* BiFC strain sufficiently similar to the signal of the positive control was observed. While the glycine linker length may also be the problem in the *AprA/AprB* BiFC, it is possible that other issues explain the lack of signal. For example, the complete BiFC protein constructs (the protein partner, plus the glycine linker, plus the mCherry fragment) may not be expressed well in Msm. Also, the temperature and time required to reconstitute mCherry in Msm may differ from those required in the Vero cells in which it was originally studied. An alternative approach might first select a different split FP that is known to reconstitute readily at 37°C to eliminate the potential for this problem and then test a variety of molecular linkers of different sizes.

**Figure 27: AprA/AprB M-PFC Data.** The *AprA/AprB* PPI is strong when the C-terminal of *AprB* is free to interact with *AprA*. 

<table>
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<tr>
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<th>Msm 4°C</th>
<th>Vero 20°C</th>
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Figure 28: Red Fluorescent Signal Produced by the *AprA/AprB* BiFC Over Time When Strains Were Incubated at Room Temperature and at 4°C.
CHAPTER EIGHT: DISCUSSION

The persistent and growing threat to global health of TB and drug-resistant TB is exacerbated by the impractical drug treatments currently available and a lack of new, more effective antibiotics to replace them. Though efforts have been made to develop better treatments, the pipeline of TB drug development remains slow, and innovative, optimized drug-screening platforms are needed. Knowledge about the PPI necessary to Mtb’s pathogenesis is still largely lacking, but great potential lies in developing drug screening techniques that are amenable to HTS and combine the benefits of target-based screening for novel PPI with the benefits of whole cell screening. Therefore, engineering tools that improve our ability to validate PPI necessary for Mtb to cause disease under host-like conditions and also improve our ability to screen for compounds that disrupt these PPI is a promising step toward discovering novel drug therapies to treat TB.

This project’s aims were focused on creating tools to advance the study of PPI in Mtb and to improve the quality of current PPI-based TB drug screening platforms. First, a new PPI assay applicable to HTS was generated: the GyrA/GyrA M-PFC. Gyrase A is an essential target in Mtb that is of interest for drug screening. Future work will optimize this assay further to improve its quality, after which it can be used to study the efficacy of a library of compounds at disrupting the GyrA/GyrA dimer interface. With this future application in mind, attempts were made to streamline the M-PFC assay readout.

To transition away from a viability readout requiring the addition of Alamar blue dye and an additional incubation, the GyrA/GyrA M-PFC plasmids were endowed with GFP following a constitutive promoter region. The fluorescent signal produced by the GFP_GyrA episomal plasmid alone was not yet sufficient to apply the strain to a fluorescent M-PFC assay. Future work on this aspect of the project would test the fluorescent signal produced when GFP was present on both the
episomal and chromosomal plasmids of the GyrA/GyrA M-PFC, and a stronger promoter region may be inserted in front of the GFP gene to increase the signal further. If the signal of the improved fluorescent M-PFC strain reached a sufficiently detectable level, then the quality of the fluorescent M-PFC would be compared alongside the original GyrA/GyrA M-PFC. A high quality GFP GyrA/GyrA M-PFC could later be applied to HTS.

Two different red-fluorescent tools were also assessed: LSS mCherry and the mCherry BiFC. The spectral properties of LSS mCherry hold the potential to improve the sensitivity of assays that rely on mCherry for their readout, like the mCherry-based M-PFC. Mutations to the mCherry gene were made to mimic those that generated an LSS mCherry protein in *E. coli*; however, mutating the mycobacterial-optimized mCherry gene and transforming this construct into Msm did not produce a strain exhibiting LSS mCherry fluorescence. Future work on this project might determine whether other mutations to the mCherry gene previously shown to generate LSS mCherry in *E. coli* can generate LSS mCherry in Msm. Alternately, mutations shown to produce a different LSS FP may be investigated to determine whether an LSS FP other than mCherry can be engineered and will be expressed more readily in Msm.

The final tool investigated was the mCherry BiFC, originally demonstrated in Vero cells. BiFC in theory provides advantages compared to current Mtb PPI assays like the M-PFC because its readout directly reflects the presence of PPI. Though BiFC has been successfully applied to many cell types and a variety of protein partners, proof of principle was not achieved in mycobacteria during this project. Two distinct protein pairs (GCN4/GCN4 and *AprA/AprB*), which show strong interaction in the M-PFC assay, were used to test the mCherry BiFC. Neither of these partnerships produced the expected fluorescent readout, and much of the future work on this project would likely be focused on determining the explanation for this through trying alternative split FPs that
more easily reconstitute at 37°C, testing different linker regions, and ensuring expression levels of the protein partner constructs are not compromised.

The final outcome of this project is a functional PPI assay for the Gyrase A homodimer, which can swiftly be translated to a new HTS drug screening platform for this highly relevant target. This may lead to the discovery of new treatment options for drug-resistant TB in the future, which would lend further credibility to the idea that developing TB drug screening platforms that target PPI is an innovative and favorable endeavor. While the fluorescent tools investigated during this project are not yet functional, much of the work necessary to determine whether their application to Mtb is ultimately feasible has been completed. Additionally, the process of engineering them provided helpful insight into the difficulties of designing tools like this and applying them to Mtb in the future.
REFERENCES


43. Hodges, H., *Identifying Protein-Protein Interactions in Mycobacterium tuberculosis Using Mycobacterial Protein Fragment Complementation (M-PFC)*. 2015, University of Central Florida College of Medicine.