2015

Roles of transcription factors, RBPA and SIGF, in the mycobacterium tuberculosis

https://hdl.handle.net/2144/16201

Boston University
BOSTON UNIVERSITY
SCHOOL OF MEDICINE

Thesis

ROLES OF TRANSCRIPTION FACTORS, RBPA AND SIGF, IN THE MYCOBACTERIUM TUBERCULOSIS

by

STEVEN SCOTT POPE II

B.A., The Johns Hopkins University, 2013

Submitted in partial fulfillment of the requirements for the degree of Master of Science 2015
Approved by

First Reader
Dr. Barbara Seaton, Ph.D.
Professor of Physiology and Biophysics

Second Reader
Dr. Christina Stallings, Ph.D.
Professor of Molecular Microbiology
Washington University in St. Louis, School of Medicine
ACKNOWLEDGMENTS

I would like to acknowledge and give thanks to the various people that helped me with my research and assisted in the completion of my thesis.

To Dr. Christina Stallings, Ph.D., I would like to thank for giving me the opportunity to complete my thesis in her laboratory and providing the guidance and enthusiasm that was crucial in my everyday work. She has been an invaluable mentor and I have learned so much in my time under her supervision. I thank her for always keeping her door open to the many questions I had along the way.

To The Stallings Laboratory, I thank everyone in the laboratory for introducing me to bench work and being so helpful and willing to teach. Their assistance and upbeat attitude has made for a very positive lab atmosphere which made all the difference in my day to day experiments.

To Dr. Jeffrey Marsh, MD, I would like to thank for being my mentor over the course of many years and always being an inspiration. I aspire to one day to exhibit the same great qualities as a physician.

To my family, I would like to thank for being the most supportive and loving family I could ask for throughout my academic career.
ROLE OF TRANSCRIPTION FACTORS, RBPA AND SIGF, IN THE
MYCOBACTERIUM TUBERCULOSIS

STEVEN SCOTT POPE II

ABSTRACT

The mechanism of prokaryotic transcription has been characterized primarily in the classic system, *Escherichia coli*, and cannot be confidently extended to include other prokaryotic species, such as those of the Actinobacteria phylum. Actinobacteria represents a diverse group of Gram-positive species that range from soil dwellers to obligate pathogens, such as *Mycobacterium tuberculosis* (Tb). These species encode RNA polymerase (RNAP) binding proteins that are not present in model organisms, and therefore present a unique lens through which the basic mechanism of transcription can be further explored outside of model systems. In addition, these mechanisms of transcriptional regulation can be studied in the context of *M. Tuberculosis* pathogenesis. The model we use for tuberculosis is *Mycobacterium Smegmatis*, a homologue, which has a faster doubling time and is only Biosafety level 1.

Within Actinobacteria, notable conserved RNAP binding proteins include RNA polymerase binding protein A (RbpA) and CarD. RbpA is specific to Actinobacteria, binding the β subunit of RNAP and primary σ factors. CarD binds to the β subunit and associates with DNA. Both proteins are upregulated upon exposure to stress, and have implications in the initiation of rRNA transcription.
Each is proposed to stimulate the formation of transcriptionally competent RNAP-holoenzyme open promoter complexes, and CarD is thought to act as a global transcriptional regulator. RbpA and CarD are believed to be essential in *M. Tuberculosis* and *M. Smegmatis*. Recent structural analyses of RbpA and CarD suggest the two proteins may share a region of similarity that could compete for binding to the β subunit, and brings into question whether the two proteins are capable of coordinately modulating transcription or antagonize each other’s activity. This was investigated through purification of CarD and RbpA and *in vitro* studies performed with [α-\(^32\)P] Uridine triphosphate used to measure the level of transcription. These experiments led to the conclusion that RbpA and CarD are able to associate with the same RNAP and have an additive stabilizing action on the polymerase. Whether or not RbpA is an essential protein was also investigated genetically, and by using a Tetracycline on/off system.

Sigma factors play an important role in transcription due to their ability to recognize promoter regions and initiate transcription. One connection that we have preliminary data for, through DNA pull downs, is that sigF binds rRNA promoters, and CarD and RbpA are both studied in the context of rRNA transcription. Therefore sigF is another factor that could be regulating rRNA transcription, possibly during stress. SigF is also the sigma factor that responds to oxidative stress, and CarD is involved in oxidative stress. Sigma F is a member of a family of 13 different sigma factors that are preset in *M. Tuberculosis*. There are two different types of sigma factors: primary, which are
essential for normal growth, and alternative, which are typically expressed during differing environmental conditions. Sigma F has been shown to be upregulated during oxidative stress, which is why it was of particular interest to us. To investigate the roles of sig F, we exposed sig F deletion mutants and wild type strains to oxidative stress and measured ribosomal RNA production by reverse transcription quantitative real time PCR. It was concluded that sigF is a probable suppressor of rRNA when exposed to oxidative stress.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>TITLE</td>
<td>i</td>
</tr>
<tr>
<td>COPYRIGHT PAGE</td>
<td>ii</td>
</tr>
<tr>
<td>READER APPROVAL PAGE</td>
<td>iii</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>iiv</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>v</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>x</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xi</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Specific Aims/Objectives</td>
<td>8</td>
</tr>
<tr>
<td>METHODS</td>
<td>9</td>
</tr>
<tr>
<td>RESULTS</td>
<td>16</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>32</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>35</td>
</tr>
<tr>
<td>CURRICULUM VITAE</td>
<td>38</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Multi-Round <em>In Vitro</em> Assay</td>
<td>23</td>
</tr>
<tr>
<td>2</td>
<td>Multi-Round CarD + RbpA Assays</td>
<td>26-27</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Estimated Tb incidence: top-ten countries, 2012</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>Model of RNAP &amp; RbpA Interaction</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>Southern Blot of RbpA Mutants and Wild Type <em>M. smegmatis</em></td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>Southern Blot of ΔsigF Mutants and Wild Type <em>M. smegmatis</em></td>
<td>14</td>
</tr>
<tr>
<td>5</td>
<td>Csm275 + pDB19 or pDB19 RbpA on Kanamycin or Zeocin</td>
<td>17</td>
</tr>
<tr>
<td>6</td>
<td>RbpA Depletion Growth Curve</td>
<td>18</td>
</tr>
<tr>
<td>7</td>
<td>ATc On/Off strains plated for survival</td>
<td>19-21</td>
</tr>
<tr>
<td>8</td>
<td>Autoradiographs of transcripts</td>
<td>20-21</td>
</tr>
<tr>
<td>9</td>
<td>Single Round <em>In Vitro</em> Assays</td>
<td>21-22</td>
</tr>
<tr>
<td>10</td>
<td>Single Round CarD + RbpA Assays</td>
<td>24-25</td>
</tr>
<tr>
<td>11</td>
<td>SigF Gene Expression Pre and Post Oxidative Stress</td>
<td>28-29</td>
</tr>
<tr>
<td>12</td>
<td>16S – 23S region Pre and Post Oxidative Stress</td>
<td>30-31</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

ATc.......................................................................................................................... Anhydrotetracycline
ATP.............................................................................................................................. Adenosine Triphosphate
CTP............................................................................................................................... Cytidine Triphosphate
DNA............................................................................................................................. Deoxyribonucleic Acid
GTP.............................................................................................................................. Guanosine Triphosphate
PCR............................................................................................................................... Polymerase Chain Reaction
RbpA............................................................................................................................ Ribosomal Binding Protein A
RNA............................................................................................................................. Ribonucleic Acid
RNAP.......................................................................................................................... Ribonucleic Acid Polymerase
rRNA........................................................................................................................... Ribosomal Ribonucleic Acid
SigF............................................................................................................................... Sigma Factor F
Tb................................................................................................................................. Tuberculosis
UTP............................................................................................................................... Uridine Triphosphate
WHO............................................................................................................................ World Health Organization
INTRODUCTION

Tuberculosis (Tb) is a disease that affects millions of people worldwide each year and thus is a great research concern. Tuberculosis is caused by the *Mycobacterium Tuberculosis* bacteria and usually attacks the lungs, although it can attack other parts of the body. Once inside the body, the mycobacterium is flagged as foreign and ingested by macrophages. Macrophages try and eliminate the pathogen by using oxidative reagents, but tuberculosis has become tolerant to these methods and eventually the macrophage will be killed by the mycobacterium (Houben Et al., 2006). Tuberculosis can be highly contagious and is spread through the air by the coughing or sneezing of infected persons. If the disease is left untreated it can be deadly, but with treatment the survival rates can be as high as 85% (WHO 2015). Normal symptoms of Tuberculosis are unrestful coughing, coughing up blood, chest pain, trouble breathing, fatigue, weight loss, and night sweats (Bennett 2010). There are many ways a person can be tested for Tuberculosis including a chest X-Ray, a blood test, and a skin test (Escalante 2009). These practices are all readily available in the developed world but unfortunately less available in the developing world where they are needed most.

Tuberculosis is so prevalent in developing countries because of the large number of people with compromised immune systems due to malnutrition, HIV/AIDS, or other compromising factors. In 2013 the World Health Organization
estimated 1.5 million deaths from tuberculosis and 9.0 million new cases (WHO 2015). The burden of tuberculosis is placed on developing countries with 85% of all tuberculosis cases occurring within Asian and African countries (WHO 2015). The Tb problem is further complicated by the fact that Tb often becomes resistant to drugs in lower income areas due to lack of access and adherence to appropriate drugs.

Figure 1. Estimated Tb incidence: top-ten countries, 2012. The problem can be seen in a range of countries and is most prevalent in India but has a much higher incidence in Sub-Saharan African countries. (Figure taken from WHO Global Tuberculosis report 2015).
Tb can manifest itself in two possible ways, active Tb and latent Tb. A person who has Tb and is sick is considered to have active Tb. On the other hand, a person can have Tb but the body’s defenses are keeping the person healthy and this person is said to have latent Tb. Tb is most often passed between people by close contact or close living quarters. This is part of the reason it can be so dangerous in a compact community with low access to healthcare. Within these vulnerable populations many people have alternative factors that can cause Tb to progress into its disease state. One of the most prevalent and dangerous factors is being HIV-Positive. There were 1.5 million death in 2013, and 360,000 of them were due to the patient being HIV-positive (WHO 2015). The HIV-positive cases of Tb are highly concentrated in the African region with four of every five cases occurring in that region (WHO 2015). One of the most important tools in the survival of Tb patients is HIV testing as this reveals crucial information that is key to the patients' survival.

Another very serious factor that can influence the treatment of a Tb patient is whether or not the infecting bacterium has developed a drug resistance to antibiotics. Multidrug resistant tuberculosis is resistant to two of the most widely used anti-tuberculosis drugs, Isoniazid and Rifampicin (Dalton Et al, 2012). Multidrug resistant tuberculosis develops when the normal dose of antibiotics is interrupted. This happens because there is not enough of the antibiotic in the body to kill 100% of the bacteria leading to the bacteria developing a resistance. This interruption can be caused by a lack of supply, misinformation, a
displacement, or other reasons. Tuberculosis becomes resistant to these first-line drugs by altering the bacteria cell wall to block drug entrance, using enzymes that modify or inactivate, or mutating the genome which makes the bacterium resistant (Louw Et al., 2009). Furthermore, a resistance to the injectable second line of tuberculosis drugs can complicate matters even more. The success rate of treatment with rifampicin and isoniazid in non-drug resistant Tb is generally 85% or higher. The success rate of treating multidrug resistant strains is much lower and treatment lengths can be doubled due to less successful treatments (WHO 2015). Antibiotics are currently the most successful treatments against Tb due to the lack of a vaccine. The existence of Multidrug resistant strains brings the need for new first line antibiotics that can help tilt the war on Tb in our favor.

The current drugs, Rifampicin and Isoniazid, function differently but are both effective in Tb treatment. Rifampicin is a RNA synthesis inhibitor which binds to a site on the RNA polymerase that is adjacent to the active site (Calvori Et al., 1965). This binding causes an inhibition of RNA synthesis and cripples the bacteria. Unfortunately resistance to Rifampicin occurs rapidly which is why it must be given in combination with other drugs (Feklistov Et al., 2008). On the other hand, Isoniazid is a prodrug, a drug that is inactive, that is activated by catalase-peroxidase which is upregulated in response to oxidative stress (Suarez Et al., 2009). Once activated, the drug blocks the synthesis of fatty acids in the cell which includes the synthesis of mycolic acid. Mycolic acid is required for cell wall synthesis, so its blockage stunts bacterial growth.
Tb has become such a successful pathogen because of its ability to resist stress while invading the host. Much of this stress resistance is dependent on transcription, and therefore transcription factors are often targets for pharmaceutical interventions because they can be essential in a functioning mycobacterium (Raman Et al., 2004). If these transcription factors are discovered and the structure, mechanism, and interacting partners can be determined it can prove to be an effective site for intervention.

Transcription of a gene is initiated by the binding of a promoter element to DNA and the formation a closed complex. Isomerization then occurs and the DNA unwinds and is exposed, which is referred to as an open complex. RbpA is thought to play a key role in the stabilization of this process. This is why RbpA has become such an interesting protein that has been identified as an essential gene in Tb. RbpA is a highly conserved gene in actinobacteria that was first discovered in *Streptomyces coelicolor* where it was shown to stimulate transcription *in vitro* (Newell et al., 2006). RbpA interacts with a RNA polymerase and creates a protein-protein interaction. RbpA is thought to associate with the Beta and Sigma subunits of the holoenzyme and initiate the forming of an open complex (Tabib-Salazar et al., 2013).
Figure 2. Model of RNAP & RbpA Interaction. The sigma and beta units of a holoenzyme are represented in this model and with the addition of promoter DNA and RbpA, represented in red, an open complex can be formed to initiate transcription (Figure Taken from Hu et al., 2014).
Sigma subunits are responsible for recognizing the -10 and -35 promoter regions which allow the holoenzyme to bind to the promoter DNA and form a closed complex. With the help of RbpA these closed complexes can stabilize in an open complex configuration, which exposes single stranded DNA for transcription to occur. As can be seen from Figure 2, RbpA is essential in forming the open complex to initiate transcription. CarD is thought to have a similar function and has been proven to interact with RNAP and stabilize the holoenzyme which leads to increased transcription (Garner et al., 2014).

SigF is an alternative sigma factor that is often associated with stress. SigF has not been labeled as essential because deletion mutants do not have decreased growth in broth culture or human macrophages (Gebhard et al., 2008). However, sigF has been proven to affect hydrogen peroxide sensitivity and therefore oxidative stress sensitivity (Provvedi et al. 2008). Hydrogen peroxide or oxidative species are often produced by the host in response to an invading pathogen which subjects the pathogen to oxidative stress as a defense mechanism. Tuberculosis has developed the ability to resist this oxidative stress, which is the reason it is a topic of interest.
Specific Aims/Objectives

RbpA is an essential transcription factor in *Tuberculosis* but there is still much that can be learned from the exact mechanism by which the protein regulates transcription as well as interacting partners and the effect on transcription. SigF is already known to be an effector during times of stress which is why it is such an interesting research target. Tuberculosis undergoes stress while in the human lung so sigF is most likely expressed during infections.

The specific aims and objectives of this thesis are as follows.

1. Obtain further evidence as to whether or not RbpA is essential in *mycobacterium smegmatis*.
2. Determine if CarD and RbpA can associate with the RNAP at the same time.
3. Determine the extent to which the RNAP-promoter complex is stabilized with RbpA and in conjunction with CarD.
4. Investigate the effect of sigF on transcription in stressful environments.

By investigating these objectives we can better understand transcription in Tb and more specifically we can better understand the role of RbpA alone, and in conjunction with CarD, and the role of sigF in transcription.
METHODS

Investigation of RbpA essentiality

Whether or not RbpA is truly essential was researched in two different ways – the attempt to create a clean deletion of RbpA from the genome and the use of a tetracycline system to deplete the protein and monitor the effects on growth. The attempt to create a clean deletion of RbpA was performed by a series of cloning techniques and checked by southern blot. Rv2050c is the gene that encodes RbpA in the Tb genome and was amplified from the genome using PCR. This PCR product was verified to be the correct size by gel electrophoresis and extracted from the gel and purified. The purified PCR product was then ligated into the pmsg430 vector which is used to insert a gene, RbpA, into the attB site. This would allow for the deletion of the endogenous RbpA site by using the pDB88 vector.

By using the pDB88 vector we were able to create regions that flanked the endogenous RbpA and use homologous recombination to remove RbpA and leave only one copy of RbpA inserted at the attB site. We were able to select for the correct product that had successfully deleted the endogenous RbpA. Southern blot was used to determine which product was present, the wild type or the strain with RbpA at the attB site. Using a radiolabeled probe that contained a portion of the flanking region we were able to distinguish which samples were the mutants and which samples were wild type. The sample that contained the correct deletion was given the name Csm275.
There were 2 correct mutants of the numerous samples tested. These mutants contained RbpA and a Kanamycin resistance cassette at the attB site with the endogenous RbpA deleted.

We were then able to perform two transformations with the pDB19 plasmid through homologous recombination. One transformation contained the pDB19 plasmid with RbpA, which had previously been transformed in, and one transformation contained empty pDB19. With successful transformations this would yield one mutant with empty pDB19 and one mutant with pDB19 containing RbpA. With a successful transformation the first mutant with empty pDB19 would have lost all copies of RbpA.

Another experiment was done to test the effect of losing RbpA by using a depleting Tetracycline system. Two new strains were made using Csm275, one
which contained a Tetracycline-On operator and another which contained a Tetracycline-Off operator. These strains were made by using a pre-purified vector that had the ability to suppress the gene at the attB site. This vector did not insert directly into the same plasmid as RbpA but the operator controlled the promoter at the attB site which would allow us to control the expression of RbpA. In an ATc-On system the tetracycline protein is only allowed to bind to the promoter at the attB site if bound to ATc and in the ATc-Off system the protein is only bound to the promoter in the absence of tetracycline. Four cultures were prepared – an ATc-On strain with ATc added, an ATc-On strain without ATc added, an ATc-Off strain with ATc added, and finally an ATc-Off strain without ATc added. These strains represented two depleting strains and two healthy strains, one for each of the On/Off systems. With these four cultures we could monitor the effect of the depletion of RbpA over the next 48 hours. Samples were taken to measure the optical density at a wavelength of 600nm every 3 hours during the day, then back diluted to an optical density of 0.2 the next morning. The experiment was carried out until 52 hours. At the same time points dilutions were plated at concentrations of undiluted, $10^{-1}$, $10^{-2}$, $10^{-3}$, and $10^{-4}$. These dilutions were plated to check for survival over the course of the growth curve.
**CarD and RbpA interaction in vitro**

RbpA and CarD are thought to have similar functions, similar structures, and possibly compete for the same binding site (Kaur 2013). To investigate the interactions between RbpA, CarD, and the RNAP an *in vitro* assay was used. The core RNAP, sigma factors, RbpA, and CarD were purified previously. The proteins were purified from pETSUMO protein-expressing BL21 E. Coli cells using nickel affinity chromatography. In the final reaction the concentrations of core, sigma, RbpA, and CarD were 80nM, 320nM, 320nM, and 320nM respectively. The core RNAP was first incubated with RbpA/CarD/dialysis buffer, or CarD and RbpA together for 10 minutes. The Sigma Factor is then added and the reaction is moved to 37°C for 10 minutes. A combination of 40mM NaCl transcription buffer, 50ng of a template DNA, and nucleotide free water is added to bring the reaction volume up to 10.25µl. The reaction is then allowed to incubate another 10 minutes at 37°C at which point it is initiated by 2.25µl of a nucleotide mix which contains ATP, GTP, CTP, and \([\alpha-32P]\) UTP. In a single round reaction there is a double stranded DNA competitor which competes for the holoenzyme and only one round of transcription is allowed to take place. In a multi-round reaction there is not a competitor and the reaction is allowed to complete multiple rounds of transcription. Both types of assays were performed in my research as both were of interest. After the reaction has run for 15 minutes it is stopped by a 7M Urea stop buffer and placed on ice.
The reaction is then run on a 5.5% denaturing acrylamide gel and the gel is allowed to dry for 1 hour at 80 °C and placed on a phosphoimaging screen which detects radioactive signals. The signal is displayed as bands and we were able to quantify the signal of the band and compare the stabilization of the RNAP.

**SigF regulation of transcription**

SigF was investigated under stressful conditions in order to mimic conditions present *in vivo*. A sigF deletion mutant was cloned in order to appropriately study the effects on transcription. To create this mutant, two flanks, one on each side of *M. smegmatis* sigF, were created and cloned into pDB88. As with RbpA, the endogenous sigF was then deleted using flanking regions in pDB88 and homologous recombination. But unlike RbpA, sigF is not essential so it was not necessary to insert at the attB site using pmsg430, and a clean deletion could be made. The clean deletion was tested by southern blot and we were able to differentiate the wild type from the sigF deletion mutants. The sample that contained the correct deletion was given the name Csm278.
Figure 4. Southern Blot of ΔsigF Mutants and Wild Type M. smegmatis.

There were 5 sigF mutants that were recorded after checking by Southern Blot. These mutants contained a clean deletion of sigF and had no antibiotic resistance.

With this mutant we were able to conduct experiments which exposed the mutant and wild type to oxidative stress and use reverse transcription PCR to monitor the exposure to oxidative stress, the ribosomal RNA, and the regulation of CarD. In the experiment, six cultures of wild type M. smegmatis, and six cultures of the sigF deletion strain were grown to logarithmic phase. Mycobacterium initially grow in a logarithmic fashion until they reach a certain confluence at which point they plateau and maintain a stable optical density. We performed our experiments when the bacterium was in logarithmic phase because this is when the most transcription and replication is happening.
After the bacterium reached logarithmic phase, three cultures of each strain were collected to isolate RNA for reverse transcription PCR. The remaining three for each strain were treated with 10nM Hydrogen Peroxide and allowed to incubate for 1 hour. After 1 hour these strains were collected to isolate RNA. RNA was isolated using Trizol reagent and a chloroform extraction technique. After the RNA for each sample was isolated the samples were treated with reverse transcriptase to make complementary DNA. With complementary DNA the sample could be safely stored and reverse transcription PCR could be performed. Real time reverse transcription PCR uses SYBR Green which emits a florescent light upon creation of PCR product and intensifies with accumulation of the product.
RESULTS

Investigation of RbpA essentiality

Whether or not RbpA is essential was investigated by using the strain mentioned above which contained RbpA at the attB site with a Kanamycin resistance. RbpA was cloned into pDB19 and with this vector containing RbpA we were able to replace the Kanamycin resistance with a Zeocin resistance and the resulting strain was resistant to Zeocin. Another transformation that was attempted was transforming empty pBD19 vector, which would replace the Kanamycin resistance and RbpA with a Zeocin Resistance. This transformation would remove RbpA from the attB site and leave the strain without a copy of RbpA. Both transformations were performed and both transformations were plated on Kanamycin and Zeocin.

This type of transformation has been used by the Stallings lab previously to determine the outcome of a probable essential gene. It is reliable because it is a common transformation that should be successful and if unsuccessful then a likely mutation occurred or the transformation was not 100% clean.
Figure 5. Csm275 + pDB19 or pDB19 RbpA on Kanamycin or Zeocin. pDB19 or pDB19 RbpA was transformed into Csm275 and then plated on either Zeocin or Kanamycin and growth was recorded.

These plates were indicative of RbpA being essential due to the inability of the mycobacterium to lose the gene. If RbpA was not essential the transformation that contained empty pDB19 would have successfully transformed into the strain and left the resulting bacteria without a copy of RbpA. Instead the
resulting strain became Zeocin and Kanamycin resistant which meant the bacteria had not lost RbpA.

Another experiment was done to test the growth of different strains when RbpA was repressed by an ATc operator. We expected the ATc-On system to grow better in the presence of ATc and the ATc-Off system to grow better in the absence of ATc.

Figure 6. RbpA Depletion Growth Curve. In the growth curve the defect can be seen in the ATc-Off + ATc culture and the ATc-On –ATc culture, with the defect being more prominent in the ATc-ON –ATc.

The growth defect present in the two strains that repressed RbpA can be seen in the above graph with the defect in ATc-On – ATc being more prominent. After a certain period of time the Tetracycline operator can become suppressed
because the bacterium needs the gene to survive. This is most likely what happens in the ATc-Off + ATc strain. There is an initial growth defect but the strain seems to recover near the 50 hour time point. In either example it can be seen that there is some sort of defect in both of these strains which is indicative of RbpA being an essential gene. Had RbpA not been essential then there would not be a growth defect in any strains due to the ability of the bacteria to survive without RbpA.

This experiment was also replicated by plating the same cultures for survival at the same time points the ODs were recorded. The dilutions that showed the best difference in colony formation was the $10^{-4}$ dilution after 2 days of growth. The colonies were counted and graphed to visualize the survival of different strains.
Figure 7. ATc On/Off strains plated for survival. A similar trend can again be seen on the survival plates with the two non-depleting strains having more colonies than the depleting strains.

This is indicative of a growth defect due to the depletion of RbpA in the depleting strains. This graph only confirms what was previously seen when the optical densities were measured at the same time points.

**CarD and RbpA interaction in vitro**

CarD and RbpA were placed in an *in vitro* assay with RNAP and the transcription was measured by radiolabeled [α-32P] UTP. RbpA and CarD were first measured with the RNAP separately in a single round assay. These assays contained a double stranded DNA competitor which was used to limit the RNAP to a single round of transcription. The bands were quantified on a gel which showed increasing intensity with increasing levels of transcription.
Figure 8. Autoradiographs of transcripts. These bands were seen on a
denaturing polyacrylamide gel. Bands were quantified and represent stabilized
RNAP.

Each experiment yielded a similar gel in which bands were quantified and
then graphed to visualize the effect of the protein on the RNAP. These bands are
recording the activity of the AP3 promoter which was the promoter used in these
particular *in vitro* assays.
Figure 9. Single Round In Vitro Assays. It can be seen from these assays CarD is the most efficient at stabilizing the RNAP while RbpA is slightly less efficient, but more efficient than the no factor.
Multi Round assays were also performed which did not contain a double stranded DNA competitor. For reasons unknown RbpA has a better effect in these assays and CarD seems to be less efficient. This effect was also seen in the Multi Round assays that were performed with CarD and RbpA together. With both CarD and RbpA combined the results showed an additive effect of CarD and RbpA with the final result producing more transcription than CarD alone but less than RbpA alone.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Transcription Normalized to No Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Factor</td>
<td>1</td>
</tr>
<tr>
<td>CarD</td>
<td>0.876468796</td>
</tr>
<tr>
<td>RbpA</td>
<td>1.484158381</td>
</tr>
</tbody>
</table>

**Multi Round In Vitro Assay**

Table 1. Multi Round *In Vitro Assay*. In the Multi Round assays there seemed to be a consistently larger amount of transcript produced when RbpA was added and less when CarD was added.

The final experiments added CarD and RbpA together to observe the effects they could have on the same RNAP. This was to test whether they had additive effects or whether they compete for the same binding site. It was also to test whether they could associate with the same RNAP. These experiment were
done with both single and multi-round assays. These assays only measured a single time point, 10 minutes, which we determined was an appropriate time point that allowed us to distinguish the differences between half-lives of the differing proteins and RNAP.
Figure 10. Single Round CarD + RbpA Assays. In the Single Round assays that combined CarD and RbpA the combination of the proteins tends to stabilize the RNAP longer than either protein alone.

The results of this experiment were not as consistent as we would have liked due to much troubleshooting. But we came to the conclusion that CarD and RbpA can associate with the same RNAP and have additive effects on the stabilization of the RNAP. Both of these proteins had been proved to stabilize RNAP in the past but never together. This was very exciting because it showed two very similar proteins that have similar functions cooperating with the same RNAP instead of competing for a single binding site. This discovery gave us
some insight into the action of these proteins in vivo as they are both present and likely have some sort of interaction.

Multi-Round assays were also performed with both proteins and the effect was again additive. In these reactions the stabilization was not at its best when both proteins were combined because CarD does not have a strong effect in multi-round assays for unknown reasons. But this again proved that CarD and RbpA could both interact with the same RNAP at the same time and have an additive effect.

### Multi-Round CarD + RbpA Assay 1

<table>
<thead>
<tr>
<th>Protein</th>
<th>Transcription Normalized to No Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Factor</td>
<td>1</td>
</tr>
<tr>
<td>CarD</td>
<td>0.588923079</td>
</tr>
<tr>
<td>RbpA</td>
<td>1.009847012</td>
</tr>
<tr>
<td>CarD + RbpA</td>
<td>0.77503738</td>
</tr>
</tbody>
</table>

### Multi-Round CarD + RbpA Assay 2

<table>
<thead>
<tr>
<th>Protein</th>
<th>Transcription Normalized to No Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Factor</td>
<td>1</td>
</tr>
<tr>
<td>CarD</td>
<td>0.369185205</td>
</tr>
<tr>
<td>RbpA</td>
<td>1.513660701</td>
</tr>
<tr>
<td>CarD + RbpA</td>
<td>0.497764142</td>
</tr>
</tbody>
</table>

### Multi-Round CarD + RbpA Assay 3

<table>
<thead>
<tr>
<th>Protein</th>
<th>Transcription Normalized to No Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Factor</td>
<td>1</td>
</tr>
<tr>
<td>CarD</td>
<td>0.895770116</td>
</tr>
<tr>
<td>RbpA</td>
<td>3.624335085</td>
</tr>
<tr>
<td>CarD + RbpA</td>
<td>1.152380424</td>
</tr>
</tbody>
</table>
Table 2. Multi-Round CarD + RbpA Assays. The additive effect of CarD and RbpA can be seen in the above tables. The RNAP with RbpA were the most stable and the RNAP with CarD were the least stable. When the two were added the resulting stability was intermediate.

Without the double stranded DNA competitor CarD seems less efficient and it is unknown whether the multi or single round assay is more likely to replicate in vivo conditions. Regardless of which is more indicative of how tuberculosis act, the action of CarD and RbpA together can be clearly seen to be cooperative. This gives an insight on how these two proteins interact during transcription and clearly show that both have an important impact on the stabilization of the transcription bubble and the RNAP as well as an impact on the amount of transcript made.

SigF regulation of transcription

SigF is another factor that was studied due to its role in stressful conditions. The role sigF plays in transcriptional activity was studied by exposing M. smegmatis to hydrogen peroxide to emulate oxidative stress. The cells were
then prepped and RNA was isolated which could be used in reverse transcription PCR to measure gene expression. The genes measured were CarD, KatG, and rRNA. KatG is a gene that has been proved to be upregulated during stress which is the reason it was tested in this experiment. KatG is a gene that codes for the enzyme catalase-peroxidase which assists in the decomposition of hydrogen peroxide (LeBlanc Et al., 2006). CarD was monitored due to the interest in possible CarD and sigF interactions during oxidative stress.
**Figure 11. Gene Expression Pre and Post Oxidative Stress.** KatG is clearly upregulated post oxidative stress which is what is expected. CarD seem to be generally unaffected and rRNA could potentially be slightly elevated.

We were unable to draw definitive conclusions from this experiment due to time constraints but further investigation is required. There is a possibility that sigF is a repressor of rRNA due to the slight increase in rRNA in the sigF mutant compared to the wild type. Reverse transcriptase PCR was used on the same samples but amplifying the area in the genome between the 16S and 23S ribosomes. This area would be upregulated in the sigF mutant if sigF was indeed a repressor of rRNA.
16S - 23S Region Pre Hydrogen Peroxide

Fold Change

Sample 1 | Sample 2 | Sample 3

16S - 23S Region Post Hydrogen Peroxide

Fold Change

Sample 1 | Sample 2 | Sample 3
**Figure 12. 16S – 23S region Pre and Post Oxidative Stress** The region between the 16S and 23S was slightly elevated in the sigF mutant after exposure to oxidative stress which is similar to the previous experiment.

These results are suggestive of sigF being a suppressor of rRNA during stressful conditions but the results are not statistically significant and further study would be required to investigate the viability of this hypothesis. But since both regions of the genome produced similar results it is predictive of sigF being a suppressor of rRNA.
The investigation of transcriptional regulators in Tb is needed because of the implications these discoveries can have on the creation of more effective Tb drugs. Tb is able to develop resistance to many first line drugs and once that resistance is established it can be very difficult to continue a successful treatment regimen. Transcriptional regulators have been proven in the past to be effective in treating Tb as one of the most successful, Rifampicin, is a first line drug. The drawback is that many strains of Tb are now resistant to Rifampicin and other drugs, which introduces the need for new and improving drugs.

CarD is a protein that is heavily studied in the Stallings Laboratory and has been proven to stabilize the RNAP and increase its half-life. RbpA is a new protein that is thought to have a similar function and from my experiments it has been confirmed that RbpA is essential and that RbpA and CarD can cooperate on the same RNAP and have an additive effect. RbpA being essential is an important factor because it allows us to realize the impact the protein is having on the overall life cycle of the bacterium. If the bacteria is unable to live without this protein then it may be a good target due to its essential nature, but it may also result in mutations that allow the bacterium to persist. The combination of my experiments, previous experiments and attempts to create a RbpA deletion mutant from other groups indicate that RbpA is very likely to be essential. The inability to lose the gene even though it is being transformed out of a plasmid and
the growth defect noted in two strains of the Tetracycline experiment both indicate that RbpA is an essential gene.

Since RbpA has been identified as essential it is necessary to understand how it interacts with the RNAP. The exact mechanism is still not understood but the investigation on how it interacts with CarD is intriguing due to their similar natures. RbpA has been previously shown to interact with the RNAP and stabilize the RNAP to allow for increased transcription (Hu et al., 2014). We wanted to reproduce these effects and further the experiment by testing both CarD and RbpA together. The effects of CarD and RbpA were both shown to be similar in the fact that they stabilized the RNAP but at varying levels of effectiveness. When added together in the same assay it became apparent that they cooperated on the RNAP instead of competing for a similar binding site as previously hypothesized. This did not reveal the mechanism but it did shed light on the interactions between the two proteins and proved they were capable interacting partners.

The in vitro assays were by no means perfect and many days of troubleshooting went into reproducing the reported effects of RbpA and CarD. A large problem was inconsistency in the effects of the two proteins as can be seen in the data. Despite these inconsistencies, the combination of the two proteins almost always created a more stable RNAP which led us to conclude with a degree of certainty that the proteins were in fact interacting on the same RNAP during the same transcription cycle. This is an important fact going forward with
RbpA research because it has verified a possible interacting partner during transcription.

The final experiment that focused on the transcriptional role of sigF hinted at sigF being a repressor of rRNA during times of stress. The fact that two different regions of the genome that are involved in transcription were both slightly upregulated in the sigF mutant could indicate a role of sigF. Further investigation would be needed to delve into this theory but it is a starting point regarding the role of sigF. Future directions would repeat the experiment and expose the wild type and sigF mutant to varying concentrations of hydrogen peroxide and visualize the effects on transcription.
REFERENCES


CURRICULUM VITAE

Steven Scott Pope II
68 Danforth Rd. Alton, IL 62002 • spope3@gmail.com • Birth year: 1991

Education
Boston University School of Medicine – May 2015
Boston, MA
Master of Science in Medical Sciences candidate

The Johns Hopkins University – May 2013
Baltimore, MD
Bachelor of Arts in Public Health Studies
Entrepreneurship & Management Minor

• Honors: Johns Hopkins University – Deans List – Fall Semester 2012
• Relevant Coursework: Biochemistry & Cell Biology, Biostatistics, Epidemiology, Histology, Leadership Theory, Medical Sociology, Molecular Endocrinology, Organic Chemistry, Physiology, Pharmacology, Risk Sciences & Policy.

Research, Work, & Volunteer Experience

The Stallings Laboratory at The Washington University School of Medicine
St. Louis, MO
Graduate Research Assistant
August 2014-April 2015
• Conducted research on essential and non-essential transcriptional factors RbpA, CarD, and SigF of the Mycobacterium Tuberculosis. Investigation included PCR, basic cloning techniques, in-vitro assays, protein purification, Southern Blot, phage recombination, Tetracycline-controlled transcriptional activity, and Beta-Galactosidase assays

Boston Medical Center
Boston, MA
Volunteer Ambassador
January 2014-Present
• Greeted visitors and patients to the hospital and assisted anyone who was in need of directions or information. Escorted elderly or disabled patients to various clinics or offices around the medical campus. The amount of patient interaction and gratitude gained from this experience is unparalleled.
St. Johns Mercy Hospital in St. Louis  
St. Louis, MO  
*Medical Observation*  
January 2009-August 2013  
- Observed Dr. Jeffrey Marsh, a pediatric plastic surgeon, perform surgeries related to craniofacial birth defects, gained experience in a surgical environment, witnessed first-hand the inner workings of a hospital’s pediatric department.

Alan Mason Chesney Medical Archives of The Johns Hopkins Medical Institutions  
Baltimore, MD  
*Research Assistant*  
May 2012-December 2012  
- Assisted researchers by gathering and organizing appropriate materials based on their topics of research, took inventory and cataloged archival materials regarding cancer epidemiology and embryology while screening for sensitive information in accordance with the IRB.

**Additional Leadership Activities**

**Congressional Award: Bronze Medal**  
St. Louis, MO – June 2013-April 2014  
- *Medal Recipient* – My application for a Congressional award at the Bronze Medal level was accepted in November 2014. This includes 100 hours of voluntary public service, 50 hours of personal development, 50 hours of physical fitness, and 1 expedition.

**Universities Allied for Essential Medicines (UAEM)**  
Baltimore, MD – June 2011-May 2013  
- *Johns Hopkins Chapter Member* – Helped lobby on behalf of UAEM for global public health interventions regarding essential medicines through meeting with President Daniels, petitions, and events. Managed the Johns Hopkins/Yale co-sponsored screening of the tuberculosis documentary “They Go to Die” at the annual UAEM conference held at Johns Hopkins.

**Sigma Phi Epsilon Fraternity**  
Baltimore, MD – January 2012-September 2012  
- *Dorm Wars Chairperson* – Organized an orientation event for the entire incoming freshman class that promotes student interaction and provides an innovative way to raise money for charity. Gained the support of various businesses and student groups and raised over $2,000 for the Maryland Chapter of the Crohn’s and Colitis Foundation.
Project Prevent
Baltimore, MD – July 2011-July 2012
• **Baltimore Community Volunteer** – Visited inner city schools to help organize and setup educational fairs that promoted prevention through means such as education in dietary and sexual health among students.

Aviation
St. Louis, MO – February 2009
• **Private Pilot** – Received Private Pilot’s License.

Boy Scouts
Alton, IL – January 2009
• **Eagle Scout** – Received Eagle Scout Rank.