Mycobacterium Tuberculosis Regulation of Efflux Pump Tap By Transcriptional Activator WhiB7

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MYCOBACTERIUM TUBERCULOSIS REGULATION OF EFFLUX PUMP TAP BY TRANSCRIPTIONAL ACTIVATOR WHIB7

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of Bachelors of Health Sciences Pre-Clinical Track in the College of Health and Public Affairs and in the Burnett Honors College at the University of Central Florida Orlando, Florida

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Abstract

Tuberculosis, caused by *Mycobacterium tuberculosis* (*Mtb*), remains a debilitating disease that affects the health of millions annually. Understanding its ability to persist within host and resist eradication by antibiotics is of utmost importance in the effort to develop new interventions. This study will focus on the transcriptional activator WhiB7 and its regulation of the multidrug Tap efflux pump encoded by Rv1258c. WhiB7 is thought to respond to redox stress induced by antibiotics and a variety of *in vivo* stresses by activating multiple genes including Rv1258c. Much remains to be determined regarding the role of WhiB7 and downstream genes in *Mtb* virulence and drug resistance. We will create a tool for studying WhiB7-mediated gene regulation by engineering a strain of the nonpathogenic bacterium *Msm* expressing the mCherry fluorescent protein controlled by the Rv1258c promoter. Knocking out the native WhiB7 gene in *Msm* via homologous recombination will allow clear introduction of wild type and mutant versions of *Mtb* WhiB7. Changes in the fluorescent activity of Rv1258c promoter fusion to mCherry will indicate the effects of WhiB7 mutagenesis. Secondly, we can also use this system to confirm additional genes identified by microarray analysis that are potentially regulated by WhiB7. This will be done by cloning other promoters in front of mCherry in the *Msm* strain containing wild-type *Mtb* WhiB7. Understanding WhiB7’s role in *Mycobacterium tuberculosis* macrophage survival and antibiotic resistance may provide new strategies for developing drugs that can lead to a cure.
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Introduction

*Mycobacterium tuberculosis* (*Mtb*), the etiologic agent of tuberculosis, remains a debilitating disease that affects the health of millions annually. *Mtb* is transmitted in airborne droplets from one infected individual to another where it will reside in characteristic lesions known as granulomas within pulmonary tissue. The mycobacteria bacilli survive in granulomas formed by the host white blood cells, primarily macrophages and T lymphocytes. In the case of active infection, the body's immune system fails to contain the bacilli within granulomas, resulting in pulmonary damage and spread to other tissues within the body leading to systemic impairment or death [1]. Treatment of tuberculosis requires a cocktail of four drugs taken over the course of six to nine months. Tuberculosis is particularly difficult to treat due to the nature of its intrinsic resistance mechanisms such as its cell wall structure [2]. *Mtb* has a characteristically thick mycolic acid-containing cell wall which makes it resistant to antibiotic penetration [3]. Additionally, *Mtb* regulates inducible efflux pumps and activates enzymes that assist in making it resistant to antibiotics and persist within macrophage [4]. The emergence of Multi-Drug Resistant (MDR) and Extremely Drug Resistant (XDR) strains of *Mtb* further complicates the problem [3]. Understanding *Mtb*’s ability to persist within immune cells and pulmonary tissue and resist eradication by antibiotics is of utmost importance in the effort to develop new drug interventions.

This study will focus on the transcriptional activator WhiB7 and its regulation of the multidrug Tap efflux pump encoded by Rv1258c. WhiB7 is thought to respond to redox stress induced by antibiotics and a variety of in vivo stresses by activating multiple genes including
Rv1258c. Much remains to be determined regarding the role of WhiB7 and downstream genes in *Mtb* virulence and drug resistance. We will create a tool for studying WhiB7-mediated gene regulation by engineering a strain of the nonpathogenic bacterium *M. smegmatis (Msm)* expressing the mCherry fluorescent protein controlled by the Rv1258c promoter. Knocking out the native WhiB7 gene in *Msm* (WhiB7$_{Msm}$) via homologous recombination will allow clear introduction of wild type and mutant versions of *Mtb* WhiB7 (WhiB7$_{Mtb}$). Changes in the fluorescent activity of Rv1258c promoter fusion to mCherry will indicate the effects of WhiB7 mutagenesis. Secondly, we can also use this system to confirm additional genes identified by microarray analysis that are potentially regulated by WhiB7. This will be done by cloning other promoters in front of mCherry in the *Msm* strain containing wild-type WhiB7$_{Mtb}$. Understanding WhiB7’s role in *Mtb* macrophage survival and antibiotic resistance may provide new strategies for developing drugs that can lead to a cure.
Introduction

*Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB), continues to be prevalent in today’s society. Dating back to 1882, microbiologist Dr. Robert Koch was the first to characterize the tuberculosis infection as caused by *Mycobacterium tuberculosis* (*Mtb*). In 1882, tuberculosis was said to take the life of one out of seven individuals in the United States and Europe alone. In present day, we have seen this number greatly reduced, although there are still nine million new cases and 1.3 million deaths due to TB each year. This problem is exacerbated by the emergence of multidrug-resistant (MDR) strains [1]. 95% of infected individuals are in developing countries. Tuberculosis, which infects all ages, is the second greatest killer of individuals caused by a single infectious agent worldwide, second to HIV/AIDS [2]. HIV co-infection with TB kills one out of five HIV patients annually. It is important for us to be able to understand the microbiological components found in *Mtb* and how and why this disease still persists in today’s society. The purpose of this section is to provide relevant background information on this bacterial pathogen, its antibiotic resistance mechanisms, and provide relevant innate immunological information about the pathophysiological response to tuberculosis.

Who is at risk?

There are multiple factors that increase the risk of individuals contracting tuberculosis. Persons at particular risk are individuals with a re-activation of poorly treated infections, persons who illicit drugs, and persons with human immunodeficiency virus (HIV). People coinfected with HIV-TB have the worst prognosis than any of the other listed populations [3].
HIV is a retrovirus that brings on immunodeficiency, which makes individuals more susceptible to opportunistic infections such as tuberculosis. 12% of HIV patients die as a result of tuberculosis infection [4]. When an individual with HIV is exposed to active tuberculosis, tuberculosis infection is harder to diagnosis with sputum smear microscopy, and is more likely to reactivate [5]. Co-infected individuals are treated using antiretroviral therapy, ART. ART is a treatment plan designed to suppress the HIV virus. When HIV patients are given treatment for tuberculosis while starting ART, pharmacokinetic interactions with rifamycins tend to occur. Thus, treatment for HIV patients co-infected with tuberculosis tend to raise the mortality rate of infected persons [6].

Vaccines

The tuberculosis vaccine is available to individuals at risk for incidence of infection. The available vaccine called Bacillus Calmette-Guérin, BCG, is a live attenuated bovine strain of tuberculosis (M. bovis). The vaccine is usually given to children who are consistently exposed to parental caregivers with tuberculosis, or are at risk of exposure due to geographic factors. Immunizations are also given to healthcare providers that are at risk of occupational exposure [7]. Risk factors prompting immunization include occupational exposure to Mtb, high frequency of hospital acquired infections, and poor facility infection control.

Immunocompromised individuals are not provided with the vaccine due to subsequent opportunistic infection that may be possible if given. Pregnant women are omitted from vaccination as precaution. Specifically in developed countries, the vaccine is not administered to the general population; if given, it will give false positive results for the tuberculin purified
protein derivative (PPD) skin test. Although, success of the current BCG vaccine is limited: approximately 0-80% of the individuals that receive the vaccine are resistant to the life-threatening miliary and meningeal infections [7].

**Diagnostics**

Currently the most common method of diagnosis is the acid-fast sputum smear. In this method, a sputum sample taken from suspected patients is stained and examined through microscopy for the presence of acid-fast bacilli. This procedure is only able to identify approximately 50% of infections. Bacterial culture and identification is the gold standard to positively identify TB. However useful, this is a dangerous and slow method given the hazardous and slow-growing characteristics of *Mtb*. NAAT (nucleic acid amplification tests) or polymerase chain reaction based methods are more sensitive and specific, but are underutilized because they are expensive and not feasible in resource limited settings.

Patients suspect for tuberculosis infection, active or latent, are given a PPD skin test. The PPD is an extract from *Mtb* and is injected into patient's skin intradermally. When the body has been previously exposed to *Mtb* antigens, such as in an infected or vaccinated state, local inflammation at the site of injection will occur due to the innate immunological response to the foreign agent. The response to the antigen can be detected on the skin by studying the radius of inflammation. If the skin test is positive, the individual will be then given a chest x-ray to determine if the individual has an active, or latent form of tuberculosis. Post-diagnosis, the patient will start on a rigorous antibiotic regimen for up to twelve months depending on the severity of the infection.
Treatment
Currently there are two treatment plans to cure tuberculosis which is determined by the strain of *Mtb* that has caused the infection. Treatment of active drug-sensitive tuberculosis involves the use of antibiotics such as isoniazid, rifampin (or rifapentine), ethambutal, and pyrazinamide over the course of six to twelve months. Treatment of multidrug-resistant tuberculosis infection (MDR TB), and extensively-drug resistant tuberculosis infection (XDR TB) is more complex. Treatment of XDR and MDR strains is difficult because these strains are resistant to the frontline drugs, rifampicin and isoniazid, and some second-line drugs in the case of XDR TB. The second line fluoroquinolone and injectable drugs are then utilized for MDR, and XDR strains [1, 8]. In most cases, the drugs are unable to permeate the granulomas in the lung, or *Mtb* has become inherently phenotypically drug tolerant. Additionally, due to the harshness of the antibiotics on the system, patient compliance is reduced. Treatment of MDR and XDR strains of *Mtb* have adverse effects on the health of the patient; this results in six out of ten infected patients dying at the same rate as an infected individual not taking the treatment [9].

Microbiologic characteristics of *Mycobacterium tuberculosis*
Mycobacteriaceae is a non-endospore forming, acid-fast positive organism, which means the bacterium contains a thick peptidoglycan and mycolic wax layer in the outermost leaflet of the cell wall. Mycobacterium is a non-motile family of organisms dependent on the presence of oxygen for growth and production of energy. They have an unusually high content of guanine and cytosine, over 60%, in their deoxyribonucleic acid, DNA. This study focuses on
the most deadly pathogenic member of this genus, *Mycobacterium tuberculosis*. *Mtb* is known for its very slow growth rate, structural tolerance to their environment, and mechanisms that inhibit the antimicrobial response of macrophages.

The peptidoglycan layer of this acid-fast bacterium is capable of making the organism structurally resistant to osmotic lysis. This structure is a polymer of sugars N-acetylglucosamine and N-acetylmuramic acid and amino acids such as L-alanine, D-alanine, D-glutamic acid and diaminopimelic acid. The peptidoglycan layer is formed by a chain of sugars that run parallel to each other while forming amino acid cross-links that acts as an interbridge between sugar chains. The rigidity of this cell wall is able of providing the organism with the resistance to external aqueous environments, where gram-negative organisms might have susceptibility to these influences; thus aiding in the transmission of *Mtb* through liquid droplets.

Targeting the peptidoglycan layer with drugs is a traditional way to treat diseases caused by gram-negative bacteria. Antibiotics such as penicillin block the formation of the N-acetylglucosamine and N-acetylmuramic acid bonds, leaving the bacterium susceptible to eventual osmotic lysis [10]. But targeting this structure in *Mtb* is ineffective due to the presence of the mycolic acid layer.

In addition to the peptidoglycan, the cell wall of *Mtb* has a lipid-wax complex that provides additional resistance to environmental and antibiotic stress. Mycolic acids are the outer most surface of the mycobacterial cell wall. This layer is a complex of hydroxylated lipids that provide a lipophilic surface covalently bound to the cell wall. This mycolic wax surface
reduces permeability to antibiotics which narrows the spectrum that is available to target and treat the bacteria.

Mycolic acid is a primary contributor to the cell wall’s resistance to the degrading activity of the macrophage’s cationic, lysozyme and oxygen radical activity. Additionally, the cord factor component of *Mtb*’s cell wall is toxic to mammalian cells and is abundant in virulent strains of *Mtb* [11, 12]. Cord factor is an inhibitor of polymorphonuclear leukocyte migration and fusion with infected macrophages, thus slowing the activity of the granuloma [12]. Granulomas are formed in response to the presence of *Mtb* in the lung, and will be discussed in subsequent sections.

**Transmission**

The transmission of *Mtb* is a topic of heightened concern. Tuberculosis is spread in liquid droplets contained inside what is referred to as droplet nuclei. Droplet nuclei are small micro-droplets about the size of a micrometer that can contain an infectious agent, in this case *Mtb*, which can be maintained in the air for hours. These droplet nuclei carry the infectious agent from one host to another through expulsion from the infected pulmonary or laryngeal tissue by means of sneezing, coughing or talking [13]. The *Mtb* droplet nuclei are taken into the lungs, and dispersed throughout the lung tissue in various places. After inhalation of infected droplets by a non-infected host, infection is determined by the strength of the host’s immune system, number of the bacilli in the droplet, and the exposure of the bacterium to UV light [14, 15]. If a bacterial presence can be maintained inside the pulmonary tissue of the host, there is an increased likelihood the bacillus can be transmitted to other tissues through the lymphatic
and circulatory systems. Systemic impairment and death will occur if the infection is not contained before spreading. The subsequent section will explain the pathophysiology of the initial immunological response to foreign agents as it relates to tuberculosis infection.

**Innate immunology: Macrophage Infection**

It is imperative in this study to understand the natural immunological response mediated by macrophages in order to understand the pathophysiology of a tuberculosis infection. This section will cover the basic immunological response to an infectious agent in the lungs, then follow with a section that relates specifically to tuberculosis infection.

During inhalation, an infectious agent will first encounter physical barriers of the innate immune system, the non-specific first line of defense. Initially the infectious agent is exposed to the pseudostratified ciliated columnar epithelium which lines the trachea. This type of epithelium is responsible for the secretion of mucus, composed of glycosylated proteins that function to trap foreign particles, and utilizing its characteristic cilia to move the secreted mucus in a particular direction. In the case of the trachea, the objective of this mucociliary escalator is to push trapped particle upward to the mouth for expulsion. This is an effective system of physical immune barriers that is able to rid the body of most airborne and ingested contaminants [16]. If the liquid droplet can bypass the mucus physical barrier, the agent will travel down the lung and into the airways of the lung. Typically, the sight of initial infection is found in airways such as the alveoli of the lung. These small air pockets are lined with squamous epithelium that are the sight of the next immunologic agent in the sequence, called the macrophage [14, 16].
Macrophages are the key players in the innate immune response, serving as a frontline of defense against pathogens [17]. Macrophage are professional phagocytes capable of ingesting and destroying most pathogens and initiating and coordinating an adaptive immune response. Macrophages recognize and phagocytose foreign bodies by binding to specific sites called pathogen-associated molecular patterns (PAMPs). These patterns are traditionally specific structures (i.e. surface proteins or carbohydrates) of a pathogen that bind to a specific pattern recognition receptor (PRR). For example: a macrophage will recognize the lipoarabinomannan glycolipid located in Mtb’s cell wall, the PAMP in this case, and bind to its PRR. Once the macrophage recognizes the PAMP on the foreign agent the cell membrane will extend and engulf the phagosome creating a phagosome[18].

Infected macrophages release hydrolytic enzymes, cationic proteins and oxygen radicals from lysosomes. These degradative agents are released into the phagosome after the macrophage’s lysosome fuses with the vesicle containing the engulfed bacterium, forming a phagolysosome [12]. Following phagocytosis of a pathogen, macrophages will initiate the body’s immunologic response by releasing cytokines such as monocyte chemoattractant proteins to attract other macrophages and leukocytes to the site of infection [19]. After signaling the immunologic response, the infected alveolar macrophage becomes surrounded by mononuclear cells in an attempt to sequester any foreign agent from the rest of the surrounding pulmonary tissue. This complex of non-infected macrophages and infected macrophages is the containment structure of the immune system called the granuloma. After the formation of the granuloma, the bacterial components are degraded and utilized or
exocytosed for removal from the tissue. In the case of most bacteriological infectious agents, this disposal method is sufficient to maintain homeostasis, and allows the body to be innately resistant to foreign agents.

In the case of tuberculosis, the innate immune response may be unable to completely eradicate this infectious agent. Failure to kill all *Mtb* is due to TB’s system of resistance to external stresses which allow the bacterium to survive when exposed to host-derived antimicrobial mechanisms. In 9 out of 10 infections, the immune response successfully controls the infection, forcing the pathogen into a latent stage. In the latent stage, the bacterium will not grow, but will persist inside the pulmonary system without harming the host until the host becomes immunocompromised. If the granuloma’s structural integrity is compromised in an immunocompromised host, the sequestered *Mtb* will become active, replicate, and spread [20].

If the active stage of infection is not treated successfully, the infection may lead to systemic failure and death. Active tuberculosis can disseminate to other parts of the body through the bloodstream or lymphatic system. Once active tuberculosis has entered the circulatory system it is said to be in the milliary stage of infection. In this stage lesions, also referred to as granulomas, can start to appear all over the body impairing host function. In the advanced stages of infection, these tubercles for unknown reasons liquefy and spread to other regions of the body, infecting the host on a greater scale. In the case of a severe infection, the bacillus will reach the nervous system leading to tubercular meningitis, which will result in death if left untreated [20].
**Mtb’s Survival in the Macrophage**

Inside of a macrophage *Mtb* is exposed to a variety of stressors that influence its growth and development. Stressors include acidification and hypoxic stress, iron starvation, nutrient deprivation, and other environmental cues such as reactive nitrogen and oxygen species. Sensing and survival of stresses is imperative for *Mtb*’s sustained presence inside of a macrophage. In addition to the structural components outlined in *Mtb* microbial section, efflux pumps, and gene expression regulators are utilized by *Mtb* to combat macrophages.

There may be many ways in which *Mtb* is able to sense its environment. One is the utilization of two component systems (TCS) that consist of a receptor, usually on the cell membrane, and an effector that activates downstream genes. One such example is the PhoP-PhoR system. Activation of PhoP-PhoR system is what is primarily responsible for responding to changes in metabolic and respiration regulation [21]. *Mtb* is equipped with over a hundred stand-alone transcription factors that modulate gene expression.

Changes in the redox state of the cytoplasm can occur when the phagosome containing *Mtb* fuses with the lysosome to expose it to acidifying agents, or when changes in nutrient metabolism causes the production of harmful waste products. Responding to these stressors is done in part by regulation of efflux pumps. Efflux pumps are membrane bound transmembrane transport proteins that are stress induced pumps designed to transport potentially harmful substances out of the bacterial cell [22]. For example, the activity of the efflux pump allows *Mtb* to inhibit the fusion of lysosome with the phagosome through *Mtb*’s secretion of potassium through a potassium proton efflux antiport that alters maturation of the phagosome-lysosome...
complex [23]. *Mtb* must combat these influences by regulating downstream genes in times of stress.

Efflux pumps also play a significant role in drug resistance by providing the ability to pump drugs out of the bacterial cytoplasm. It is the activity of these transporters that is responsible for multidrug resistance strains of bacteria, including *Mtb*. Understanding the role of efflux pumps, and the regulation of such in times of macrophage survival may lead to new strategies to develop drugs that can lead to a cure.
Chapter Two: Transcriptional Regulator WhiB7

Introduction
An essential aspect of microbial pathogenesis is the ability of pathogens to carefully regulate the expression of virulence factors during infection in response to host-derived cues. Virulence factors may include a wide variety of components such as structural, genetic and enzymatic activity that provides the infectious agent with a proclivity to cause disease. The ability of *Mtb* to survive within macrophages, remain dormant in granulomas for decades, and resist antibiotic therapy hinges on its appropriate genetic regulation of virulence mechanisms. This chapter will focus on the genetic regulation of virulence factors that contribute to *Mtb* pathogenesis, with specific emphasis on one particular regulator mechanism that is the basis of this study.

Regulation of gene expression in bacterial systems
In bacteria, the regulation of gene expression primarily occurs at the level of transcription. Thus, increasing or decreasing the production of a specific gene product occurs by controlling the level of mRNA transcripts produced. This is accomplished by regulatory proteins known as transcription factors (TF) which sense and respond to environmental cues by binding to promoter regions of specific genes. Knowing how bacteria regulate virulent gene expression will increase our understanding of *Mtb*’s pathogenesis and subsequent disease.

In normal biological systems organisms must make messenger ribonucleic acid, mRNA, from deoxyribonucleic acid, DNA, in order to form long peptide chains that may fold to form a protein. In prokaryotes, such as *Mtb*, gene expression is carried out by the use of a single RNA polymerase that binds to DNA through the use of a sigma factor that acts as a transcription
initiation factor. Binding of the sigma factor to a specific promoter recruits RNA polymerase to initiate mRNA transcript synthesis. The resulting mRNA is then translated by a ribosome to synthesize a protein product [10]. Some proteins are then able to regulate genes by acting as repressors and activators.

Regulatory proteins, transcription factors, regulate the transcription of genes in response to environmental conditions, a process that is crucial for virulence. Transcription factors are small DNA specific binding factors that regulate the genetic flow of information. There are two different types of transcription factors, one that inhibits the regulation of a gene called the repressor, and another factor that promotes the regulation of a gene called the activator. Transcriptional regulators work to enhance the assembly of the transcription machinery at promoters by recruiting sigma factors and the RNA polymerase in order to control transcription [24].

Transcriptional activators are sensing molecules that are able to detect changes to the status, or environment of the bacterium, and facilitate adaptation to those stresses in order to survive. Changes in the carbon source, introduction of sub-inhibitory antibiotic concentrations, heat shock, iron starvation, reductive, and oxidative stress, and hypoxia, causes changes in gene expression to counter-act these stresses. Therefore it is the principal responsibility of transcription factors to regulate a particular set of genes in order to maintain homeostasis for the survival and growth of the organism [25]. The focus of this study is a particular family of proteins called WhiB proteins, which are identified as transcriptional activators and are responsible for stress resistance.
The Role of WhiB proteins

The WhiB family of proteins mostly restricted to a group of filamentous, branching, gram-positive bacteria called actinomycetes [26]. First identified in *Streptomyces coelicolor* mutants, the WhiB genes were shown to have a direct involvement in the formation of spores; *S. coelicolor* whiB mutants lost the ability to form spores [27]. Further studies revealed that the WhiB proteins in *Mtb* are specifically involved not only in cell division, but also nutrient starvation, pathogenesis, antibiotic resistance, and stress sensing [28].

The *Mtb* genome encodes seven different WhiB proteins, each responding in a different way when exposed to redox signals [28]. WhiB protein sequence analysis shows that all members of this family contain four conserved cysteine residues and an AT-hook DNA binding domain. The AT-hook is a DNA binding motif located on the C-terminal end of regulatory proteins which is consistent with the WhiB family. This region facilitates the formation of the transcription complex to initiate transcription.

Cysteine residues are significant to the structure of proteins due to their primary function of holding the tertiary and quaternary structure of proteins together. Cysteine residues contain a thiol group that link to each other to create a disulfide bridge. These intramolecular bonds can bind to iron to form an iron sulfur cluster. The iron sulfur cluster is able detect changes to the oxidative and reductive environment in the cytoplasm [29]. Studies show that in the time of oxidizing stress, the iron sulfur cluster disintegrates, but in the presence of reductive environment the iron-sulfur cluster is maintained. The strength and duration of the redox environment will alter the disulfide bond which will change the
conformational shape of a protein, therefore changing its function. In summary WhiB proteins help counteract intracellular changes caused by the environment by activating different downstream genes that play a role in bringing the bacterium back to homeostasis [28].

**WhiB**

The function WhiB7 is of particular interest due to its strong correlation with inducible drug resistance in *Mtb*, and macrophage survival [30]. Learning how this protein is able to interact with downstream genes that regulate antibiotic resistance, and resistance to macrophage stress will allow us to confirm its activity as it relates to *Mtb* virulence and its ability to cause disease.

whiB7 is a gene that encodes the WhiB7 transcriptional activator which responds to changes in the redox environment of the bacterium [30]. The redox balance is changed when *Mtb* resides in macrophages and granulomas, a low-oxygen, low-nutrient environment, and when exposed to antibiotics. Inducible drug resistance is suggested to be regulated by WhiB7 when exposed to antibiotics such as kanamycin, erythromycin, and streptomycin [30]. In times of macrophage infection stress such as iron starvation, or entrance into the stationary phase, WhiB7 is significantly upregulated causing activation of downstream genes responsible for countering the effects of these stressful conditions [31].

Additionally, WhiB7’s activity is upregulated during macrophage infection which is probably due to nutrient starvation. This occurs when the macrophages phagosome limits the *Mtb*’s access to carbon sources, forcing the bacillus to change its carbon source to utilize host lipids and cholesterol. When metabolized, lipid eating contributes to reductive stress and
creates harmful byproducts such as propionyl-CoA and its toxic intermediates [31]. Harmful by-products, like these, may be pumped out of the cytoplasm by efflux pumps. Interestingly, the WhiB7 transcriptional activator regulating drug resistance, is also responsible for pushing harmful metabolic waste out of the cell [30].

The whiB7 genetic locus consists of three main regions of importance which we will address in this study: the whiB7 promoter, a region called MT3290.2, and the whiB7 open reading frame itself (figure 1). The whiB7 promoter contains the -10 and -35 sigma factor binding site, as well as an A-T rich region of DNA that acts as a WhiB7 binding site upstream of the -10 and -35 promoter region. A WhiB7 binding site on the promoter of WhiB7 allows for positive feedback on the levels of WhiB7. This means the WhiB7 protein has an auto-regulatory ability, as well as the ability to regulate promoters of downstream genes [32]. This WhiB7 binding site region is conserved throughout the WhiB7 regulon [33]. MT3290.2 is encoded immediately upstream of whiB7 and is co-transcribed from the same promoter. It is currently considered as the untranslated region of DNA in between the WhiB7 promoter, and the whiB7 gene itself. This region does not have a known function, but may have activity as non-coding cis-regulatory elements affecting WhiB7 transcription, or may function as a small regulatory protein to assist whiB7 regulation. Strains containing a series of mutations in this region cause an increase in the transcription levels of WhiB7, thus making this region appear to be playing a role in regulation [33]. The last region in the WhiB7 locus is the whiB7 gene, a 279 base pair open reading frame encoding conserved features of WhiB proteins including four cysteine residues and the A-T hook motif.
There are currently 12 known genes putatively regulated by WhiB7, including three genes implicated in inducible drug resistance: Rv2416c, Rv1258c, and Rv1988 [30]. These genes were identified by comparing the microarray expression profiles of a WhiB7 knockout strain, a WhiB7 overexpressing strain, and parental M. tuberculosis H37Rv. The study showed a strong correlation between the transcription levels of WhiB7 and the transcription levels of the three listed genes. Rv2416c, also known as Eis, is an acetyl transferase that modifies kanamycin so that it can no longer inhibit ribosomal activity [34]. Additionally, in time of macrophage stress Eis is shown to contribute to enhanced survival of Mtb in macrophages [35]. Rv1988c is correlated with the resistance to MLS (macrolide, lincosamide, and streptogramin). The Rv1258c gene, which is a focus of this study, encodes an efflux pump gene called Tap which confers resistance to low concentrations of antibiotics [35]. Tap is responsible for Mtb’s intrinsic resistance to ethambutol, rifampin, and isoniazid, the frontline drugs used in treatment of tuberculosis. Understanding WhiB7’s role in the regulation of Rv1258 is the emphasis of this study, in addition to identifying other promoters in the WhiB7 regulon.
Figure 1: WhiB7-mediated regulation of stress resistance mechanisms
Chapter Three: Objectives

Objective

It is imperative that we understand the mechanisms that allow *Mtb* to survive in macrophages and resist antibiotics. The transcriptional activator WhiB7 has been seen to play a role in regulating *Mtb* macrophage survival and drug resistance [30, 36-38]. Our hypothesis is that WhiB7 is a dual-function regulator involved in macrophage survival and drug resistance that controls a larger regulon than previously thought. Our aim is to understand mechanisms of WhiB7-mediated adaptation to these stresses and characterize the role of downstream effector genes. The focus of this study will be on WhiB7’s ability to regulate a known promoter Rv1258c which encodes a multidrug efflux pump [30]. We will create a tool for studying WhiB7-mediated gene regulation by engineering a strain of the nonpathogenic bacterium *M. smegmatis* (*Msm*) expressing the mCherry fluorescent protein controlled by the Rv1258c promoter. Using *Msm* as a surrogate for the *Mtb* WhiB7 circuit will allow us to study the inducers of WhiB7, essential components necessary for WhiB7 activity, and the activity of WhiB7-regulated promoters. Studying this gene will provide insight into the activation properties of WhiB7 and downstream genes in *Mtb* virulence and drug resistance. Understanding WhiB7’s role in *Mtb* macrophage survival and antibiotic resistance may provide new strategies for developing drugs that can lead to a cure.
Specific objectives:

To create an *Msm* surrogate for the study of *Mtb* WhiB7 gene regulation
We will generate a strain of *Msm* with its native WhiB7 homologue deleted that will allow the clear introduction of *Mtb* WhiB7 without interference from the native *Msm* WhiB7.

Cloning the *Mtb* Rv1258c promoter fused to the fluorescent protein mCherry into the *Msm* WhiB7 knockout strain will allow us to detect the changes in Rv1258c transcription induced by WhiB7 mutagenesis described in objective two.

Determine required elements for WhiB7-mediated gene regulation
We will use bioinformatics tools to analyze WhiB7 genomic sequences to identify the necessary components of the WhiB7 system for DNA binding. A series of altered WhiB7 variants will be introduced into the WhiB7 knockout strain of nonpathogenic *Msm* containing the promoter fusion of Rv1258c and mCherry. Changes in fluorescence will serve as an indicator of the level of WhiB7 activation.

To confirm genes in WhiB7 regulon
Preliminary microarray analysis of mRNA levels in a WhiB7- mutant and WhiB7 overexpressor vs. wild-type revealed more genes whose expression is affected by the levels of WhiB7 present. To validate and confirm their control by WhiB7, we will clone the promoter regions upstream of expressed target genes in front of mCherry. These reporter fusions will be introduced into our *Msm* surrogate strain expressing various forms of active or inactive WhiB7 (Figure 2).
Figure 2: Exploiting M. smegmatis to construct Mtb regulatory circuits. Figure represents a summary of the three main goals of the project. A) Represents the Msm homologous knockout of whiB7. B) Represents the fluorescent reporter introduced to the chromosome of Msm used to study WhiB7Mt. C) Depicts our introduction of mutant and recombinant WhiB7 constructs.
Chapter Four: Materials and Methods

Introduction
The goal of this study is to understand the molecular mechanisms of WhiB7-mediated gene regulation in response to antibiotic- and macrophage-derived stresses. Using the methods previously described, we will make a series of mutant and recombinant versions of \textit{Mtb} WhiB7 and study their activity using a fluorescent reporter system. The regions of particular interest when looking at WhiB7 activity involve its promoter, the MT3290.2 gene immediately upstream, and the WhiB7 gene itself. This gene cassette has the ability to regulate itself and regulate other downstream promoters in order to maintain \textit{Mtb} virulence and resistance to different stresses. This study is designed to understand the WhiB7 cassette. Creating various forms of this cassette containing engineered mutations and modifications will allow us to characterize its function. There are a total of four mutant strains that will be created and studied. Each strain was created using one of two cloning methods, Round the Horn, and FastCloning.

General Methods
Bacterial Growth and Strain Handling
Two bacterial agents used in this study were \textit{Escherichia coli} (\textit{E. coli}) and \textit{Mycobacterium smegmatis} strain mc2155 (\textit{Msm}), \textit{E. coli} is utilized as a host for plasmid replication during routine cloning and recombinant DNA procedures. Due to the slow growing and potentially dangerous aspects of \textit{Mtb}, \textit{Msm} was chosen to act as a surrogate in the place of \textit{Mtb} based on
their level of genetic similarity. Various mutations and plasmids will be added to the strain of *Msm* as described in detail below.

**Culture conditions:**
E. coli was cultured at 37°C in Luria-Bertani (LB) broth shaken at 250 revolutions-per-minute (rpm), or on LB agar plates. *Msm* was cultured at 37°C in LB broth containing 0.05% Tween80, or on LB agar plates.

**Transformation conditions for E. coli**
Transformation in *E. coli* was done by using chemically competent cells from New England Biolabs (NEB). NEB cells were treated with DNA for thirty minutes, heat shocked at 42°C for thirty seconds, followed by addition of 250μL of SOC (super optimal broth) recovery media before shaking for two hours at 37°C. 150μL of this mixture was plated onto selection LB agar plates.

**Electrocompetent cells:**
15mL of *Msm* cultures were pelleted at 4,300 rpm at 4°C. Supernatant was removed and then the pellet was resuspended in one-half of the starting volume of the previous suspension, using 10% ice-cold glycerol. This process was done four times leaving the final volume at 0.6 mL left over from the starting volume of 15mL from the overnight culture. 100μL of the final glycerol washed pellet was aliquoted into microcentrifuge tubes. Tubes were then sealed and treated with dry ice and ethanol to snap-freeze the cells for storage and subsequent transformation.
Transformation conditions for Msm:
Transformation of Msm required using electroporation by adding approximately 500ng of plasmid DNA to 100μL of target strain of Msm electrocompetent cells. After 1 minute, cells were transferred to a 1-mm electroporation cuvette. Cells were pulsed at 2.5kV, 200 Ohms and 25μF. 250μL of SOC media was then added to electroporation cuvette. Media and electrocompetent cells were transferred to a sterile tube to be shaken for 2 hours then plated onto selection LB agar plates.

Vector selection:
Hygromycin (Hyg), streptomycin (Strep), or kanamycin (Kan) was added to liquid and solid media. For E. coli cultures: Hyg and Kan were used at a final concentration of 50μg/mL in liquid cultures, and 250μg/mL for Hyg and 50μg/mL for Kan in solid media. For Msm cultures: Hyg and Kan were used at a final concentration of 50μg/mL in liquid cultures, and for Kan 50μg/mL and streptomycin 100μg/mL in solid media.

FastCloning
Method Overview:
One method that is routinely used in the lab is a DNA cloning method coined as FastCloning [39]. This FastCloning method is used to clone various fragments into a vector, or, depending on the vector used, utilized to form gene knockouts. FastCloning allows us to do this by negating the need to use restriction endonucleases and ligase to make genetic mutations and recombinant DNA constructs. FastCloning relies on polymerase chain reaction (PCR), to amplify a desired DNA fragment and target vector sequence such that they can be combined without utilizing restriction enzymes and ligase to cut and paste constructs. This approach
introduces a great degree of flexibility into the design of cloning experiments. The key is designing primers to amplify your target gene, referred to as an insert, to include DNA “overhangs” complementary to regions of the plasmid on either sides of an engineered cloning insertion site. The cloning site is created by PCR of the entire plasmid DNA using primers designed to linearize the once circular plasmid, creating a vector fragment. When these two PCR products are combined, the complimentary ends on the insert anneal to the vector causing them to hook together to form a make-shift plasmid, in which the insert and vector fragments are not ligated together. They are held together by complementary base pairing. The mixture of vector and insert PCR produces is treated with an enzyme called Dpn1 which digests the methylated plasmid DNA. Any plasmid used as templates for vector or insert PCR is methylated during its replication in *E. coli*. Digesting this methylated DNA reduces carry over parental plasmid which will reduce the frequency of false-positive clones. After Dpn1 treatment, the make-shift plasmid is transformed into *E. coli*, where it is ligated by *in vivo* DNA repair mechanisms. Colonies are then selected if they contain the antibiotic resistance gene making them resistant to an antibiotic in agar plates. Colony PCR will then be utilized for further sequencing (Figure 3).

**Method Protocol:**

Fast cloning involves three steps: PCR, fragment assembly/template digestion, and transformation. Insert and vector fragments were amplified by PCR using Phusion polymerase. A typical FastCloning PCR reaction will involve the initial use of a template that contains the genomic sequence that is to be amplified. This template was usually *Mtb* chromosomal DNA to
synthesize the insert fragment. A standard reaction mx for the insert PCR would be: 1μL of chromosomal DNA [17-20 ng/μL], 1μL forward primer [10ng/μL], 1μL reverse primer [10ng/μL], 5μL GC buffer, 0.5μl dNTP [10mM], 0.25 μl Phusion polymerase, and 16.25 μL sterile water. The reactions were ran with standard Phusion PCR cycling (see table two) with an extension time of 15 seconds, a calculated annealing temperature using the New England Biolabs (NEB), Tm calculator: https://www.neb.com/tools-and-resources/interactive-tools/tm-calculator. The extension time and annealing temperature is variable depending on the insert fragment size, and primer annealing temperature. Extension time is determined as 15 seconds per kilobase of insert needing to be amplified, whereas the primer annealing temperature is determined by nucleotide content. For the synthesis of the vector fragment, the reaction is essentially the same. Instead of using chromosomal DNA as the template, we would use a plasmid engineered in the lab. Using approximately 15-20 ng/μL of the template plasmid, we amplified the vector using a 1.5 minute extension time for vector fragments as they are usually six kilobases. 5μL of the PCR reaction of both the insert and vector are run on a 1% agarose gel and stained with GelRed nucleic acid stain. A digital photograph is taken utilizing an ultraviolet light source and imager to confirm product synthesis.

Fragment assembly and template digestion: After confirmation of product syntheses, insert and vector products are mixed at various ratios to allow for optimal annealing. Volumetric ratios of 4:1 (6.4μL vector + 1.6μL insert), 1:1 (4μL vector + 4μL insert), and 1:4 (1.6μL insert + 6.4μL insert) vector: insert is used. FastCloning reactions are treated with Dpn1 at 37°C for two hours to digest methylated template plasmid DNA used in initial vector PCR.
Eliminating template plasmid DNA reduces false positives caused by template plasmid transformation. 2μL of FastCloning reaction is mixed into 25μL of NEB chemically competent heat shock E. coli. E. coli is plated onto LB agar plates with the antibiotics appropriated for selection marker. Colonies are then selected from the plate for colony PCR to confirm cloning.

**Project Relevance:**
The FastCloning method was used to synthesize three out of our total four clones. First, we cloned the native Mtb whiB7 locus which contains the promoter, the putative MT3290.2 gene upstream of whiB7, and the whiB7 gene itself. These three components make up the native form of the whiB7 locus. This clone will be referred to as Native-MT+WhiB7. The second clone eliminated the native whiB7 promoter by amplifying the region of the cassette containing just MT3290.2 and whiB7. That fragment was cloned in front of the strong constitutively active hsp60 promoter. This clone will be referred to as hsp60-MT+WhiB7. The third clone contains only whiB7 cloned downstream of a strong constitutively active promoter and will be referred to as hsp60-WhiB7. These mutant and recombinant versions of whiB7 were FastCloned into a shuttle vector called pVVG.2, a derivative of the pVV16 plasmid. pVVG.2 is a vector that includes a green fluorescent protein (GFP) driven by a constitutively highly active hsp60 promoter.
Figure 3: Summary of FastCloning method. A) PCR vector B) PCR insert C) Mix in vector to insert D) Digest with DpnI E) Transform into E. coli
**Round the Horn**

Another method of cloning that is routinely used in the lab is referred to as Round the Horn [40]. This method is used to further alter clones synthesized using the above FastCloning method. This method is particularly useful for creating defined deletions or introducing insertions to DNA at a particular region of interest. For instance, you have regions A, B, and C in a vector and you want to delete region B. Primers are designed to be on opposite sides of the target deletion, in this example region B. Prior to use, primers are phosphorylated to facilitate the subsequent ligation step. PCR will amplify regions starting with A, and then amplifying around the plasmid through to region C. This will essentially delete the regions of the plasmid that are no longer desired for the new vector. Once the vector fragment is amplified, the phosphorylated blunt ends of the linear vector fragment are ligated to form a plasmid. Once ligated, the vector is then transformed into *E. coli* where clones will be grown onto agar plates and selected for colony PCR.

**Method Protocol:**
Round the Horn is broken up into four essential steps: primer phosphorylation, PCR, product ligation, and then transformation and screening. Phosphorylation is done to each individual primer by utilizing T4 Polynucleotide Kinase (PNK): 37 μL sterile water, 5 μL 10x kinase reaction buffer, 1 μL [50mM] MgSO4, 5 μL primer at [100 μM], 1 μL [100mM] ATP, and 1 μL T4 PNK for a final volume of 50 μL. Reaction incubation occurs for one hour at 37 °C. Heat inactivate at 95°C for five minutes. PCR utilizing the phosphorylated primers: 1 μL of template vector at 17-20 ng/μL, 1 μL phosphorylated forward primer, 1 μL phosphorylated revere primer,
5 µL GC buffer, 0.5 µL dNTP, 0.25 µl Phusion polymerase, and 16.25 µL sterile water. We would amplify the vector using a 1.5 minute extension time, vector fragments are approximately six kilobases. 5 µL of the reaction is run on a 1% agarose gel, stained with GelRed. A digital photograph is taken utilizing an ultraviolet light source and imager to confirm product synthesis. Product is then treated with Dpn1 to digest template methylated plasmid. Product ligation using Fast-Link ligase: 1.5 µL 10x Fast-Link ligation buffer, .75 µL [10mM] ATP, 3µL Dpn1-ed PCR product, 8.75 µL sterile water, and 1 µL Fast-Link DNA ligase. The reactions were incubated at room temperature (25°C) for 30 minutes before heat inactivation at 70°C for 15 minutes. A portion of this reaction was transformed into NEB heat shock E. coli using the transformation protocol described in Fastcloning section and plated onto LB agar plates with the appropriate selection marker. Colonies are then selected from the plate for colony PCR to confirm cloning.

**Project Relevance:**
Mutagenesis of the WhiB7 promoter and leader sequences is of particular importance in order to characterize the cis regulatory elements required for WhiB7 to regulate downstream genes. Bioinformatics tools have identified WhiB7 binding motifs that can be altered. Truncating the sequences in between the promoter and the gene itself are expected to alter WhiB7’s ability to activate promoters, including its own. This will increase our understanding of how WhiB7 acts to maintain virulence and drug resistance. The Round the Horn method was used to construct our fourth clone which is a derivative of the Native-MT+WhiB7 clone. This construct was synthesized to essentially delete the MT3290.2 gene, leaving the WhiB7
promoter fused to WhiB7 and will be referred to as Native-WhiB7 (Figure 4). This mutant version of WhiB7 was FastCloned into a shuttle vector pVVG.2.

Figure 4: Summary of the Round the Horn method. 1) Phosphorylated primers amplify and linearize vector 2) Linear vector is ligated 3) Ligated product ready for subsequent transformations
Colony PCR and Sequencing

Method Overview:
The colonies that reside on the agar plate are selected and resuspended into a small tube of water. Once in the tube, the colony is lysed to form a crude mix of DNA and cellular debris. The crude lysate is then centrifuged to pellet the debris, and the supernatant is used as a DNA template for screening PCR.

Method Protocol:
Colony PCR has two essential steps, lysing colonies, and then PCR. Colonies were selected and resuspended in a microcentrifuge tube containing 20 μL sterile water. The resuspended colonies were then incubated at 100°C for five minutes. The tube was then centrifuged at 13,000 RPM to pellet cellular debris. Supernatant contains chromosomal and plasmid DNA, which was used as a template for PCR amplification. We used 1 μL of colony lysate as template into a 10 μl Phusion PCR using standard PCR reaction conditions that were utilized in FastCloning method. Primers flanking the insertion site of the vector were used to amplify the region in-between the primers. 5 μL of the reaction was run on a 1% agarose gel and stained with GelRed. A photograph was taken to analyze product synthesis in comparison to a negative control. An increase in size compared to a negative control was deemed a positive clone for FastCloning. A reduction in size in comparison to a negative control is deemed a positive clone when making deletions using the Round the Horn method. Once “positive” clones are confirmed by this method, they are grown in culture of LB broth containing 50 μg/mL of antibiotic selection marker overnight. Each plasmid construct was purified using an alkaline
lysis protocol consisting of buffers made in-house coupled with Eco spin purification columns. Isolated plasmids were then used for sequence analysis, further manipulations, or transformation.

**Sequence analysis**

The accurate assembly of recombinant constructs was confirmed by dideoxy sequencing of plasmids from positive clones at an offsite company. An adapted no cycling, single extension PCR is done utilizing one specific primer to decode a region of DNA whose sequence needs to be confirmed. The reaction is done in the presence of dideoxy terminator nucleotides mixed in with normal nucleotides which will cause synthesis termination at various stages, each fragment being a single nucleotide bigger than the previous. The products are then run through a glass capillary gel electrophoresis to separate terminated sequences based on size. That gel will be exposed to a laser that will cause the dideoxy terminator nucleotides to fluoresce. The fluorescence is recorded by a computer that analyzes the colors, a different color per base, based on size. This makes a nucleotide readout that places them in sequential order from the start of the primer binding, through the mutated sequence of DNA in question [41]. That sequence data is then compared in silico with the designed genomic DNA for confirmation.

**Method Protocol:**

Sequencing is done at GENEWIZ, Inc. - DNA Sequencing Services. A reaction of plasmid, and a sequencing primer is prepared for shipment. A typical reaction for sequencing includes 700-800 ng of plasmid DNA, and 25pM of sequencing primer mixed to a final volume of 15 µL. Prepared mix is then sent to GENEWIZ. A detailed sequence analysis is retrieved and compared
with in silico models of positive clones. Positive clones are confirmed as exact sequence with hypothesized in silico models.

**FastCloning Knockout**

**Method overview:**
The WhiB7 homologue in *Msm* (WhiB7<sub>Msm</sub>) could potentially interfere with studies of WhiB7<sub>Mtb</sub> function. Thus, knocking out the native WhiB7<sub>Msm</sub> gene is necessary in order to have a clean background prior to the introduction of *Mtb* WhiB7 (WhiB7<sub>Mtb</sub>). Utilization of the FastCloning method previously described can be done to synthesize various knockout constructs to inactivate genes of interest, in this case WhiB7<sub>Msm</sub> (*MSMEG_1953*). In this method, we used a plasmid called pFCKO which stands for “FastCloning Knockout plasmid”. The pFCKO plasmid contains a hygromycin selection marker (HYG) with flanking loxP recombination sites, and an *rpsL* counterselectable marker. The knockout procedure involves the amplification of regions approximately 1 kb upstream and downstream of the target gene *MSMEG_1953*, WhiB7<sub>Msm</sub> and Fastcloning them onto either side of a hygromycin (Hyg) selection marker. When this construct crosses into the chromosome it will delete any region in between them, while adding a hygromycin selection marker. Positive colonies that have integrated our genetic mutations can then be identified based on resistance to the hygromycin antibiotic when growing on agar plates. This will allow the introduction of WhiB7<sub>Mtb</sub> without interference of WhiB7<sub>Msm</sub> (figure 5).
*rpsL* is a gene coding a wild-type ribosomal protein which confers dominant streptomycin sensitivity, strepS. This can be used to select bacteria which undergo double homologous recombination versus single homologous recombination. Single crossover events will result in integration of the entire pFCKO plasmid including the *rpsL* cassette onto the chromosome making the bacterium strepS [11]. A double cross recombinant mutant will effectively inactivate a target gene, maintain native resistance to streptomycin, while adding a hygromycin selection marker. Streptomycin and hygromycin resistance will allow us to select for clones containing a double-cross recombination, and be determined as positive for knocking out the target gene.

Once the pFCKO has successfully undergone double homologous recombination we will utilize the loxP sites flanking the HYG marker. A recombinase enzyme called Cre can resolve out anything between loxP sites. We will transform a plasmid expressing Cre into our ΔWhiB7 hygR strain of *Msm*. Doing this will cause the HYG gene to be resolved out of the chromosome. We will then select for the loss of HYG by plating on agar plates, then moving the colonies to a hygromycin containing agar plate. Death of colonies after moving them to a Hyg agar plate would indicate positive colonies. Resolving out the HYG marker using a Cre expressing plasmid will allow us to utilize HYG as a selection marker for further mutagenic introductions.

**Method Protocol:**
The pFCKO knockout is done in two stages. The upstream fragment is amplified by PCR using the standard conditions described above. It is then cloned into the pFCKO vector and then transformed into *E. coli*. Positive clones are verified through colony PCR and sequencing.
Similarly, the amplified downstream fragment is Fastcloned into the resulting vector on the other side of the Hyg marker. Once positive clones in *E. coli* are found to contain both upstream and downstream fragments using PCR primers to amplify the cloning region of pFCKO, the pFCKO vector is then electroporated into the target bacterial strain, in this study *Msm*. The *Msm* possibly containing the pFCKO plasmid is plated on hygromycin and streptomycin containing LB agar plates. Colonies growing on the double antibiotic plates are then selected as positive for WhiB7*<i>Msm</i>* knockout, ΔWhiB7<sub>*Msm*</sub>.

To remove the hygromycin cassette, a plasmid expressing the Cre recombinase is transformed by electroporation into the knockout strain followed by plating on LB agar plates. Colonies on the LB plates are then transferred to a hygromycin LB plate. Death of colony on hygromycin plate indicates resolution of hygromycin selection marker from positive knockout strains.
Figure 6: Summary of FastCloning Knockout method. A) FastClone DNA flanking target gene on either side of HygR marker. B) Electroporate into Msm. C) Select for double crossover knockout based on HygR and StrepR.
Fluorescent reporter system

Method Overview:
The FastCloning method described above was used to create a fluorescent assay that can detect promoter activity through the use of a plate reader. Our double-fluorescent reporter system will be able to detect activation levels of a promoter that can be cloned upstream of a fluorescent protein called mCherry, and compare it to the basal level activation of another fluorescent protein called green fluorescent protein (GFP). The mCherry protein is a protein that can be detected at an excitation of 580nm and an emission at 615nm. This protein is resistant to photo-bleaching, and is detectible almost immediately after its activation. GFP is a protein similar to mCherry, immediately detectable, excitation of 475nm and an emission at 508nm, and resistant to photo-bleaching. The system will be designed to have the mCherry protein integrated to the bacterial chromosome driven by an promoter that is the focus of current study, and have GFP on an expression plasmid driven by a constitutive hsp60 promoter that is always active in vivo. When the promoter:mCherry fusion is on the chromosome, and the GFP on an expression plasmid in the cytoplasm of the same bacterium, the mCherry signal can be normalized. If a transcription factor known to activate the promoter of the mCherry reporter is placed on same expression plasmid of the hsp60:GFP, changes in the ratio of mCherry signal compared to GFP signal can be attributed to the activity of the transcription factor. High levels of mCherry signal in comparison to GFP signal will provide strong evidence of transcription factor-promoter communication.
Construction of WhiB7-activated fluorescent reporter:

Putting the Rv1258 promoter upstream of mCherry will allow mCherry expression to serve as an indicator of WhiB7<sub>Mtb</sub> activity. The Rv1258c promoter consisting of 500bp upstream of the well-characterized Tap efflux pump was Fastcloned into a shuttle vector called pUAB400. The promoter was inserted upstream of mCherry and the resulting construct, Rv1258c:mCherry, was confirmed by sequence analysis to ensure proper placement of Rv1258c (Figure 7). Rv1258c:mCherry was then transformed into wild-type <i>Msm</i> to confirm the activity of the promoter based on detectable mCherry fluorescence in the plate reader. This vector will be electroporated into the WhiB7 homologue knockout strain in <i>Msm</i> once that strain is completed, where it will cross into chromosomal DNA. When WhiB7 becomes activated, the Rv1258 promoter will cause the transcription of mCherry and synthesis of mCherry which will be detectable by a plate reader.

**Method Protocol:**

Synthesizing the promoter-reporter system involves two steps, reporter integration on the chromosome, and introduction of a plasmid containing the transcription factor and GFP normalizer. Using the FastCloning method, Rv1258c was cloned into an integrating shuttle vector called pUAB400 that has the ability to replicate in <i>E. coli</i> and integrate into the mycobacterial chromosome. Once cloned, the integrating plasmid is electroporated into our surrogate <i>Msm</i>. pUAB400 contains a bacteriophage gene called Int that integrates the entire
plasmid onto the chromosome, including a gene located on vector encoding kanamycin resistance. Positive colonies will be confirmed for growth on kanamycin LB agar plates.

The normalizer will be synthesized using the FastCloning methods that have been previously described. The GFP protein was FastCloned with its hsp60 promoter into a plasmid called pVV16 which is a shuttle plasmid that has the ability to replicate in *E.coli* and mycobacterium. Positive clones will confer strong fluorescence compared to background detectable by plate reader, and then electroporated into competent cells of the strain of *Msm* positive for mCherry-promoter fusion. The normalizing pVV16 plasmid with GFP built into it, (pVVG.2), will create ratio between mCherry signal in comparison to GFP signal which is analyzed to determine interactions of the mutant and recombinant versions of WhiB7 activation of the Rv1258c promoter.

A culture of *Msm* containing the integrated promoter reporter fusion and the pVV16G.2 expression plasmid was grown overnight in 5mL cultures of LB broth containing 0.05% tween80. Cultures were grown until log phase. Cultures are pelleted at 4,300 rpm, and washed in 1mL of PBS (phosphate buffered saline) containing 0.05% tween80. 100µL of the washed culture is placed in a clear-bottom 96-well plate. Plate reader settings are set to read optical density (600nM), mCherry signal (Excitation=580nm, Emission=615nm), and GFP signal (Excitation=475nm, Emission=508nm). Fluorescent signals are compared in silico.

**Project Relevance:**
The mutant and recombinant versions WhiB7 were FastCloned the pVVG.2 shuttle vector. The presence of the GFP in this shuttle vector will provide an internal baseline to
normalize mCherry signal, driven by the Rv1258c promoter, within the strain of nonpathogenic *Msm*. Changes in the mCherry expression, compared to the expression of GFP, will allow us to characterize the changes in mCherry being caused by WhiB7 activity and not that of other variables such as bacterial growth phase. ∆WhiB7 strain of *Msm* that contains the Rv1258c promoter fusion to mCherry will be referred to as *MsmΔ 1258c::mCherry* (Figure 8).
Figure 8: WhiB7 activated reporter system
**KanR** | gene conferring resistance to kanamycin  
---|---  
**HygR** | gene conferring resistance to hygromycin  
**mCherry** | fluorescent protein; Excitation=580nm, Emission=615nm  
**GFP** | fluorescent protein; Excitation=475nm, Emission=508nm  
**rpsL** | gene confers dominant streptomycin sensitivity  
**Int** | bacteriophage chromosomal integration gene
Chapter Five: Results

Nonpathogenic Msm surrogate: ΔwhiB7Msm

The knockout of the WhiB7 homologue in Msm is yet to be completely confirmed as positive. Recall that this method involves two separate cloning steps: cloning a fragment upstream of the hygromycin selection marker, and cloning a fragment downstream of the hygromycin selection maker. Currently we have the downstream fragment cloned into the pFCKO vector that can be positively identified through colony PCR analysis, data not shown. Unfortunately the cloning of the upstream fragment into the pFCKO vector is yet to be complete. It is due to time constraints and “trouble shooting” that have been inhibitory of this goal currently, but in the future we will complete this goal. Screening with the M13 primers, lane two shows the pFCKO base plasmid containing no insert, lane three represents the pFCKO plasmid containing the downstream fragment. Lane one represents the standard one kilo base GeneRuler ladder.

1258c::mCherry

To generate a fluorescent reporter strain of the nonpathogenic wild type Msm that could be used to monitor WhiB7 activity, we cloned the Rv1258c promoter upstream of mCherry. When plated on kanamycin plates, positive colonies were identified based on resistance to the kanamycin bactericidal agent. Colony PCR screening followed by plate reader analysis of fluorescence further confirmed that we had successfully cloned the Rv1258c promoter into pUAB400, and integrated our construct onto the chromosome. Figure ten represents the pUAB4001258cmCh chromosomal construct as compared to the wild type
mc^2 155 strain of *Msm*. When comparing the fluorescence of the reporter to the wild type strain of *Msm*, we can confirm the presence of our reporter on the chromosome (Figure 10).

![Fluorescence of Rv1258c:mCherry reporter](image)

Figure 10: Validation of Rv1258c fluorescent reporter. This graph positively confirms pUAB400Rv1258mCh integration.
WhiB7 mutant and recombinant constructs:
All four constructs proposed were confirmed to contain the desirable gene construct based on size analysis (Figure 11). Plasmid DNA was isolated and then sent to GENEWIZ for sequencing, which revealed 100% positive alignment with the in silico model of the construct. Of the four WhiB7 constructs proposed in the methods section, all four have been successfully cloned and confirmed by sequenceing using in silico alignments (Figures 12-15). This success was not met without difficulty. The GFP protein that was thought to be present on the pVVG.2 plasmid was not present as we originally thought; this was due to false positive sizing analysis and inaccurately reading culture fluorescence screening assay done in experiments previous to this project. Identification of this issue was recently discovered during plate reader assay optimization. Circumvention of this issue is currently in the works, and is left as a story for another time.
Figure 11: WhiB7 mutant and recombinant constructs

A) Native-MT+whiB7: Native Mtb whiB7 locus including promoter, MT3290.2 ORF, and whiB7T8.

B) hsp60-MT+whiB7. MT3290.2 ORF and whiB7T8 driven by strong constitutive hsp60 promoter.

C) Native-whiB7. whiB7T8 expressed from native promoter, MT3290.2 deleted.

D) hsp60-whiB7. whiB7T8 expressed from strong constitutive hsp60 promoter, MT3290.2 deleted.
Figure 12: The sequence alignment above represents the Mtb native version of WhiB7. Sequence alignment is done to represent the comparison of GENEWIZ sequence data, to the in silico model of the Native-Mt+WhiB7 construct. Blue box: Putative WhiB7 binding site. Red box: The core promoter -10 and -35 sigA binding region. Orange box: MT3290.2 gene. Green box: whiB7 open reading frame.
hsp60-MT+WhiB7

Figure 13: This sequence represents the native whiB7 locus with the whiB7 promoter region removed, then cloned downstream of the constitutively active hsp60 (not shown). Sequence alignment is done to represent the comparison of GENEWIZ sequence data, to the in silico model of the hsp60-Mt+ WhiB7 construct. Orange box: MT3290.2 gene. Green box: whiB7 open reading frame. Sequence analysis showed incomplete GENEWIZ sequencing to the end of the WhiB7 open reading frame depicted by the green box extended passed aligned sequences.
Figure 14: The sequence alignment above represents the Mtb in vivo version of WhiB7 locus with the MT3290.2 open reading frame removed. Sequence alignment is done to represent the comparison of GENEWIZ sequence data, to the in silico model of the Native-WhiB7 construct. Blue box: Putative WhiB7 binding site. Red box: -10 and -35 sigA binding region. Green box: whiB7 open reading frame.
**hsp60-WhiB7**

Figure 15: The sequence alignment above represents the *Mtb* in vivo version of WhiB7 open reading frame doned downstream of hsp60 (not shown) with the WhiB7 promoter, and MT3290.2 removed. Sequence alignment is done to represent the comparison of GENEWIZ sequence data, to the in silico model of the hsp60-WhiB7 construct. Green box: *whiB7* open reading frame.
Fluorescent reporter system: preliminary results

To identify genetic elements required for WhiB7 mediated, we created a series of recombinant and mutant strains of WhiB7 and cloned them into the pVV16 expression vector. We then transformed the WhiB7 constructs into the *Msm* strain containing the 1258c::mCherry reporter. We then were able to test WhiB7 activity on the known activated Rv1258c promoter promoting the tap efflux pump. Results are shown in figure 16.

Figure 16 Fluorescent reporter system. Graph depicts the changes in the levels of fluorescence based on the four different whiB7 clones.
Chapter Six: Discussion

Understanding WhiB7’s ability to regulate *Mycobacterium tuberculosis*’ homeostatic state during macrophage stress and antibiotic resistance is a system of particular interest when developing new strategies for drug intervention. Unfortunately not enough data has been generated to confidently understand the cis regulatory elements required for WhiB7 mediated gene regulation. Due to incompletion of the \( \Delta \text{WhiB7}_{Msm} \) 1258c::mCherry model, the WhiB7 clones were instead transformed into the mc\(^2\)155 strain of *Msm*. Due to the lack of GFP to act as a normalizer we instead utilized ocular density of cultures to normalize the mCherry signal. Although this is not the ideal set up we proposed, we have found expression of the reporter when exposed to our WhiB7 clones.

The Native-MT+WhiB7 recombinant construct was shown to have the most activity on the Rv1258 reporter compared to any of the constructs. When we compare the Native-MT+WhiB7 construct to the hsp60-MT+WhiB7 construct, we see reduced activity on the reporter. Due to the elimination of the WhiB7 promoting region, we also eliminated the possibility of the positive feedback activity of WhiB7, disabling the autoregulatory ability of WhiB7. This leads us to assume the reduction of reporter fluorescence of hsp60-MT+WhiB7 as compared to the Native-Mt+WhiB7 clone is caused by disabling this autoregulatory ability.

Recall the function of MT3290.2 has not yet been determined, but data suggests it plays a role in whiB7 regulation. The Native-WhiB7 mutant in comparison to the Native-MT-WhiB7 clone suggests the deletion of MT3290.2 from the native gene locus has caused a reduction in WhiB7 activity. This data may suggest that the deletion of this region results in poor *whiB7*
regulation, which may be attributed to deletion of a required element. Further data and analysis will be required to confidently confirm MT3290.2’s role in whiB7 regulation.

The level of fluorescence of the pUAB4001258cmCh Msm control is relatively high in comparison to the wild type Msm strain. This suggests that the native Msm whiB7 is likely interacting with the Rv1258c promoter and reaffirms the necessity of the Msm WhiB7 homolog knockout (ΔWhiB7Msm) to reduce the level of background fluorescence when we introduce our WhiB7

Mtb constructs. When comparing the activity of the Native-WhiB7 and the hsp60-WhiB7 recombinant constructs, we see a reduction in the level of fluorescence. But, due to the lack of the ΔWhiB7

Msm, we cannot deduce any phenotypic generalizations when the levels are below that of the control pUAB4001258cmch Msm.
Chapter Seven: Future works

Due to the issue of time constraints, and unforeseen issues that were previously described, we were unable to fully address the goal of identifying promoters related to WhiB7 regulation using our promoter reporter system. Genes potentially activated by WhiB7 were identified by microarray analysis as genes with higher expression levels in a WhiB7 overexpressor strain, and lower expression in the WhiB7 knockout strain.

These studies identified 12 genes that were regulated by WhiB7, but these have yet to be cloned. In the future, the identified promoters driving these genes will be fused to the mCherry fluorescent protein in the same fashion described in promoter fusion method in objective one. Once the promoter fusion is in the chromosomal integration vector, pUAB400, it will be transformed into the ΔWhiB7_{Msm} as described in objective one. Expression of the promoter fusion will be regulated by the native WhiB7_{Mtb} if the promoter proves to be in the WhiB7 regulon. Inducing promoter mCherry expression will correlate with WhiB7_{Mtb} activated transcription of the promoter, linking the promoters function with WhiB7. Characterization will be done by comparing the expression levels of the promoter fusion via plate reader assay analysis. In the future, we hope to clone these promoters into the pUAB400 vector upstream of mCherry to identify WhiB7 communication.
Chapter eight: Summary

Creating a strain of nonpathogenic \textit{Msm} to act as a surrogate for the introduction of \textit{Mtb} genes for expression studies will allow us to isolate and characterize WhiB7’s ability to activate promoters responsible for drug resistance and macrophage survival. Introducing the native WhiB7 and truncated series of WhiB7 into the Rv1258c reporter knockout strain of \textit{Msm} will allow us to define the essential elements required by WhiB7 to maintain full functionality in its role in the activation of downstream promoters. Confirming additional genes identified by microarray analysis that are potentially regulated by WhiB7 will provide additional information about the role of WhiB7. Understanding WhiB7’s ability to regulate \textit{Mycobacterium tuberculosis’} homeostatic state during macrophage stress and antibiotic resistance is a system of particular interest when developing new strategies for drug intervention.
References


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