

**THE MYCOBACTERIUM TUBERCULOSIS TWIN-ARGININE  
TRANSLOCATION PATHWAY**

An Undergraduate Research Scholars Thesis

by

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Submitted to the Honors and Undergraduate Research  
Texas A&M University  
in partial fulfillment of the requirements for the designation as an

UNDERGRADUATE RESEARCH SCHOLAR

Approved by  
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May 2014

Major: Biomedical Sciences

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## ABSTRACT

The *Mycobacterium tuberculosis* twin-arginine translocation pathway. (May 2014)

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*Mycobacterium tuberculosis* (MTb) is the causative agent of tuberculosis. It is difficult to study biochemically because of its slow growth rate and its pathogenicity, which requires BSL3 conditions. The twin-arginine translocation (Tat) pathway of MTb is essential for its growth and virulence<sup>1</sup>. The Musser lab has worked on the *Escherichia coli* Tat system for over 12 years and has developed numerous biochemical and biophysical assays. This expertise will now be applied toward the MTb Tat machinery. Our hypothesis is that the Tat system is a good drug target because it is essential for the growth and function of MTb. The proposed work seeks to express the MTb Tat proteins in *E. coli* to more easily functionally study the MTb Tat machinery. The major outcome of a successful project will be a functional in vitro MTb Tat transport assay that can be used for biochemical studies, and, in particular, for drug development.

## **DEDICATION**

I would like to dedicate this research to my parents, teachers and friends for their unrelenting support throughout my educational experience.

## **ACKNOWLEDGEMENTS**

I would like to thank Dr. Musser for giving me the opportunity and pleasure of working in his lab and giving me the knowledge and experience to make an impact on the scientific community.

I would like to thank all the staff members of the Musser lab, particularly Dr. Umesh K.

Bageshwar, for the countless hours that they spent training, teaching, and supporting me. I would also like to thank Ankur Annapareddy who worked on this project with me in Summer 2013.

## NOMENCLATURE

EDTA	Ethylenediaminetetraacetic acid
LB	Luria-Bertani
MTb	<i>Mycobacterium tuberculosis</i>
IMV	Inverted Membrane Vesicles
PCR	Polymerase Chain Reaction
SDS	Sodium dodecyl sulfate
TB	Tuberculosis
Tat	Twin-arginine translocation

# CHAPTER I

## INTRODUCTION

### **Tuberculosis: an epidemic**

Tuberculosis is an airborne disease that predominantly affects the human respiratory system. About one-third of the world's population is infected with tuberculosis and in 2011, 9 million people became sick with tuberculosis worldwide (10,500 of which were reported in the United States)<sup>2</sup>. Most of the individuals afflicted by this disease reside in third world countries<sup>3</sup>. The Tuberculosis-causing bacteria *Mycobacterium tuberculosis* primarily targets the lungs; however, it is capable of spreading to the kidney, spine and even the brain. There are two primary types of tuberculosis-latent tuberculosis and tuberculosis disease. In latent tuberculosis, the tuberculosis bacteria live in the body but do not cause the individual to become sick because the individual's immune system is capable of fighting off and preventing the bacteria from multiplying. The individual, at any time, can lose the ability to fight off the bacteria, causing full-blown tuberculosis disease. Tuberculosis disease is the state during which the individual's body cannot control the growth of the multiplying tuberculosis bacteria. Individuals afflicted with this condition are typically infectious<sup>4</sup>.

There has been an alarming increase in multi-drug resistance (MDR) strains of tuberculosis. Currently, MDR is defined as strains that are resistant to two out of the four most effective drugs (isoniazid and rifampin). Within the category of MDR strains, there is a subcategory known as extensively drug resistant (XDR) strains. These strains are not only resistant to isoniazid and rifampin, but also to fluoroquine and one out of three injectable anti-tuberculosis drugs such as

kanamycin, capreomycin and amikacin. 3.7% of individuals who are diagnosed with tuberculosis and 20% who have already been diagnosed with tuberculosis have MDR tuberculosis, which translates to about 630,000 individuals. Of all the individuals with MDR tuberculosis, approximately 9% have XDR tuberculosis. The alternatives for those with MDR strains are limited and are often harmful and require extensive procedures such as sputum culture conversion with 5 or 6 drugs. These treatment methods are much more expensive and time consuming than those with first-line drugs<sup>5</sup>.

In recent decades, there has been a global decline in spending for research and the development of drugs. This is because of the limited economic payoff for pharmaceutical companies, which result in part from the high cost of research and clinical trials. There is little incentive to develop new drugs because of the drug resistance to new strains. Antibiotics are costly and time consuming to produce and by the time they are available to the market, there are few individuals to which the antibiotics will cater. Scientists are looking for drug targets that will work with multiple strains of TB. Third world countries are heavily affected, as they have limited resources to pay for drugs and have highly infected populations<sup>6</sup>. We believe that the TB Tat system is a target for new drug therapies.

### **Twin Arginine Translocation Pathway**

The Tat pathway was first identified in the thylakoids of plant and was later found to be orthologous in gram-negative bacteria such as *E. coli*. The Tat pathway is said to be similar to the Sec pathway because they are both secretory pathways. Unlike the Sec pathway, the Tat protein transports folded proteins. In gram-negative bacteria, such as *E. coli*, the Tat system



usually secretes proteins to the periplasm; however in some cells, proteins can even be secreted extracellularly with the aid of other organelles. Also in *E. coli*, the Tat A proteins are said to be necessary for protein transport. The Tat system typically transports folded proteins, cofactors and virulence factors in numerous pathogens. The Tat system translocates proteins from the cytoplasm to the periplasm, the first step in secretion extra-cytoplasmically<sup>4</sup>. The Tat pathway is not found in any human cells and is known to secrete virulence factors<sup>4</sup>.

*E. coli* is a good vector to incorporate in the MTb Tat proteins because of the similarity in amino acid sequence identity and similarities in shapes of the hydropathy plots. Figure 1 shows the amino acid sequence identities between MTb and *E. coli* Tat systems. The TatA, TatB, and TatC proteins have 29%, 32%, and 36% sequence identity, respectively. Figure 2 shows hydropathy plots that are similar in shape for Tat A, B and C. The similarities in amino acid positions make *E. coli* a good vector to express the MTb Tat proteins. Figure 3 shows the differences between the *E. coli* Tat system and the MTb Tat system. The order of the Tat proteins is different in *E. coli* versus in MTb. The MTb Tat proteins are controlled by two promoters, one for Tat A and Tat C and the other promoter controls the expression of Tat B. The MTb Tat proteins we have chosen to express in *E. coli* are controlled by two T7 promoters that can be induced with IPTG.

```

E.coli_TatA      MGGISIWQLLIIAVIVVLLFEGTKKLGSI GSDLGASIKGFKKAMSDDEPKQDKTSQDADFT 60
MTb_TatA        MGSLSPPHWAII LAVVVIVLFGAKKLPDAARSLGKSLRIFKSEVR--ELQENKAEASIIET 58
                **.* * : *.*.*:***:*** . . .** *:: ** . : * : : : : : *

E.coli_TatA      AKTIADKQADTNQEQAKTEDAKRHDKEQV 89
MTb_TatA        PTPVQSQRVDP SAASGQDSTEARPA---- 83
                .... : : : * . . . : . *

E.coli_TatB      -MFDIGFSELLLVFIIIGLVVLGPQRLPVAVKTVAGWIRALRSLATTVQNELTQELKIQEF 59
MTb_TatB        MFANIGWWEMLVLMVGLVVLGPERLPGAIRWAASALRQARDYLSGVTSQLREDIG-PEF 59
                : : ** : * : : : : * : : : : * : : * : * : * : * : * : * : *

E.coli_TatB      QDSLKKVEKASLTSLTPELKASMDL RQAESMKRSYVANDPEKASDEAHTIHNPPVKDN 119
MTb_TatB        DD-----LRGHLGELQKLRGMPRAALTKHLLDGDDSLFTGD----- 96
                : *                * : . : * : : * : : : . . . * . * .

E.coli_TatB      ETAHEGVTAAAQTQASSEEQKPEETPEPVVKAADAEPKTAAPSPSSSDKP 171
MTb_TatB        --FDRPTPKPDAAGSAG--PDAT-----EQIGAGPIPEFSDAT 131
                . : * * . : : * : * : * : * * * * . * * * * .

E.coli_TatC      -----MSVEDTQPLITHLIELRKRLNLCIIAVIVIFLC-LVYFANDI 41
MTb_TatC        MRAAGLLKRLNPRNRRSRVNPDATMSLVDHLTELRTLLISLAAILVTFIFGFVWYSHSI 60
                . : . * . * : * * * * . * : * : * : * : : * : : : *

E.coli_TatC      YHLVSA-----PLIKQLPQGS-----TMIATDVASPFPTPIKLTFMVSLILSAPV 86
MTb_TatC        FGLDSLGEWLRHPYCALPQSARADISADGECRLLATAPFDQFMLRLKVGMAAGIVLACPV 120
                : * *                * * : :                : * * . * : * : : . : : * : *

E.coli_TatC      ILYQVWAFIAPALYKHERRLVVPLLVS SLLFYIGMAFAYFVVFP LAFGFLANTAPEGVQ 146
MTb_TatC        WFYQLWAFITPGLYQRERRFAVAFVIPA AVL FVAGAVLAYLVLS-KALGFLLTVGSDVQV 179
                : * : * * : * . * : : * * : * : : : : * * * : * : * * : * : * : *

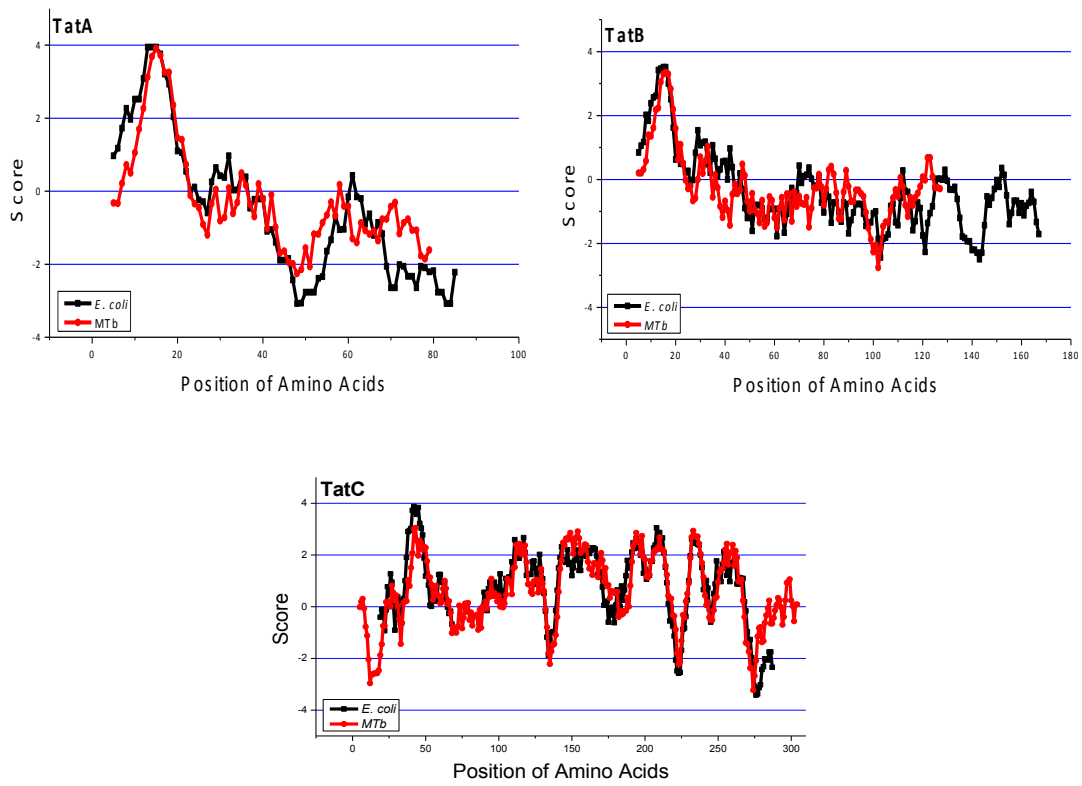
E.coli_TatC      VSTDIASYLSFVMALEMAFGVSEFV PVAIVLLCWMGITSPEDLRKKRPYVLVGA FVVGML 206
MTb_TatC        TALSGDRYFGFLLNLLVVFVGVSEF PLLIVMLNLAGLLTYERLKS WRRGLIFAMFVFAAI 239
                . : . * : * : : * : : * * * * . * : * * * : * : : . . . * . . :

E.coli_TatC      LTP-PDVSQTL LAIPMYCLFEIGVFFSRFYVKGGRNREEENDAEAESEKTEE----- 258
MTb_TatC        FTPGSDPFSMTALGAALTVLLELAIQIARVHDKRKAKREAAIPDDEASVIDPPSPVPAPS 299
                : * * . * * * * . : : * : : : : * : : : : * * : : *

E.coli_TatC      -----
MTb_TatC        VIGSHDDVT 308

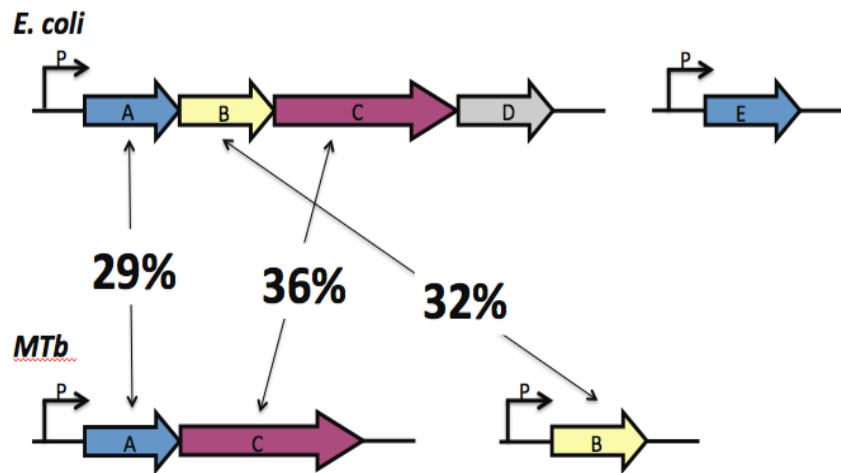
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Figure 1: Amino Acid Sequences for TatA, TatB and TatC from *E. coli* and MTb.



**Figure 2: Hydropathy plots for TatA, TatB and TatC from *E. coli* and MTb.**

### Operon Structure of *E. coli* versus MTb Tat genes



**Figure 3: Gene organization of the Tat proteins in both MTb and *E. coli*.**

The Musser lab has substantial experience in the Tat transport and is ideally suited to develop an in vitro transport assay for the MTb Tat system. In particular, the Musser lab developed the first efficient in vitro Tat transport assay that uses overexpressed precursor protein. This assay has been used to determine the energetic requirements for transport, to characterize lipid and translocon-bound forms of the precursor protein, and to determine real-time interaction kinetics between the precursor protein and the Tat-BC receptor complex. This expertise will now be applied for the MTb Tat system<sup>1,6,8</sup>.

## Objectives

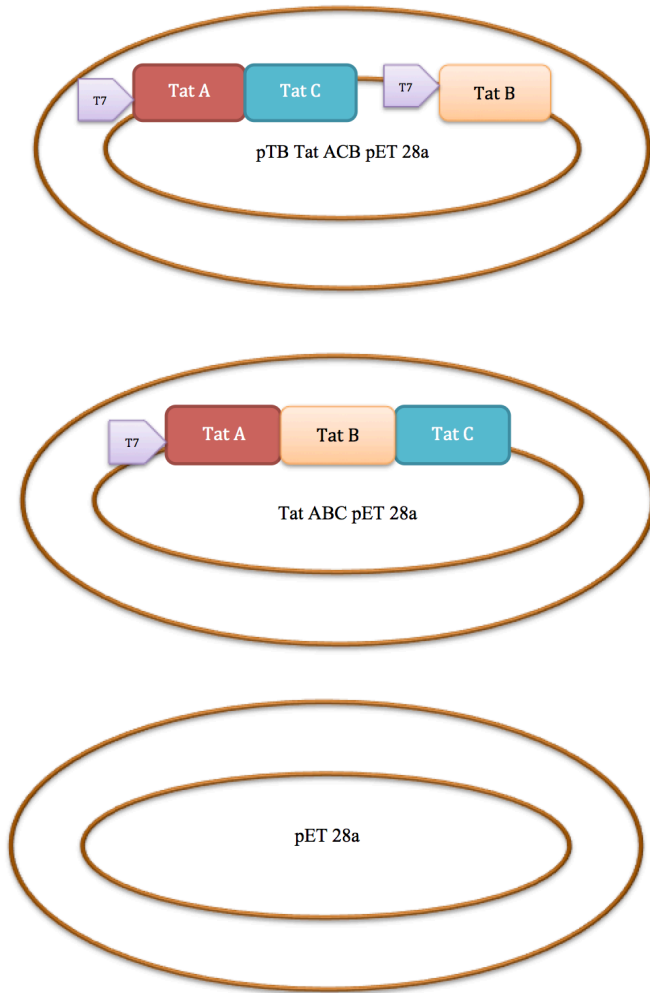
Based on work performed in Dr. Musser's laboratory by multiple people, including myself, during the past summer, all three MTb Tat proteins (TatA, TatB and TatC) were expressed in *E. coli*. We discovered that the Tat protein is partially complemented in *E. coli*. We consider two explanations for only the partial complementation: (i) The *E. coli* pre-SufI was not recognized by the MTb Tat machinery due to differences in signal peptide recognition; or (ii) MTb Tat proteins were not fully functional.

Our goal is to build a tractable model system for in vitro biochemical study of the MTb Tat system. Since the MTb Tat system at least partially functionally replaces the *E. coli* Tat system, we know that it is at least partially active in *E. coli*. To test whether the incomplete complementation arises from substrate recognition (issue 1 above), we will use a model MTb Tat substrates BlaC and chimeric substrate plcB-BlaC, which are efficiently transported by the MTb Tat system<sup>9</sup>. A second explanation for the poor complementation observed thus far (issue 2), i.e., that the MTb Tat system is not fully functional in *E. coli*, is more challenging to address since numerous explanations are possible. The first issues we will address, if necessary, are: i) which of the three Tat proteins are inactive; and ii) can we increase functional incorporation by overexpression of *E. coli* SecYEG and/or YidC.

## CHAPTER II

### METHODS

**Preparation of Strains.** *E. coli* strains CJMS2 pTB Tat ACB 28a, CJMS2 Tat ABC pET 28a, CJMS2 pET 28a have been described earlier (Reference 15). Overexpression cultures were grown in Luria-Bertani (LB) medium at 37°C supplemented with appropriate antibiotics<sup>6</sup>. Figure 4 shows diagrams of the plasmids we constructed and used.



**Figure 4: Plasmid Construction.**

**Creation of Inverted Membrane Vesicles (IMVs).** Creation of IMVs followed the protocol described earlier in reference 8. Cells were grown in LB medium overnight. *E. coli* strain cells were grown at 34°C whereas MTb strain cells were grown at 37°C. Cells were then subcultured and grown for around 5 hours. Cultures were then harvested by centrifugation at 4,000 g for 9 minutes at 4°C. The pellet was then suspended in a buffer A (composed of Magnesium Sulfate, polyvinylpyrrolidone, mannitol, DNase, RNase, KCl, Tricine, lysozyme, EDTA, 1 mM MgSO<sub>4</sub>, 0.5% polyvinylpyrrolidone, 450 mM mannitol, 2 mM DTT, 50 µg/ml DNase I, 10 µg/ml RNAase, 1 mM KCl, 100 mM Tricine, pH 7.5 with 0.4 mg/ml lysozyme, 0.5 mM EDTA, and protease inhibitors). This mixture was then cooled on ice for approximately 20 minutes and was then sedimented via centrifugation at 4,000 g for 10 minutes and resuspended in the Buffer A. The mixture was then passed through a French Pressure cell at high pressure to produce IMVs. The solution was centrifuged as explained above to remove unlysed cells and cell debris. The supernatant fraction was loaded over a 2.3M sucrose cushion and membranes were collected by ultracentrifugation at 108,000 g for 90 min at 4°C. The IMVs collected from the interface were diluted with IMV dilution buffer in 1:3 dilution and then were centrifuged in an ultracentrifuged as described above for 30 min. The IMV pellet was resuspended and finally stored in IMV storage buffer (1 mM KCl, 1 mM MgSO<sub>4</sub>, 2 mM DTT, and 10 mM HEPES, pH 7.0 with 2.2 M sucrose at -80°C for long-term storage in 50% glycerol)<sup>8</sup>.

**Light Microscopy.** The morphology of *E. coli* stains in the absence and presence of the MTb Tat pathway was examined using light microscopy. The cells were viewed at a 100 X magnification using a Zeiss Axiovert 200 M microscope.



**Western Blotting.** The incorporation of MTb Tat proteins into the *E. coli* inner membrane was determined by Western blot analyses against MTb TatA, TatB and TatC using rabbit derived polyclonal antibodies. Goat polyclonal anti-rabbit IgG-HRP conjugate was used as the secondary antibody<sup>8</sup>. The cells were fractionated via centrifugation using ultracentrifuged at 50,000g for 10 minutes to obtain portions of the total cell extract, cytoplasm, and inner membranes.

**Outer Membrane Integrity Assays.** Sensitivity assays were used to determine the functional complementation of the *E. coli* Tat pathway by the MTb Tat pathway<sup>10</sup>. Sensitivity to EDTA and SDS were determined by culturing *E. coli* CJMS2 (pTB-Tat-ACB-28a) and CJMS2 (pET28a) on a soft agar plate in the presence of various concentrations of EDTA and SDS. Filter paper disks with various concentrations of EDTA and SDS were placed on the agar plate overnight.

**Overexpression of Tat Machinery.** The Tat machinery was expressed in CJMS2 (pTB-Tat-ACB-28a) in LB media with various concentrations of IPTG for a 5-hour growth period. Samples were then collected (1 ml/OD) and centrifuged for 4 minutes at 9,000 g. A Western blot was performed using the samples against MTb TatA and TatB antibodies. An IPTG induction time course was then performed by inducing strain with 100  $\mu$ M IPTG. Samples were taken at one-hour increments for 9 hours (1 mL/OD). A Western blot was performed using the samples against MTb Tat A and Tat B antibodies.

**In vivo Transport of B-lactamase.** This sensitivity assay was performed in a similar manner to the EDTA and SDS sensitivity assays however we used the plasmids pPlcB-BlaC and pBlaC in a compatible plasmid. The *E. coli* CJMS2 ( $\Delta$ TatABCDE) was used as a host while doing this assay. Disks are dipped in various concentrations of Ampicillin and grown on a lawn of *E. coli* culture. Cells are then grown at 37°C overnight.

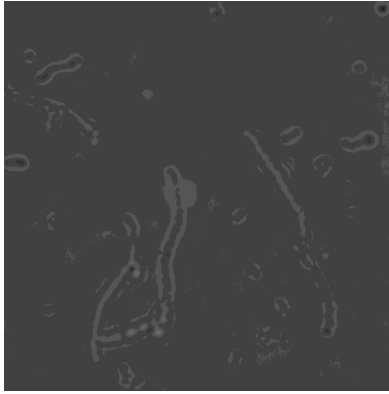
**pre-SufI Binding Assay.** The concentration of bound *E. coli* authentic Tat precursor pre-SufI (fluorescently labeled to Atto 565 dye) to *E. coli* and MTb Tat machinery were determined by performing a binding assay using IMVs. Standard in vitro translocation assays used a 35- $\mu$ l reaction volume containing 50 nM pre-SufI and 4 mM NADH in Translocation Buffer (TB; 5 mM MgCl<sub>2</sub>, 50 mM KCl, 200 mM sucrose, 57  $\mu$ g/ml BSA, 25 mM MOPS, and 25 mM MES, pH 7.0). Solutions were prewarmed at 37°C for 5 min before the addition of IMVs (to a typical final concentration of A280 = 5). After a 30 min incubation at 37°C, reactions were quenched in an ice bath for 2 min. Samples were digested with 0.73 mg/ml proteinase K for 40 min at RT. Digestions were quenched with 68 mM PMSF, diluted twofold with 2 $\times$  Gel Buffer (4% SDS, 10% glycerol, 0.04% bromophenol blue, 0.4%  $\beta$ -mercaptoethanol, 10 M urea, and 200 mM Tris, pH 6.8), and incubated in a boiling water bath for 10 min. Samples were centrifuged briefly at 16,000 g, and then were resolved by 8% SDS-PAGE with known standards. Gels were electroblotted onto PVDF membranes and immunoblotted with SufI antibodies and examined using a Bio-Rad phosphorimager<sup>12</sup>.

## CHAPTER III

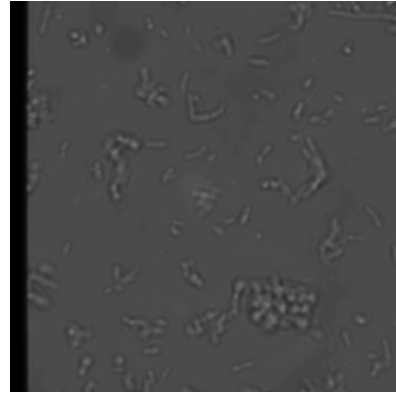
### RESULTS

**Project Design.** The first series of experiments performed was to see if the Tat system was functional in the *E. coli* vector. We performed a microscopy to show the role of the Tat proteins in the growth and cell division of the CJMS2 strain. We then progressed where the Tat proteins were located within *E. coli* and then progressed to show the role of Tat proteins in maintaining the cell wall integrity. We finally progressed to the most important issue, which is to find out if two authentic MTb Tat precursors BlaC and PlcB-BlaC can be efficiently transported by the MTb Tat pathway expressed in *E. coli*.

**Role of Tat Protein Incorporation within Inner Membrane.** Tat machinery is imperative for the growth and development of cells<sup>1</sup>. As shown in Figure 5, in the absence of Tat machinery, cells grown are long, thin, unhealthy and not well divided. In the presence of Tat machinery, cells are well divided and appear healthier. This shows that Tat machinery is essential for the growth and development of bacteria.



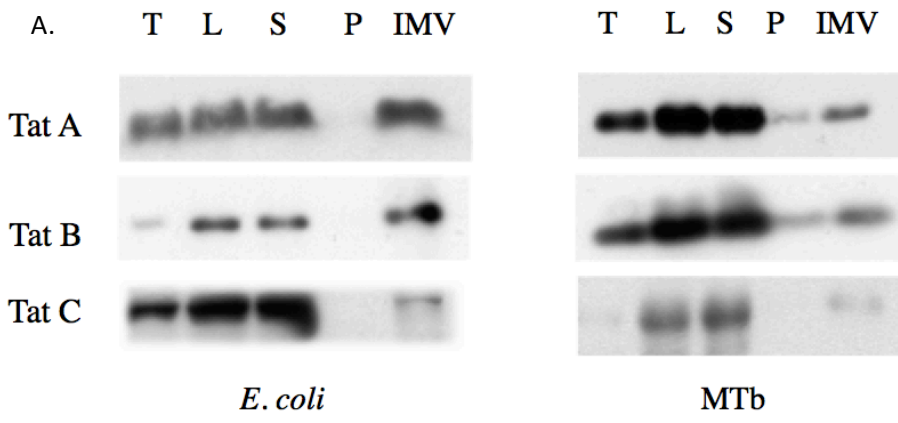
*E. coli* CJMS2 (pET 28a)



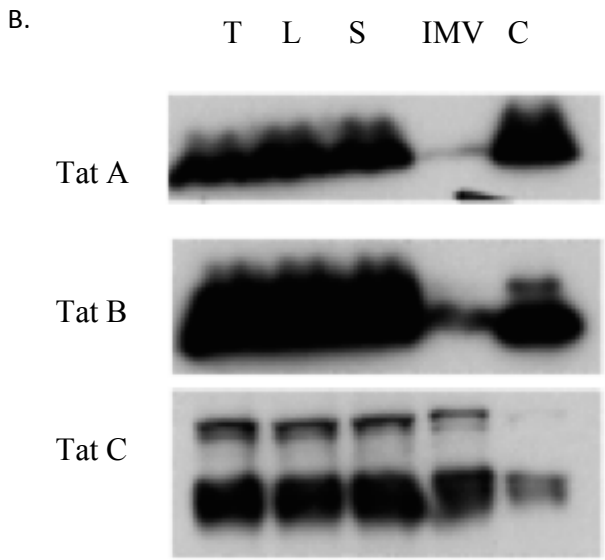
*E. coli* CJMS2 (pTB-TatACB-28a)

**Figure 5. Complementation of the *E. coli* Tat machinery by the MTb Tat pathway.**

**Incorporation of Tat Proteins in the Inner Membrane:** Cellular fractionation indicates that the MTb Tat proteins are incorporated into the inner membrane. Figure 6A shows the results obtained during the summer of 2013 and 6B shows the results obtained during the spring of 2014. Figure 6B shows that Tat A and B were localized in the cytoplasm whereas Tat C was found in the inner membrane. This contradicts what was found previously. Further studies must be performed for conclusive results.



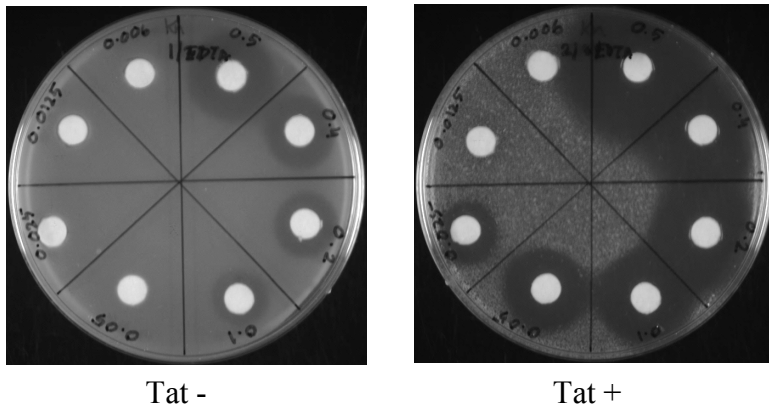
T- Total Cell Extract  
 L- Total Lysed Cell Extract  
 S- Supernatant of Lysed Cell  
 P- Pellet of Lysed Cell  
 IMV- Inverted Membrane Vesicles (Inside out cell)



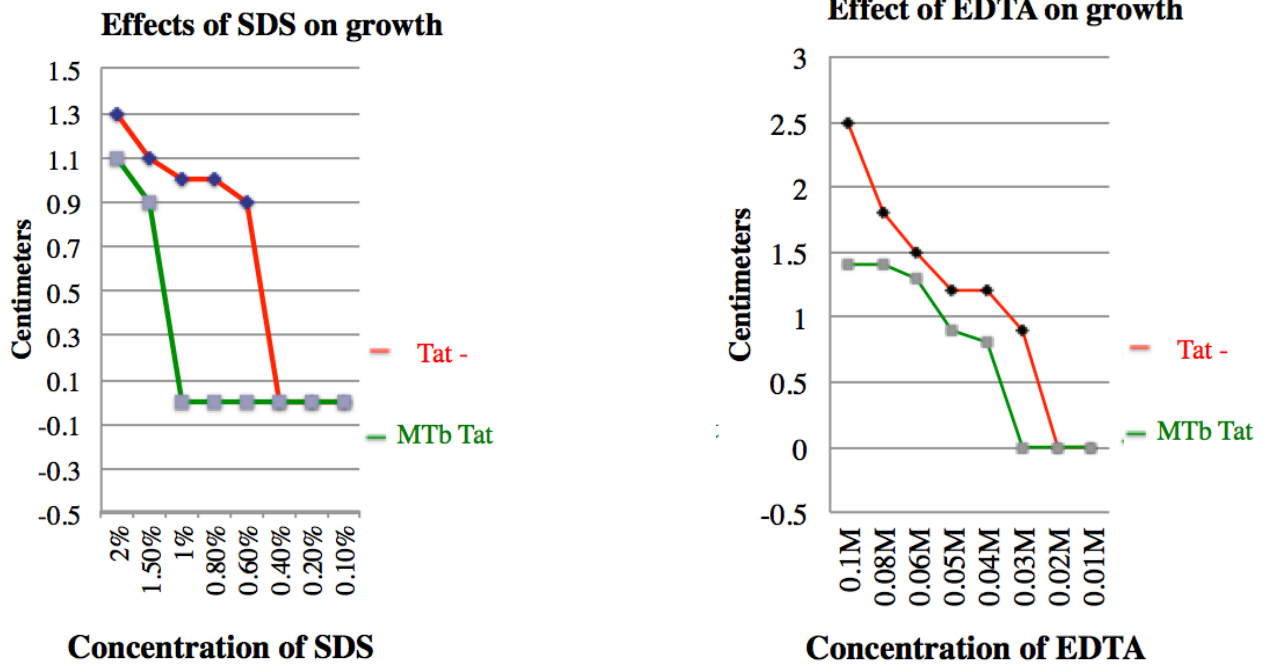
T- Total Cell Extract  
 L- Total Lysed Cell Extract  
 S- Supernatant of Lysed Cell  
 C- Cytoplasmic Fraction  
 IMV- Inverted Membrane Vesicles (Inside out inner membranes)

**Figure 6. Western Blotting was used to confirm the presence of all three MTb Tat proteins within the cytoplasmic membrane (IMV lane).**

**EDTA SDS Sensitivity Assay:** Some of the amodases transported by the Tat system, AMIA and AMIC, are involved in cell wall maintenance<sup>14</sup>. Thus, Tat deficient strains have higher cell envelope permeability<sup>13</sup>, and thus are more susceptible to chemical reagents such as EDTA and SDS. Figure 7 shows that in the presence of Tat Machinery, colonies are formed in moderate amounts of EDTA; however, in the absence of Tat machinery, colonies are not formed. Figure 8 shows that in large concentrations of EDTA and SDS, colonies cannot be formed, regardless of the presence of Tat machinery. This experiment was successful in showing that the MTb Tat machinery was at least partially functionally in *E. coli* because of the growth of colonies in the presence of ampicillin.

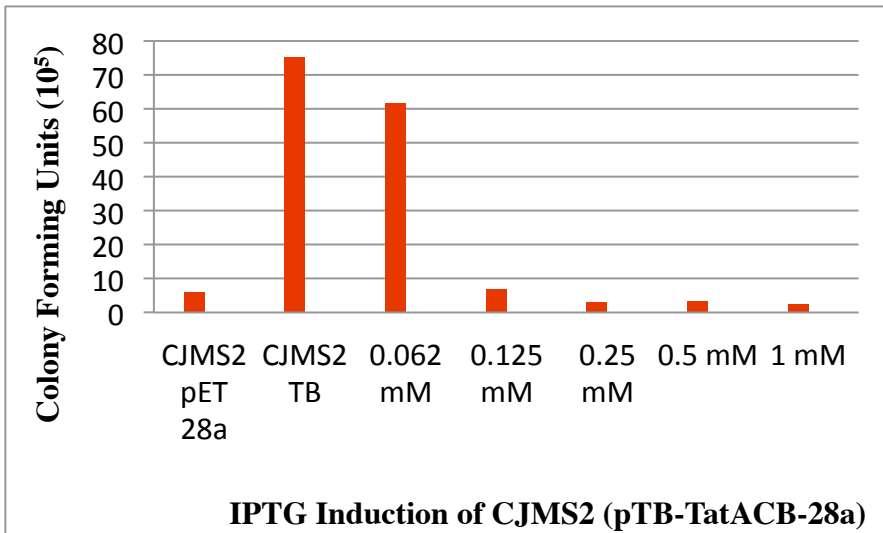
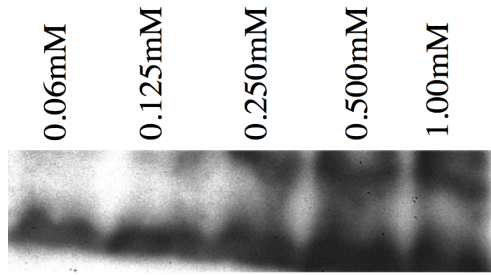


**Figure 7: EDTA Sensitivity Assay**

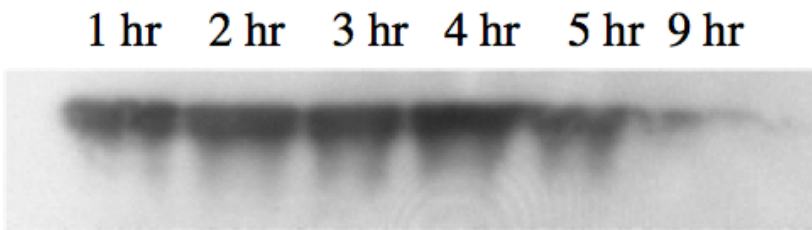


**Figure 8: Effect of SDS and EDTA on Growth**

**IPTG Induction of Tat Machinery.** The T7 promoter controls the Tat proteins incorporated into E. coli. IPTG can induce the T7 promoter, which allows that translocation of Beta Lactamase translocation across the inner membrane. As seen in Figure 9, a high concentration of IPTG causes the strain to not divide as well and inhibits growth rather than optimize it. The colony forming units are indicative of cell growth in the presence of IPTG. A western blot analysis determined that the minimum concentration of IPTG required for induction is 100  $\mu$ M. A western blot analysis also determined that strains must be grown at least 4-5 hours for ideal concentration of cell growth (Figure 10).



**Figure 9: Effect of IPTG Induction on Cell Growth**



**Figure 10: Time Course of IPTG**



**PreSufI Binding Assay.** We have performed experiments to see if the *E. coli* Tat authentic precursor preSufI could be translocated across the membrane. We found that preSufI was better transported with authentic *E. coli* Tat machinery rather than the MTb Tat machinery (Figure 11). For this reason, we have decided to repeat the assay with purified BlaC protein, which is authentic to the MTb Tat system.

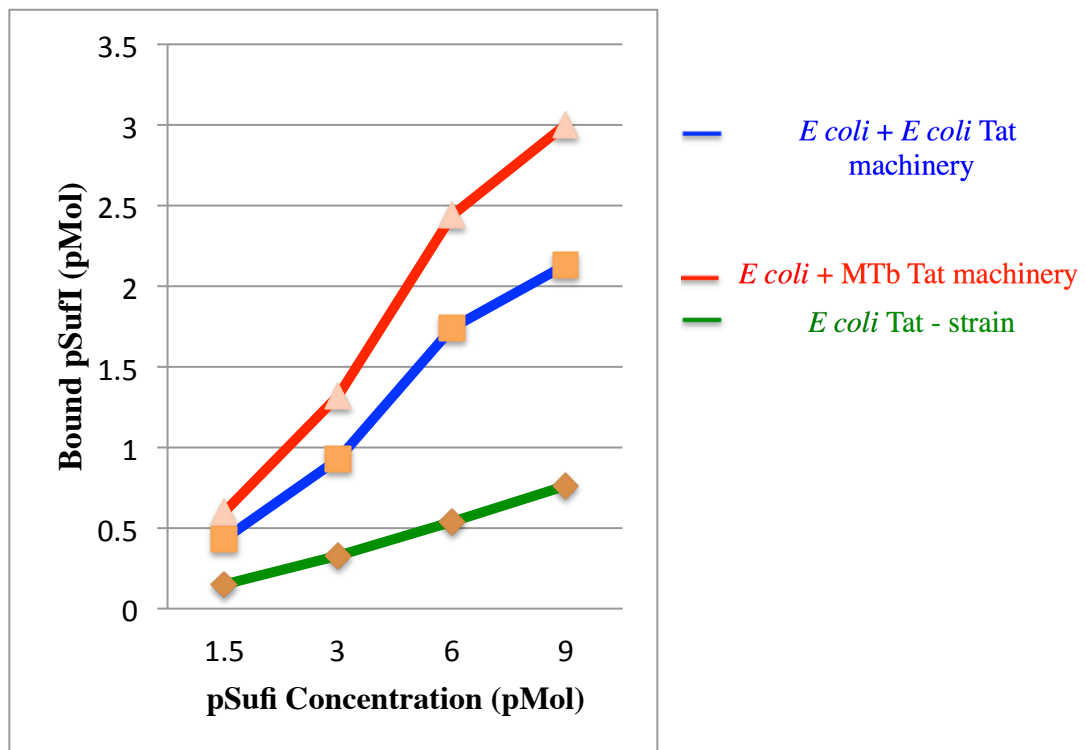


Figure 11: preSufI Binding Assay

## CHAPTER IV

### CONCLUSIONS AND DISCUSSION

The purpose of this experiment was to develop a tractable model organism in which we can characterize the MTb Tat system. The Tat machinery is essential for bacterial cell growth and development. Since we have previously discovered the MTb Tat system to partially function in *E. coli*, we have tried to develop a fully functional and complementary model. After discovering that the MTb Tat machinery was successfully expressed into *E. coli*, we determined the ideal concentration and time period for IPTG induction of the T7 promoter so that the Beta lactamase can be secreted into the inner membrane. In the presence of BlaC and Tat machinery, the cell becomes resistant to antibiotics such as ampicillin. This is imperative in showing that the Tat machine is functional after having been incorporated into *E. coli*.

Currently we are working on growing strains that will transport BlaC and show resistance to ampicillin. This assay is performed in a manner similar to the EDTA and SDS sensitivity assays. Future plans of the lab include creating strains that incorporate both *E. coli* and TB Tat proteins to see if any of the TB Tat proteins is not functional in *E. coli*. We are also currently purifying the BlaC precursor so that we can use it for binding and protein transport assays.

The CJMS2 strain we are working with can sometimes be problematic. Although the CJMS2 strain has an IPTG inducible T7 promoter sequence, we are having trouble in testing function of the MTb Tat proteins when incorporated. For this reason, we will try to clone the MTb Tat proteins in the MC4100 wild type strain. This strain will have an arabinose induced promoter

sequence. The MTb Tat proteins will be arranged in a similar manner to the *E. coli* Tat proteins to hopefully yield a more successful transport assay.

Creating a functional assay will serve as a target for drug testing. Inhibiting the TB Tat machinery will stop the growth and development of these cells. As the strains of tuberculosis become increasingly pathogenic, it is imperative that scientists find the drugs that are currently on the market. The TB Tat machinery serves as a potentially successful drug target.

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