Characterization and Crystallization of the Mycobacterium Tuberculosis trmD

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CHARACTERIZATION AND CRYSTALLIATION OF THE *MYCOBACTERIUM TUBERCULOSIS* TRMD PROTEIN

A thesis submitted in partial fulfillment of the requirements for the degree of Master’s of Science at Virginia Commonwealth University.

by

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One third of the world’s population is affected by Tuberculosis (TB), a disease caused by infection with *Mycobacterium tuberculosis*. The emergence of multidrug-resistant *MtB* makes this disease a major public health concern. New agents are needed to treat TB infections in a manner that circumvents existing pathways of resistance. One strategy is to target the organism at the translational level by inhibiting vital modifications of RNA. One gene responsible for these modifications is the tRNA (guanosine-1)-methyltransferase, *trmD*, which has been shown to be essential in several bacteria. The eukaryotic and bacterial m^1^G methyltransferases are structurally dissimilar, making this enzyme an ideal target for selective anti-TB agents.
One strategy for TrmD inhibitor design is to target the catalytic center of the enzyme. Existing inhibitors such as Sinefungin exhibit poor selectivity due to the substrate’s role, SAM, as a universal methyl donor in many biological processes. Structure/activity relationships for inhibitory compounds are sparse, impeding the design of novel antimicrobials. Crystallographic data would identify molecular features unique to TrmD, and allow design of agents complimentary to the TrmD active site with minimal differential toxicity. Presently, no crystal structure for *Mycobacterium tuberculosis* TrmD exists.

As a first step in this direction, the *MtB* gene has been cloned and expressed by using a His-tagged T7 expression vector. The recombinant protein was characterized through kinetic and preliminary inhibitor assays. The native enzyme displays a mass of 50 kDa, proving this enzyme is a dimer of two identical subunits. This is similar to data found on other TrmD orthologs. Crystallization of *MtB* TrmD has been achieved and preliminary x-ray diffraction studies conducted.
Chapter 1

1.1 General Introduction to *Mycobacterium Tuberculosis*

Tuberculosis (TB) is the most common cause of infectious disease–related mortality worldwide. According to the World Health Organization (WHO), one third of the world's population has been exposed to the tuberculosis pathogen (1). Although TB rates are decreasing in the United States, the disease is becoming more common in many parts of the world. In addition, the prevalence of drug-resistant TB is also increasing worldwide. Co-infection with HIV has been an important factor in the emergence and spread of resistance (2). New TB treatments are being developed (3) and new TB vaccines are currently under investigation (4).

Tuberculosis is a communicable disease caused by the *Mycobacterium tuberculosis* (*MtB*) bacterium, which is the causative agent of human tuberculosis. It is an aerobic bacterium that divides every 16 to 20 hours, an extremely slow rate compared with other bacteria, which usually divide in less than an hour (5). Humans are the only known reservoir. The pathogenicity of *M. tuberculosis* relies on its ability to survive and persist within the host macrophage cells during infection. The pathogen primarily infects the mammalian respiratory system but can also attack any part of the body such as the kidney, spine and brain (5). If not treated properly, TB is fatal. The mode of transmission is through air from one person to another. A person with active TB disease of the lungs or throat infects people around them. Two types of conditions exist: latent and active TB. In
latent, the pathogen lives in one’s body without showing symptoms in the individual. In active, the person shows symptoms and infects others nearby. Annually, 8 million people become ill with tuberculosis, and 2 million people die from the disease worldwide. In 2004, approximately 14.6 million people had active TB disease with 9 million new cases. The annual incidence rate varies from 356 per 100,000 in Africa to 41 per 100,000 in the Americas (6). Tuberculosis is the world's greatest infectious killer of women of reproductive age and the leading cause of death among people with HIV/AIDS. It is responsible for three million deaths annually, more than AIDS, malaria and other tropical diseases combined. Tuberculosis is one of the three primary diseases of poverty along with AIDS and malaria (1).

TB was once thought to be virtually eradicated in developed countries due to development of novel antimycobacterial drugs and improved public health management. However, the recent resurgence of multi-drug resistant (MDR) strains of TB and the immunocompromising nature of the HIV virus, which allows TB to thrive and spread in the host, make the statistics associated with TB frightening. This has led to the WHO recognizing TB as a potential global emergency for human health. After the discovery of the first anti-tubercular drug streptomycin in 1943, followed by Isoniazid (INH), Para-Amino Salicylic Acid and Rifampicin (RIF) in the early 1960s, the drug regime for TB has remained unchanged for the past 50 years (7). Patients with drug resistance are much harder to treat. Therefore, it is of importance to identify genes and pathways that are involved in the survival and persistence of *M. tuberculosis* within cells. Thus, this indicates
the urgent need for new anti-TB drugs and subsequent discovery of novel drug targets in TB.

1.2 Characteristics of Methyltransferases

A methyltransferase (Mtase) is a type of transferase enzyme which transfers a methyl group from a donor to an acceptor. A considerable number of the methyl groups in cell metabolites accept a methyl group by an ATP-activated form of methionine, S-adenosyl-L-methionine (AdoMet) (8). Mtases use a reactive methyl group bound to sulfur in S-adenosyl methionine as the methyl donor (Figure 1). It is now known that DNA, RNA, proteins, lipids and polysaccharides are methylated by AdoMet-dependent methyltransferases (8).

Figure 1: Structure of AdoMet. Sulfur bound to methyl group that is donated to tRNA. AdoMet is highlighted in yellow.

All types of cellular RNAs contain modified nucleosides, but the largest number and greatest variety are found in transfer RNAs (tRNAs) (9). tRNAs contain site-specific base and backbone modifications that are important in overall structural stability and function (10). One important modification enzyme is the \textsuperscript{m1}G37 tRNA methyltransferase which methylates the N1 group of guanine at position 37 of tRNA using S-adenosylmethionine (Figure 2). Methylation of G37 requires

Figure 2: Guanine 37 of tRNA (Anticodon Loop)
a purine (Guanine) at position 36 of tRNA.

\[ \text{m}^4\text{G37} \] tRNA methyltransferase is found in all three domains of life, Eubacteria, Eukarya and Archaea and is denoted TrmD, Trm5 and aTrm5 respectively (11, 12). This enzyme is also present in organelles (mitochondria and chloroplasts) and in the bacteria \textit{Mycoplasma spp}, which have the smallest genomes known to date (11). Mtase participates in the translational phase of protein synthesis by maintaining proper codon:anticodon alignment. Methylation of Guanine at position 37, near the 3’ of the anticodon nucleotides 34, 35, 36, provides a steric hindrance to further Watson-Crick base pairing between tRNA and mRNA molecules (Figure 3). Thus, it prevents the occurrence of frame shift events which would result in the formation of truncated or aberrant proteins (13, 14). Hence, it reduces frame shift errors during translation. Previous experiments have proven this is an essential function step for yeast and gram negative bacteria (15-17). Studies performed by Björk et al. (11, 14, 15) reported that the TrmD protein plays a vital role in maintaining the proper reading frame during translation. Having either a mutant enzyme or \textit{trmD} gene knockout will cause the bacteria to grow more slowly or even be lethal in some organisms (17). The importance of the \text{m}^4\text{G37} modification in the overall quality of protein synthesis emphasizes its conservation throughout evolution.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{Watson-Crick base pairing. A.) Nucleotides are base pairing with each other. B.) Nucleotides cannot base pair due to methylation.}
\end{figure}
1.3 TrmD Structure

"trmD is a member of the SPOUT methyltransferases which are a class of S-adenosylmethionine dependent enzymes characterized by an unusual α/β fold with a deep topological knot (18,19). This knot constitutes a major portion of the AdoMet binding pocket and is buried deep in the protein. Most S-adenosylmethionine dependent methyltransferases, including trmD, belong to the Class I family which contains the recognized AdoMet binding pocket known as the Rossman fold (20). Substrates for Class I methyltransferases include DNA, RNA, small molecules and other proteins (21).

The Holmes laboratory has performed extensive studies on the E. Coli TrmD and the structure has a 38% sequence homology identity to Mycobacterium tuberculosis trmD (Table 1). Thus, it will be used as a form of comparison to Mycobacterium tuberculosis trmD. X-ray crystallography of the E. Coli enzyme reveals two binding sites for AdoMet which are deeply buried within the interior of the enzyme (22). The E. coli monomer of trmD consists of two discrete domains, the C-terminal domain and the AdoMet binding domain, connected by an extended flexible linker region of 11 residues (Figure 4). Five major parallel beta strands and a sixth short strand extending the sheet on one edge are inserted between layers of helices and make up the core of the domain (Figure 5). The AdoMet binding site is found at one end of the beta sheet and is a central part of a deep trefoil knot structure. Beta sheets are built from a central N-terminal (NT) beta strand β1, with β2 lying along side of β1, and β4 as the next strand in the sheet which are parallel to each other (Figure 5). More than one-half of the enzyme is involved in the knot structure,
which forms the AdoMet-binding site or active site (22). The active site is located in the AdoMet Domain near the β3, β5 and α5 strands. The active site is located in between the N and C terminal domains of two monomers. This observation suggests that the dimer edges interact (22). The substrate, AdoMet, is encircled in a deep pocket surrounded by the adenine-binding loop, the β3 and β5 strand, and the loop leading into helix α5 (Figure 5). The loop structure called “the halo” extends between α3 and α5. The C-terminal domain (CT) consists of alpha coils (α7-α10) connected to the AdoMet domain by a flexible linker (Figure 5). The substrate takes into a bent conformation in the binding site. The adenine binding region curves around the adenine ring (Figure 5). *E. coli* structure analysis suggests that significant conformational changes must occur for the substrate binding and catalytic turnover (22).

Using a program entitled *Chimera*®, depictions of the active site interacting with AdoMet was generated. At first glance, certain amino acids appear to interact through hydrogen-bonds with the AdoMet substrate. The amino acids involved include Tyrosine 134, Glycine 136, Leucine 138, Glycine 140, Glycine 141, Tyrosine 86 and Leucine 87. Hydrogen bonds between Glycine 134, Tyrosine 136 and Leucine 138 to the AdoMet substrate help to stabilize the cofactor in place. Additionally, Glycine 140, Tyrosine 86 and Leucine 87 interact with the ribose of AdoMet to tether it in place (Figure 6). The ribose moiety of the AdoMet twists in a conformation that results in the methionine region that is perpendicular to the adenine ring.
**Figure 4:** Model of *E. coli* TrmD (pdb 1P9P) generated by Chimera® displaying dimer structure with AdoMet domain, C-terminal domain, AdoMet binding site, Adenine binding loop and Halo region. Homologous monomers are indicated in red and blue ribbons. The substrate, AdoMet, is represented by red ball & stick structures in each monomer.
Figure 5: Model of a monomer of *E. coli* TrmD (pdb 1P9P) generated by Chimera® displaying monomer structure with alpha 1-10 and beta 1-5 strands, AdoMet and C-terminal domains (CT), AdoMet molecule bound, flexible linker region and Halo region. NT stands for the N-terminal domain. AdoMet molecule is represented by gray spheres.
**Figure 6:** Interaction of amino acids in active site with AdoMet colored green. Pink lines indicate where hydrogen bonding occurs between amino acids. Image produced using Chimera®.
Currently, six crystallographic TrmD protein structures have been done. These structures correspond to: *Anaplasma phagocytophilum, Aquifex aeolicus, Bartonella henselae, Escherichia coli, Haemophilus influenza* and *Staphylococcus aureus*. An amino acid sequence alignment between these organisms and *Mycobacterium tuberculosis* TrmD was performed using a program entitled “Clustalw” (Figure 7). From this alignment percent sequence homologies were calculated between the organisms and *Mycobacterium tuberculosis* TrmD (Table 1). *Bartonella henselae* TrmD has a 42.9% sequence homology to *Mycobacterium tuberculosis* TrmD, which is the highest percent sequence homology. *Anaplasma phagocytophilum* TrmD has a 42.7% sequence homology. *Aquifex aeolicus* TrmD has the lowest sequence homology with a 32.6%. *Escherichia coli* TrmD has a sequence homology of 38% (Table 1). Sequence alignment of TrmD proteins of known structures indicates that TrmD from *B. henselae* and *A. phagocytophilum* have the highest shared sequence identity with *MtB* TrmD. Furthermore, *B. henselae* contains structural regions that were overlapping with *MtB* TrmD and that were not resolved in the other crystal structures. The length of the *B. henselae* TrmD (233 amino acids) is almost identical to the *M. tuberculosis* TrmD (230 amino acids). Thus, the *B. henselae* TrmD is the best choice for the phasing model, since it includes most of the protein, and has a high percent homology with *M. tuberculosis* TrmD. A multiple alignment TrmD diaphragm was generated using Chimera with *Anaplasma phagocytophilum* (yellow), *Bartonella henselae* (olive green), *Escherichia coli* (purple), *Haemophilus influenza* (blue) and *Staphylococcus aureus* (orange) (Figure 8). The flexible linker region is yet to be resolved in the pdb structures.
Figure 7: Amino Acid Sequence Alignment generated by program ClustalW of Anaplasma phagocytophilum, Aquifex aeolicus, Bartonella henselae, Escherichia coli, Haemophilus influenzae and Staphylococcus aureus. The amino acids in red depict residues that displayed no electro densities in the crystal structure, which therefore must be very mobile regions of the proteins. Some regions of these proteins are completely conserved such as the halo region. "*" means that the residues or nucleotides in that column are identical in all sequences in the alignment. ":" means that substituted conservations have been observed. ":." means that semi-conserved substitutions are observed.
<table>
<thead>
<tr>
<th>Organism</th>
<th>Percent Sequence Homology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bartonella henselae</td>
<td>42.9%</td>
</tr>
<tr>
<td>Anaplasma phagocytophilum</td>
<td>42.7%</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>39.0%</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>38.0%</td>
</tr>
<tr>
<td>Haemophilus influenza</td>
<td>34.3%</td>
</tr>
<tr>
<td>Aquifex aeolicus</td>
<td>32.6%</td>
</tr>
</tbody>
</table>

**Table 1**: Percent Sequence Homology of TmD organisms to *Mycobacterium Tuberculosis* TrmD.
Figure 8: Alignment of TrmD organisms. The flexible linker region is unsolved in the pdb files. The following organisms are shown: *Anaplasma phagocytophilum* (yellow), *Bartonella henselae* (olive green), *Escherichia coli* (purple), *Haemophilus influenza* (blue) and *Staphylococcus aureus* (orange). Image produced using Chimera®
Figure 9: tRNA Anti-codon arm and Loop
1.4 Mechanism of Action

AdoMet dependent methyltransferases catalyze the transfer of the methyl group in a SN\(^2\) type reaction scheme, which allows for only one stereoisomeric product to form due to formation of a triplet state intermediate (Figure 10). The transfer occurs via a nucleophilic attack at a sp\(^3\) hybridized carbon, which often requires a general base catalyst, followed by a thioester transition state, and the release of the product S-adenosylhomocysteine. Methylation of the N1 position of guanine is believed to occur via a deprotonation by a general base catalyst on the N1 nitrogen. tRNA (guanosine-N1) methyltransferase (TrmD) is the product of trmD genes which catalyze the transfer of the methyl group of AdoMet to the N1 position of guanine 37 adjacent the anticodon loop (Figure 9). These proteins methylate only transfer RNAs that recognize codons starting with cytosine, and therefore methylate only tRNA species with guanine at positions 37 and 36. AdoMet is the product of S-adenosylmethionine synthetase, which transfers the adenosyl moiety of ATP to the amino acid methionine. After transfer of its methyl group, AdoMet becomes S-adenosylhomocysteine (AdoHcy). Further metabolism of the product results in the cleavage of the adenosine moiety to form homocysteine. Homocysteine can either be recycled or eventually broken down into the amino acid cysteine within the cell. Methylation of Guanine at position 37, near the 3’ of the anticodon nucleotides 34, 35, 36, provides a steric hindrance to further Watson-Crick base pairing.
between tRNA and mRNA molecules (Figure 11). Thus, it prevents the occurrence of frame shift mutations which would result in the formation of truncated or aberrant proteins (13, 14). Hence, it reduces frame shift errors during translation.

![Frame shifting prevented during protein translation](image)

**Figure 11:** Frame shifting prevented during protein translation

### 1.5 Inhibition of *trmD*

One potential target site for TrmD inhibitors is the SAM binding site. The presence of an inactive compound in the cofactor site will abrogate SAM binding and deprive the enzyme of its methyl donor. Several inhibitors modeled after SAM have been designed and evaluated, however these agents have been found to be non-selective. This non-selectivity is a major drawback because SAM is utilized as a universal methyl donor in many
biological processes (23); hence inhibition of SAM binding will have broad toxicity. Furthermore, the structures considered in these studies are not diverse and hence structure activity relationships are poorly defined as of yet. One strategy to achieve selectivity and high affinity is through structure based design. However, this requires the crystal structure of TrmD. Crystallographic data will permit identification of molecular features unique to TrmD, and thus the design of agents complimentary to the TrmD active site(s). This strategy can be extended through the use of bisubstrate analogues (24); which bridge between the SAM and nucleoside sites, affording more features to exploit for modification.

The urgent need to develop new drugs due stems from the fact that resistance to antibacterial drugs is becoming more common. Drug design strategies currently underway to inhibit tRNA methyltransferase include making analogues of SAM or targeting the SAM binding site. In a study performed by Barchas and Deguchi (25) it was found that S-adenosyl-L-homocysteine (L-SAH), analogue of SAM, is a potent inhibitor of several methyltransferases including tRNA methyltransferase (26). This demethylated product inhibits many SAM-dependent methyltransferases (27). Analogues of AdoMet that either compete with substrate and/or prevent methylation can be a possible way to inactivate the enzyme. Mechanism based inhibition that targets the active site of SAM can perhaps irreversibly degrade the enzyme. Successful DNA methyltransferase inhibitors have been found. Azacitidine, an approved DNA methyltransferase inhibitor, has also been found to inhibit RNA and protein methyltransferases (28). This drug acts by incorporating itself into DNA and preventing methylation by the methyltransferase. Subsequently, cell death is induced since DNA synthesis is obstructed (28).
Selectivity is the main challenge in developing inhibitors for methyltransferases. SAM is a universal methyl donor recognized by many biological processes. Developing a compound that only targets TrmD is the major hurdle to overcome. Additionally in understanding mechanisms based inhibition, crystallographic structures of enzymes need to be determined. Drug potency is also another issue to consider. Yet, the benefit of designing a drug that effectively targets proliferation of a disease has extraordinary implications. One third of the world’s population infected with TB will be dramatically impacted. A successful RNA MTase inhibitor may also affect DNA and protein Mtase. Furthermore, selectivity over mechanistically similar enzymes can be achieved using the crystallographic data to tune the drug to the TrmD active site.

1.6 Introduction to Enzyme Kinetics

To determine the maximum rate of an enzyme-mediated reaction, a series of experiments are carried out with varying substrate concentrations [S]. The Michaelis-Menten equation can then be used to determine the constants for the data sets.

The Michaelis-Menten equation relates two observable parameters, the initial velocity of an enzymatic reaction (V₀) and the substrate concentration [S],

\[ v_0 = \frac{v_{\text{max}} [S]}{K_M + [S]} \]
From the Michaelis-Menton equation it is clear that \( V_o \) and \([S]\) do not exist in a linear relationship. One way to determine \( K_m \) and \( V_{\text{max}} \) is through the use of a Line-Weaver Burke plot, in which the \( 1/V_o \) is in a linear relationship with \( 1/[S] \).

\[
\frac{1}{v_0} = \frac{K_M + [S]}{V_{\text{max}}[S]} = \frac{K_M}{V_{\text{max}}} \cdot \frac{1}{[S]} + \frac{1}{V_{\text{max}}}. 
\]

The reciprocals of the experimental parameters \( V_o, [S] \) may be calculated and a linear fit performed in which the y intercept is \( 1/V_{\text{max}} \) and the x intercept is \(-1/K_m\). This method is problematic since it represents a linearization of an inherently nonlinear data set. Most measurements are obtained at high substrate concentrations \([S]\), and hence the data is clustered around small values of \( 1/[S] \). Thus, a fit is performed over a limited range of spatially distributed data points. Furthermore, at small values of \([S]\), small errors in \( V_o \) propagate into large errors in \( 1/V_o \) and hence large errors in \( K_m \) and \( V_{\text{max}} \). To circumvent these pitfalls nonlinear regression analysis can be used in which the data is directly fit to a Michaelis-Menton like equation.

Nonlinear regression is a form of analysis where a function which has nonlinear parameters, depends on more than one independent variable. This form is best used for enzyme kinetics due to the problems mentioned above. Actual constants of \( K_m \) and \( V_{\text{max}} \) are not determined accurately by the reciprocal Line-Weaver Burke plot. The values for the slope and intercept are not the most accurate determination of the variables. The program entitled *GraphPad Prism®* which uses nonlinear regression analysis determined the constants for the data sets in these experiments.
1.7 Introduction to Crystallography

X-ray crystallography provides the most detailed information available on the structure of molecules. A crystal is mounted between an X-ray source and an X-ray detector. In an X-ray diffraction experiment, a high quality protein crystal of appropriate size is mounted on a goniometer. An x-ray source emits a directed beam of x-rays which bombard the crystal. The x-rays pass through the crystal however they are deflected in the process. These scattered x-rays are then recorded as a diffraction pattern on an imaging plate or detector. The crystal is rotated on the goniometer and this process is repeated. Using the angles and intensities of these diffracted beams, a crystallographer can produce a three-dimensional picture of the density of electrons within the crystal (Figure 12).

Figure 12: General Schematic of X-Ray Crystallography
In an x-ray scattering experiment, a single diffraction pattern is insufficient to determine a crystal structure. The crystal must be rotated 360 degrees with an image recorded at several intermediate steps to get enough data to solve the structure. There are only certain crystallographic planes from which constructive interference leads to strong diffraction. Each of these planes provides a distinct set of data regarding spatial arrangement of atoms in the crystal. Furthermore, every diffraction pattern corresponds to a different image of the electron density map and each spot in the diffraction pattern corresponds to a different type of variation in the electron density. The electron density maps are related to diffraction data by a Fourier transform.

A Fourier transform is used to find the electron density from the diffraction pattern. This transform takes as input structure factors for each spot and their corresponding frequencies. The structure factor is proportional to the square root of the measured spot intensity and the frequency corresponds to the location of the spot in the diffraction pattern. Unfortunately, only the absolute value is available. Hence, additional information is necessary to determine phases and thus electron density maps. The following are common ways to solve the phasing problem of crystallography: Heavy Element Replacement, Anomalous Dispersion, Molecular Replacement (29).
1.8 Specific Aims of the Research

A.) To clone and characterize *Mycobacterium Tuberculosis trmD* gene.

We will clone the *trmD* from *Escherichia coli* into an appropriate expression vector. Protein made in this vector will display an added N terminal his tag sequence permitting rapid purification of recombinant protein using Ni affinity column technology. To establish that the cloned gene product is in fact a tRNA methyl transferase, synthetic tRNA<sub>1</sub><sup>Leu</sup> substrate which contains G37 and G36 residues in the molecule will be employed. It has been shown previously that this serves as a substrate for several TrmD proteins. This will involve the determination of K<sub>m</sub> and V<sub>max</sub> values at various concentrations of both S-adenosyl methionine and tRNA. We will be able to compare these values to that of other orthologues of TrmD.

Once protein is obtained and shown to be a *bona fide* TrmD methyltransferase, we will first determine its oligomeric structure and demonstrate whether or not it will exist in solution as a homodimer. All other TrmD proteins so far obtained have been shown to be dimeric (22).

B.) To Crystallize Mycobacterial TrmD protein, and ultimately obtain the crystal structure. Initial crystal screens will be done using Hampton® general screening kits. If crystals are obtained, conditions will be optimized and diffraction studies will be carried out in collaboration with Dr. Martin Safo. Once diffraction data is obtained of
sufficient resolution, attempts to solve the structure will be carried out using Molecular replacement programs available to us.

C. Ultimately, if a structure is obtained attempts to develop inhibitors of the enzyme will be initiated. This however, maybe beyond the scope of this study, but certainly a structure will facilitate this effort for rational drug design. In addition, with the protein in hand, other high throughput screens for inhibitors could be carried out.

Chapter 2: Materials & Methods

Materials:

The Novagen ligation-independent cloning vector was used for cloning and expression of *M. tuberculosis* gene DNA into suitable *Escherichia Coli* host cells. The oligonucleotides necessary for PCR amplification of trmD sequences were ordered from Integrated DNA Technologies ®. Using these oligonucleotides, Finnzymes Phusion High-Fidelity Polymerase Chain Reaction (PCR) Master Mix® was used to amplify the desired *trmD* sequence from genomic DNA. QIAquick® Gel Extraction Kit was utilized to purify DNA from the agarose gels used to isolate amplified gene sequences. Ampicillin was obtained from Sigma® and isopropyl β-d-thiogalactopyranoside (IPTG) was obtained from Molecula®. LB broth growth media was purchased from Fisher and Sigma®. NovaBlue GigaSingles Competent Cells were purchased from Novagen®. Plasmid DNA was purified using the QIAPrep Miniprep® kit was ordered from Qiagen. The DNA Core laboratory 3700xl machine for sequencing located in Sanger Hall, MCV campus was utilized. Sonication was used to disrupt cells at a small scale and French Press was used for larger
preparations. Microcolumn® Nickel-NTA columns were used for the pilot scale batch of protein.

**Methods:**

2.1 Cloning

The *trmD* gene from *Mycobacterium tuberculosis* was isolated from genomic DNA of the *MtB* CDC1551 strain (gene ID 925304/ accession number is NP_337486.1). This was provided to us by the laboratory of Dr. John Hackett. The pET46 EK/LIC ligation-independent cloning system was utilized for cloning (Novagen®) (Figure 13a). Ligation-independent cloning (LIC) exploits the 3’ to 5’ exonuclease activity of T4 DNA polymerase to strip nucleotides from a specific gene. This process continues until the polymerase utilizes and inserts a single dATP, which has been included in the reaction mixture. At this point, the polymerase activity of the enzyme overrides the exonuclease activity terminating the excision process and thus providing long sticky ends complementary to the overhangs in the LIC vector (Figure 13b). The resulting nucleic acid is readily introduced into the vector through a short annealing step, circumventing the ligation process. The pET46 vector contains an N-terminal His-tag expresses protein under the control of the T7lac promoter. Since the host cell contains an inducible RNA polymerase gene, and a lac operator, repressor system, IPTG addition leads to expression of the *trmD* gene. Furthermore, this vector contains kanamycin and ampicillin resistance genes for selection of transformed cultures.

Forward and reverse primers were specifically designed for expression using Novagen’s EK/LIC system. To ensure optimal function, primers were chosen which have
an absence of dimerization capability and avoid primer self-complementarity which may result in secondary structure formation. The following properties were used when designing the primers: 17-28 bases in length, Tm between 55-65°C, absence of dimerization capability (avoid primer self-complementarity which form secondary structures), and should have a 50-60% GC rich base ration (23). The designed primers are as follows:

**UPSTREAM:** 5’-GACGACGACAAAGATGCGCATCGATATCGTGACGATCTTC-3’

**DOWNSTREAM:** 5’-GAGGAGAAGCCCGGTCTAGTCGGGGTGGGACAG-3’

Amplification was performed using the Phusion High-Fidelity Polymerase Chain Reaction (PCR) Master Mix (New England BioLabs). Following PCR amplification, the DNA was purified on an agarose gel and extracted using an alkaline ethanol method via the QIAquick® Gel Extraction Kit. 700 base pair bands were clearly detectable in duplicate wells as judged by DNA standards. These DNA bands were next treated with T4 DNA polymerase, which exposes single stranded complementary sequences.

The isolated DNA fragment was annealed with the pET-46 vector (Figure 13a) which comes with exposed complementary strands. Next the mixture of vector and insert was mixed with competent NovaBlue GigaSingle® cells. NovaBlue is an *E. coli* K-12 strain and has high transformation efficiency with blue-white screening capability and *recA* *endA* mutations. Cells were provided in a 50 μl single-use volume tube and stored at -70°C. Consequently, 250 μl of SOC media, provided in the kit, was added to the GigaSingle tubes. The tubes were incubated at 37°C while shaking at 250 rpm for 60 minutes prior to plating on ampicillin medium. Selection for transformants was carried out
by plating cells mixed with vector and annealed insert on agar plates containing 50 μg/ml ampicillin. Plates were set on the bench for several minutes to allow excess liquid to be absorbed, inverted and incubated overnight at 37°C. Ten transformant colonies were picked from the agar plate and using a sterile toothpick and each were transferred to a 0.5-ml eppendorf tube containing 50 μl sterile water and vortexed to disperse the cells. All tubes were placed in boiling water to lyse cells and denature DNAases, then centrifuged at 12,000 x g for 1 minute to remove cell debris.

A PCR reaction mixture was setup using PCR-grade water, dNTPs, upstream primer, downstream primer, 10X NovaTaq Buffer with MgCl2 and NovaTaq DNA Polymerase. Positive and negative controls were also included in the procedure. The cycles for PCR were repeated 35 times for the purpose of amplifying the plasmid. An agarose gel was made including DNA from all 10 clones isolated, a positive control and negative control and one well for the DNA 100 base pair ladder. Phusion High-Fidelity Polymerase Chain Reaction (PCR) Master Mix was used to make a PCR product. PCR conditions included: initial denaturation at 98°C for 30 seconds, denaturation at 98°C for 10 seconds, annealing at 70°C for 30 seconds and final extension at 72°C for 10 minutes. The denaturation and annealing step continued for 35 cycles. The sample was then put into a 1% agarose gel 1X TAE. QIAquick Gel Extraction Kit® using a microcentrifuge was used to separate purified DNA from agarose gel.

Next, DNA containing inserts as judged by analysis via gel electrophoresis was purified and sent to the VCU DNA Core facility. Appropriate clones were inoculated in Luria Broth (LB) and grown for 5 hours at 37°C. After this time, the tubes were
transferred to large tubes and centrifuged for 5 minutes. The liquid was discarded and the cell pellet was left intact. The QIAprep Miniprep® extraction kit was utilized to extract DNA from the LB media in a matrix band tube. Four DNA samples and the appropriate complementary DNA primers were sequenced at the DNA Core laboratory. DNA was sequenced using a 3700xl DNA Analyzer. The concentration of DNA utilized for sequencing was 100 μg/ml, and the concentration of DNA reverse and forward primers were 20 μg/ml. T7 polymerase for chain elongation and reagents for dideoxy sequencing reactions was provided by the Core laboratory. Data were sent via email as ASCII sequence files or raw data. Sequence Scanner® was used to view the electropherograms and the Expasy® website was used to compare the sequences.

2.2 Expression

Initially, a small scale culture was processed to determine if any protein was being made by the selected constructs. Recombinant DNA from several clones were each transformed into Rosetta RS2 cells. These host cells provide rare tRNA species which might recognize rare codons in the cloned trmD gene DNA. Each transformation mixture was plated with chloramphenicol and ampicillin for positive selection. Chloramphenicol is added to maintain the second plasmid in the Rosetta strain which encodes the rare tRNA species. Ampicillin selects for the recombinant plasmid containing the trmD gene. The plates were incubated overnight at 37°C. Isolated colonies were selected from plates and each inoculated into LB test tubes. The tubes were then incubated at 37°C for 3-4 hours in a rotary shaker (200 rpm). These tubes were added to 4 ml of LB media and inoculated for 1 hour. Spectrophotometer readings were periodically taken until OD reading of 0.6 (at
600 nm) was achieved. The cells were then induced with 1mM IPTG and shaken in a rotary shaker (200 rpm) overnight at 18°C. Cells were harvested by centrifugation for 10 minutes at 10,000 rpm at 4°C. The cell pellets were suspended in resuspension or lysis buffer (300mM NaCl, 50mM pH 7.5 phosphate buffer, 10 % glycerol and 10mM Imidazole). Cells were disrupted using an Avestin Multiflex® cell disrupter. In all cases three passes through this automated French Press were carried out. Cell debris was then removed by centrifugation of the crude extract for 30 minutes at 15,000 rpm. The soluble supernatant was collected and microcolumn Nickel-NTA columns from Qiagen® were used for purification. Centrifugation and equilibration of extract through these commercial spin columns (6000 rpm for 1 minute) assisted in separating protein from cells. The columns were washed with 300mM NaCl, 50mM pH 7.5 phosphate buffer and 10mM imidazole. Protein was eluted with 300mM Imidazole, 10 mM NaCl and 50mM pH 7.5 phosphate. An SDS (Sodium dodecylsulfate) PAGE gel was done to check for the presence of protein.
Figure 13a: pET-46 Ek/LIC Vector generated by program EZ PLASMID MAP V1.9®.
Figure 13b: Diagram of Ek/LIC strategy (Adapted from Novagen®)
2.3 Protein Purification

Large scale protein expression was next performed. Each liter of culture broth was prepared by adding 25 grams of granular LB-Broth from Fisher to 1 liter of deionized water. After the media was autoclaved, ampicillin was added at a concentration of 50ug/ml and chloramphicol at a concentration of 25μg/ml. These were added because the E.coli BL-21 strain contains the pET-46 vector, which in turn has the Mycobacterium tuberculosis trmD gene and the ampicillin and the Rossetta strain contains the tRNA encoding plasmid selected and maintained in the stain by chloramphenicol resistance. The culture medium was then inoculated with an overnight 30 ml starter culture containing ampicillin and chloramphenicol. Four liters of media were grown to obtain protein. Cells were put in a shaker at 200 rpm at 37°C. Cultures were grown to an A^600^ of 0.6, then induced over night at with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) at 18°C. Cells were harvested by centrifugation at 5,000 rpm and the pellet was resuspended in lysis buffer, which contained 300mM NaCl, 50mM pH 7.5 phosphate buffer and 10mM Imidazole. Cells were then frozen at -80°C for subsequent protein isolation and purification.

The cells were then lysed using the Avestin French Press machine at 20,000 psi. In all cases extracts were passed three times through the cell disruptor. Consequently, the crude cellular lysate was centrifuged for 30 minutes at a speed of 10,000 rpm. The crude extract was then loaded onto a Qiagen® Ni-NTA nickel column. TrmDz protein has a Histag that is therefore bound tightly by the Nickel-NTA agarose beads. Wash buffer was then passed through the column, which contains 300mM NaCl, 50mM pH 7.5 phosphate buffer
and 10mM imidazole. This step was performed to remove any non specific binding protein as judged by removal of all detectable OD from the column. Following this initial wash, TrmD protein was eluted from the column with elution buffer containing a high concentration of imidazole. Elution buffer contains 300mM Imidazole, 10 mM NaCl and 50mM pH 7.5 phosphate. Washing and eluting protocols using gravity flow as outlined by Qiagen® were modified as follows: after extensive washing in buffer A, the column was washed in a buffer with lower salt (50mM phosphate buffer pH 7.5, 50mM NaCl, 250mM imidizol). Fractions were collected and protein concentration was estimated by measuring UV absorbance at 280nm. Peak fractions were pooled and analyzed for purity via SDS PAGE gels. Next the samples were dialyzed overnight in (50mM Tris-HCl pH 7.5, 300 mM NaCl, 5mM 2-mercaptoethanol, .5mM EDTA, and .04% Na-Azide) in the cold room.

2.4 SDS Polyacrylamide Gel Electrophoresis (SDS Page)

The SDS Page gel was prepared following the protocol generated by Laemmli (30). This gel has a 12.5% acrylamide separating gel and a 4.1 % stacking gel. A gel is generated with a 10x10 cm gel rack with a thickness of 0.75mm. The protein samples to be analyzed are prepared by first adding appropriate amounts of 2X SDS loading buffer, which is made up of Tris buffer, SDS, glycerol and the reducing agent beta mercapoethanol, to the protein solution containing about 10-100μg protein. Then the protein was denatured by placing the samples into boiling water for 2-5 minutes. The SDS PAGE running buffer contained Tris-base, glycine and SDS. One liter of 5X stock solution of the protein running buffer contains 15 grams of tris-base, 72 grams of glycine
and 5 grams of SDS. A 1X solution of this mixture is used to perform SDS Polyacrylamide Gel Electrophoresis. Approximately 15μl of sample solution was loaded into each well, then electrophorized at a constant voltage of ~50mV at room temperature. The 2X loading buffer contains bromophenyl blue, which determines the progress of electrophoresis. As soon as this dye reached the bottom of the gel, electrophoresis was stopped. Gels were then stained with coomassie brilliant blue solution, containing 50% H2O, 40% methanol 10% acetic acid and (v/v) 0.05% coomassie brilliant blue R250, ideally it is allowed to sit over night and subsequently destained with a destaining solution composed of 70% water, 20% methanol and 10% acetic acid.

2.5 Native Gel Electrophoresis

"Native" or "non-denaturing" gel electrophoresis was performed in the absence of SDS. In the SDS-PAGE the electrophoresis mobility of proteins depends primarily on their molecular mass, however in the native PAGE gels mobility depends on both the protein's charge and its hydrodynamic size. The resolving gel contained 10% acrylamide and Bis-acrylamide added to achieve a ratio of 1:37 and .4M Tris-HCl pH 8.8. The stacking gel contained 7.5% acrylamide and Bis-acrylamide was added to achieve a ratio of 1:37 and 0.25M Tris-HCl pH 6.8. Polymerization of gel was achieved by the addition of 10ul of Tetramethylethylenediamine (TEMED) and 40ul of 15% ammonium persulfate. Sample buffer contained 100mM Tris-HCl buffer pH=6.8, 200 mM DTT, 20% glycerol and .1% bromophenol blue. The electrophoresis buffer contained 3g of Tris base and 14.4 grams of
glycine in one liter. The final pH was 8.3. Protein stain solution consisted of .05% Coomassie Brilliant Blue R, 50% Methanol, 40% water and 10% Acetic Acid (v/v). Destaining solution contains 50% Methanol, 40% water and 10% Acetic Acid (v/v).

2.6 Methods of Kinetic Assays

Two assays were developed to assess TrmD kinetics as well as inhibition. In the process of performing a kinetic assay, the velocity of the reaction is measured at a series of incrementally increasing substrate concentrations. This permits determination of $K_m$ and $V_{max}$. For these assays, the concentration of enzyme and cofactor were optimized. In the case of TrmD, the substrates are a G36G37 tRNA (tRNA$_{Leu}^1$) and S-adenosyl-methionine (AdoMet). Enzyme activity was determined using a modification of the method developed by Hjalmarsson et al. (31). The assay conditions were optimized to ensure proper function of the enzyme for the measurements conducted. 50 µl of reaction mixture consisted of 50 µM cold SAM, 1.8 µl of $^3$H-SAM (specific activity 85 Ci/mmol), buffer (0.1 M Tris-HCl pH 8.5, 1 mM DTT, 0.1 mM EDTA, 6mM MgCl$_2$, 24 mM NH$_4$Cl, 20 µg of enzyme and variable amounts of $E. coli$ tRNA$_{Leu}^1$ transcripts (Figure 14). Using these conditions as a starting point the parameters were optimized for the $MtB$ TrmD. tRNA synthesis was performed using a combination of methods. The protocol described in Holmes et al. (32) outlined a basis for the procedure. Template preparation involved the growth of pUTL4 in XL1-Blue E. coli cells obtained from Stragene. Plasmid purification was carried out using the Qiagen Plasmid Giga kit® followed by DEAE batch column chromatography. Using these methods tRNA was produced and supplied by M. Palesis.
To ascertain an optimal enzyme concentration for the assay, tRNA and AdoMet concentrations were added in excess and the enzyme concentration varied. Activity was measured in a time course spanning 25 minutes in a 5 minute interval. Once an optimal enzyme concentration which ensured linear methylation over the time course of the assay (20 µg of protein per assay), a second series of experiments were performed in which the concentration of SAM was varied in the presence of excess tRNA concentration to allow determination of $K_m$ and $V_{max}$ for SAM. The concentrations of SAM employed were 45 µM, 65 µM, 150 µM, 155 µM. 5 minute assays were carried out. The values of [tRNA] were 5 µM, 15 µM, 18 µM, 58 µM, 78 µM and 65 µM set through a time course assay of 5 minute intervals between concentrations. The enzyme reaction was allowed to proceed for 4 minutes. Following 4 minutes, the reaction was stopped by adding 1 ml of ice cold (10%) tri-chloro-acetic acid (TCA). After 15 minutes, all of the tRNA is completely precipitated, and the reaction mixture is loaded onto a GF-A Whatman filter paper. Vacuum filtration and washing with TCA was done to remove excess labeled free AdoMet. The filter paper is then dried with 100% ethanol and activity is measured (Figure 14). The filter paper will retain the radioactively labeled tRNA which then can be counted using the Packard Tri-Carb 1500 Liquid Scintillation Analyzer. Conversely, to obtain the $K_m$ and $V_{max}$ for the tRNA excess SAM and the tRNA concentration varied. The time course of the reaction was followed to ensure the rate being observed in these assays was linear and representative of the initial reaction rate. $K_m$ and $V_{max}$ were calculated by using the program Prism® which utilizes non-linear regression analysis.
A preliminary inhibitory assay was also conducted on this enzyme. The same procedure as clone for the kinetic assays was utilized (Figure 14) except that 156 uM of inhibitory compounds were included in the reaction mixture. The two inhibitory compounds used were Congo Red and 4,4'-bis(1-anilinonaphthalene 8-sulfonate) (Bis-ANS) (Figure 16). Both these compounds are hydrophobic and have proven to be inhibitors of the *E. coli* TrmD protein (unpublished, M Palesis).
Figure 14: General Schematic for the kinetic assay reactions performed for varying substrate concentration
2.7 Methods of Crystallography

The most common setup to grow protein crystals is by the hanging drop technique. A few microliters of protein solution are mixed with an about equal amount of reservoir solution containing the precipitants. A drop of this mixture is put on a glass slide which covers the reservoir. As the protein/precipitant mixture in the drop is less concentrated than the reservoir solution (protein solution with the reservoir solution about 1:1), water evaporates from the drop into the reservoir. As a result the concentration of both protein and precipitant in the drop slowly increases, and crystals may form (Figure 16). Simply put, the hanging drop method differs from the sitting drop method in the vertical orientation of the protein solution drop within the system. It is important to mention that

**Figure 15:** Structure of Inhibitors
a.- Congo Red
b.- 4,4'-bis(1-anilinonaphthalene 8-sulfonate) or Bis-ANS
both methods require a closed system, that is, the system must be sealed off from the outside using an airtight container or high-vacuum grease between glass surfaces (Figure 17). Even smaller volumes can be handled in button-like dialysis cells which are often used in x-ray crystallography. A dialysis membrane with a cutoff molecular weight value is used to seal the buttons. However, it is important that the buttons are completely filled and without air bubbles which otherwise can prevent the contact of the solution with the dialysis membrane.

**Figure 16**: Hanging Drop Diagram
A seed provides a template for the assembly of molecules to form a crystal with the same characteristics as the crystal from which it originated. It is important to differentiate the process of crystal growth from nucleation. In general, the degree of supersaturation required for nucleation is higher than that required for crystal growth. Normally, during aggregation, there is an equilibrium between the formation of ordered nuclei (reversible) and the formation of precipitate (irreversible). When crystal seeds are added, the equilibrium can shift towards crystal formation, and avoids the random nature of spontaneous nucleation. There are three commonly used types of seeding: Microseeding which adds very small pieces of crushed protein crystals to the crystallization drop; Macroseeding which adds an intact, already grown crystal to the crystallization drop; Streak seeding which is similar to microseeding but uses a fine hair (often a cat’s whisker or horse hair is best) to pick up small protein crystal fragments.

Mounting of most protein crystals on an x-ray diffractometer is done at cryogenic temperatures, usually around 100K. A small nylon loop is used to pick up a crystal,
immerse it a cryoprotectant solution for a short period of time, and then either plunge it in liquid nitrogen (or sometimes liquid propane), or place it directly on the goniometer head in a stream of cold nitrogen gas.

Crystals of *M. Tuberculosis* TrmDz without AdoMet bound grew in sitting drop trays at 25°C at room temperature. The concentration of the protein was 12.4 mg/ml in 50 mM Tris-HCl pH 7.5, 0.1 M NaCl, 1 mM DTT. Drops were a 2μl:2μl ratio mixture of protein to precipitant. Hampton Research Crystal Screening 1 was used to screen reagents. A total of 48 reagents were included in the kit. The 24 well VDX48 crystal trays with sealant from Hampton Research were utilized for the screens. Siliconized Glass Cover Slides from Hampton Research allowed a droplet to be suspended in a position which provides near optimal conditions for vapor diffusion. The cryoprotectant used was 25% glycerol for each crystal shot.

Initially, attempts to crystallize the *M. Tuberculosis* TrmD protein were done without AdoMet in sitting drop trays at 25°C. The concentration of the protein used was 12.4 mg/ml in 50 mM Tris-HCl pH 7.5, 0.1 M NaCl, 1 mM DTT. Each Drop contained 2μl of precipitant and 2 μl of protein solution. Initially, Hampton Research Crystal Screening kit 1® was used to screen reagents. This kit provided 48 separate precipitant solutions. Hampton Research® (24 well) VDX48 crystal trays with sealant were utilized for the screens. Siliconized glass cover Slides from Hampton Research were employed as well. Most of the following steps were carried out with the help Dr. Martin Safo in the Institute. In all cases promising crystals from selected wells were suspended in 25% glycerol plus the correct selected precipitant.
Diffraction experiments were performed on a Raxis IV (Rigaku Americas Co.) diffractometer under Nitrogen cryo-cooling conditions at -100 K. Crystals were swiftly taken out of the mother liquor in the well using a nylon loop then flash-frozen under a stream of liquid nitrogen on the goniometer head. Indexing of initial frames showed different forms for buffer conditions. The diffractometer was programmed to collect 0.5° oscillation frames from φ angles 0° to 180° at 0.5° intervals (360 frames) and an exposure time of 10 minutes. The crystal-to-detector distance was adjusted to 130mm. Once the data collection was complete, the data was integrated using the d*trek program under the CrystalClear® software package (Rigaku Americas Co). The program entitled CrystalClear collects and integrates the reflection of spots.

Chapter 3

3.1 Results of Cloning

As outlined in the previous section trmD sequences have been cloned and verified by sequence analysis. Using the protocols and stated in the methods section, forward and reverse primers were designed then used with a Finnzyme Phusion High-Fidelity PCR Master Mix® to PCR trmD sequences from genomic DNA. Once PCR was performed, an agarose gel confirmed the presence of amplified trmD DNA (Figure 1). In figure 18 700 bp bands are seen as judged from a 100 bp DNA ladder which corresponds nicely with the calculated size of the amplified gene (MW 693). Consequently, the DNA was extracted and cloned into a pET-46 EK/LIC vector. Once cloned host cells were transformed as outlined above and ten colonies selected for analysis. DNA extracted from
each of these clones was analysed by analytical PCR which should determine if \textit{trmD}
inserts were present. Inserts were found in colony numbers 2, 6, 8, 10 (Figure 19).

DNA was prepared from clones 6 and 8 which had been shown to contain \textit{trmD}
inserts as judged by PCR amplification. Plasmid DNA from these two clonal isolates was
sent to the DNA Core laboratory for sequencing. The results from the sequencing lab
reported that all sequences were in-frame and contained no mutations. The sequences for
samples 6 and 8 were found in the first frame (Figure 20). These samples were used for
protein expression and named TrmDz1 and TrmDz2, respectively. Utilizing the program
Sequence Scanner v1.0® an actual sequence readout from the 3700 sequencer is shown
(Figure 21).
Figure 18: Agarose gel of PCR product: leftmost lane; 100 base pair (bp) ladder, Sample and Sample 2. Sample 1 and 2 contained 20ul of PCR product. The red arrow indicates where the 700 base pair standard runs.
**Figure 19:** Agarose Gel electrophoresis of PCR amplified *trmD* gene inserts.

Left to Right: Molecular Weight standards Ladder (L), 2, 6, 8, 10 and positive control contained inserts.
Mycobacterium Tuberculosis trmD CDC1551
Amino Acid Sequence

A. VRIDIVTFPACLDRQGKAIESGLVDLNVHDLRRWTHDVHHSVDDAPYGGGGPMetVMetKAPVWGEALDEICSSETLLIVPTPAGVLFTQATAQRWTTESLVFA CRTYGIDQRVVQDAARRMetRVEEVSGDYVLPPGESAAAVVMetVEAVRLLAGVGLNPASHQQDSSHSTGLDLLEGPSYTRPASWRGLDVPEVLLS
GDHARIAAWRREVSLQRTERRPDLSHPDStop

Sample 8 Forward Primer:
B. 5'3' Frame

PCACLDRQGKAIESGLVDLNVHDLRRWTHDVHHSVDDAPYGGGGPMetVMetKAPVWGEALDEICSSETLLIVPTPAGVLFTQATAQRWTTESLVFA CRTYGIDQRVVQDAARRMetRVEEVSGDYVLPPGESAAAVVMetVEAVRLLAGVGLNPASHQQDSSHSTGLDLLEGPSYTRPASWRGLDVPEVLLS
GDHARIAAWRREVSLQRTERRPDLSHPDStop

Figure 20: Sequencing Results a.-putative amino acid sequence for annotated gene, (gene ID 925304/ accession number is NP_337486.1) b.- Amino acid sequence derived from the DNA sequence of clone 8. Shown in red is the deduced amino acid sequence from one of the reading frames of this sequence.

Figure 21: Screenshot of Sample 8 DNA sequence using Sequence Scanner Program®
3.2 Expression and Purification

Clones 6 and 8 which have been designated TrmDz1 and TmDz2 were expressed at a pilot scale to determine if protein of the correct size had been made. As can be seen in Figure 22, protein of the correct size can be seen for both clones 6 and 8. Since this is a SDS gel then this should reflect the monomer molecular weight of the subunits for this TrmD protein. A monomer form with a molecular weight of approximately 25 kDa is seen as judged by molecular weight markers incorporated in the gel. The actual size of the TrmD protein should be larger than 25 kDa since there is now a His-tag sequence present at the amino terminus. The gel mobility reflects these added residues.

Since the protein gels displays bands of the correct size from both clones, a larger scale expression of TrmDz2 was initiated. A two liter volume of cells was induced with 1 mM IPTG once the OD at 600 nm reached 0.8. The liters were incubated in the shaker at 18°C at a speed of 200 rpm overnight.
Figure 22: Protein Gel of \textit{MtB} TrmD Sample 1 is TrmDz1 and Sample 2 is TrmDz2. Order of wells: protein after incubation trmDz1 (Cell Extract 1), protein after incubation trmDz2 (Cell Extract 2), protein centrifuge trmDz1 (Protein Centrifuge 1), protein centrifuged trmDz2 (Protein Centrifuged 2), cell lysate z1 (Cell Lysate 1), cell lysate z2 (Cell Lysate 2), protein ladder (Protein Ladder), flow through z1 (Flow Through 1), flow through z2 (Flow Through 2), wash z1 (W1), wash z2 (W2), elution z1 (EL1), elution z2 (EL2). Arrow points to 25kDa on the Protein Ladder.
3.3 Protein Purification

As outlined above, the cultures were centrifuged and the cells lysed using the French Press. Crude extracts were clarified by centrifugation, then purified using Ni-NTA columns as outlined above. Figure 24 shows stained proteins in a 12% SDS Acrylamide gel. In this case, BL21(DE3) host cells were used and excellent expression was achieved. Approximately 9 ml of purified protein was obtained containing 12 mg/ml of TrmD protein. As can be seen in all appropriate wells, a single band of 25 kDa was observed on the SDS-PAGE gel (Figure 23). In these cases, very high levels of TrmD protein were added (25 μg per well) to show the degree of purity of the protein.
Figure 23: SDS protein gel of purified Mycobacterial TrmD protein. Lanes from left to right: Protein Ladder (PL), Cell Lysate (CL1), Protein (P1), and Protein after Dialysis (D1). Sample CL2, P2 and D2 are repeats of the left side. In the case of purified TrmD samples approximately 25 μg of TrmD protein were loaded.
Figure 24: Native Gel with purified *MtB* TrmD protein concentrations in designate lanes:

Sample 1 (5ug), Sample 2 (4ug), Sample 3 (3ug) and Sample 4 (1 ug). The first well is the Protein ladder. The bands all line up to the 50 kDa line of the ladder.
It has been shown that all TrmD protein orthologues so far studied are homodimers (22). The putative subunit molecular weight of this enzyme was consistent with that of other TrmD proteins. In order to determine whether or not this enzyme is dimeric like other TrmD proteins, samples of Mycobacterial trmD protein was electrophoresed through Polyacrylamide gels without SDS or other denaturants. As shown in figure 24, samples run show a molecular weight of approximately 50,000 daltons which is consistent with a dimeric form of the enzyme.

3.4 Results of Kinetic Assays

The $V_{\text{max}}$ for SAM was 0.02818 nM product/min/ug of protein and the $K_m$ was 26 $\mu$M (Figure 26). The methylation kinetics of the tRNA displayed a $V_{\text{max}}$ of 0.001024 nM product/ min/ $\mu$g protein and a $K_m$ of 41.80 $\mu$M (Figure 27). Extensive kinetic analyses have been performed on the $E. coli$ TrmD and these results can be compared to that of the MtB TrmD. Previous data has shown that the $E. coli$ TrmD has a $K_m$ value of 5 $\mu$M for tRNA methylation and 8 $\mu$M for SAM methylation (32). A turnover number was calculated and compared with that of MtB TrmD (Table 2). The turnover number was 12.6 molecules of product/molecule of enzyme/minute for $E. Coli$ TrmD and 0.14 molecules of product/molecule of enzyme/minute for the MtB TrmD. This result reveals that the $E. coli$ enzyme is much more active than the MtB enzyme. In the inhibition studies, both compounds at 156 $\mu$M proved to significantly diminish the activity of MtB enzyme. Activity drastically dropped from a 13000 CPM$^3$H to a mere 449 CPM$^3$H for the Bis-ANS and 330 CPM$^3$H for the Congo Red assays. Thus, these compounds are potent inhibitors for this enzyme.
Figure 25: Kinetic Assay Data of enzyme, TrmDz, and varying concentration of substrate, SAM.
**Figure 26:** Kinetic Assay Data of enzyme, TrmDz, and varying concentration of substrate, tRNA.

**Turnover**

(molecules of product/molecule of enzyme/minute)

<table>
<thead>
<tr>
<th></th>
<th>E. coli</th>
<th>M. tuberculosis</th>
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<tr>
<td>Value</td>
<td>12.6</td>
<td>0.14</td>
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**Table 2:** Table comparing *E. coli* and *MtB* TrmD turnovers
Figure 27: Graph representing activity of enzyme in the presence of inhibitors Bis-ANS and Congo Red.

3.3 Results of Crystallization

As previously mentioned, all crystals were grown at room temperature. There were several conditions where crystals were observed. The first was 30% Polyethylene Glycol (PEG) 4000, 0.1M Tris-HCl, 0.2M Lithium Sulfate (Figure 28 a,b). The protein concentration was 12.4 mg/ml. Microcrystal showers were observed under this condition after one week. Optimization of this condition was performed. Larger, better crystals were never produced.

The second condition which produced crystals was 1.0M Ammonium Phosphate, 0.1M Na Citrate pH 5.6 (Figure 28 c,d). One single crystal formed in this well after three weeks. A resolution of 4.9 Å was observed for this crystal formation. The spot intensity
dropped after 7.8 Å. The crystal shape was hexagonal. The unit cell was a=b=119.765 Å, c=172.29. After optimization of conditions, larger crystals were observed in 0.8M Ammonium Phosphate 0.1 M Na Citrate pH= 5.6 and 0.9M Ammonium Phosphate 0.1M Na Citrate pH=5.6 (Figure 28 f,g). However, after diffraction it was found that the resolution was poor for this crystal formation, 3.5 Å (Table 3).

Two other conditions found were 20% Polyethylene Glycol (PEG) 3350 0.2M di-Ammonium Citrate and 20% Polyethylene Glycol (PEG) 3350 0.2 M Calcium Acetate (Figure 28h). However, even after optimization larger crystals were not produced.

The best condition for growth was 1.2M Sodium Formate, 0.1M Na Acetate pH 4.6 (Figure 28e) and many crystals were grown under these conditions. Crystals formed in approximately one week period. Multiple single crystal formations were seen in each well. These crystal formations were shot many times. The first had a resolutions of 4.5 Å. The initial shape was hexagonal and the hypothesized space group was P622. However, several different shapes were seen in each well. The next attempt had a resolution of 4.0 Å and was triclinic. The unit cell for all these crystals shot were a=b=119.765 Å, c= 172.29. The water content was 65%. Ultimately, the crystal formations yield a space group of P321. The unit cells were a=b=207.88, c=172.1269. These crystals yielded a resolution of 2.9 Å. However, using Matthew’s coefficient, 12 residues were found per asymmetric unit. A total of 300 frames were collected for the last attempt. Using Crystal Clear® the data was integrated and an R factor of 12.5% was observed. The diffraction pattern is shown in Figure 29.
Figure 28: Picture of crystal formations

a, b- 30% PEG 4000, .1M Tris HCl, .2M Lithium Sulfate
c, d- 1.0M Ammonium Phosphate, .1M Na Citrate pH 5.6
e- 1.2M Sodium Formate, 0.1M Na Acetate pH 4.6
f- .8M Ammonium Phosphate .1 M Na Citrate pH= 5.6
g- .9M Ammonium Phosphate .1M Na Citrate pH=5.6
h- 20% PEG 3350 .2 M Calcium Acetate
Table 3: Summary of crystal diffraction data for *MtB* TrmDz

<table>
<thead>
<tr>
<th>Crystal Conditions</th>
<th>Space Group</th>
<th>Unit Cell</th>
<th>Shape</th>
<th>Res. (Å)</th>
<th>R_{merge}(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2 M Sodium Formate 0.1 M Sodium Acetate, pH=4.6</td>
<td>P321</td>
<td>a=b=207.88 Å, c=172.1259 Å</td>
<td>tri-clinic</td>
<td>2.9 Å</td>
<td>12.5</td>
</tr>
<tr>
<td>1.0 M Ammonium Phosphate 0.1 M Na Citrate, pH=5.6</td>
<td>P622</td>
<td>a=b=119.765 Å, c=172.29 Å</td>
<td>Hexag</td>
<td>4.9 Å</td>
<td>N/A</td>
</tr>
<tr>
<td>0.8 M Ammonium Phosphate 0.1 M Sodium Citrate, pH=5.6</td>
<td>P622</td>
<td>a=b=207.88 Å, c=172.1289 Å</td>
<td>Hexag</td>
<td>3.5 Å</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Figure 29: X-Ray Diffraction Pattern of 1.2M Sodium Formate, 0.1M Na Acetate pH 4.6
Chapter 4

4.1 Discussion

TrmD functions catalytically as a ternary complex comprised of the trmD enzyme, a tRNA substrate and the AdoMet cofactor. In the case of the E. Coli enzyme, the binding of tRNA and AdoMet are not co-dependent, and the enzyme exhibits random sequential kinetics. Studies in the Holmes lab suggest that only one AdoMet is bound not two in the S. pneumonia TrmD protein (unpublished, M Palesis).

The kinetic assays of methylation done here show that the activity of methylation occurs much slower in MtB than in the E. coli TrmD. The turnover number relates the amount of product methylated per a unit of time, per molecule of enzyme. MtB is an aerobic bacterium that divides every 16 to 20 hours, an extremely slow rate compared with many other bacteria, which usually divide in less than an hour (5). Thus, the rate at which methylation of tRNA occurs is important for maintaining tRNA methylation which is in turn required for error free protein synthesis. The data presented here only provide an initial study of the kinetics of the TrmD from TB. More extensive studies need to be performed to properly compare rate constants of this enzyme to other known trmD organisms.

It is clear that the TrmD protein is an important target for antimicrobial agents because it has been shown to be essential in a number of eubacterial forms and is a protein unique to eubacteria. All cellular forms contain enzymes that catalyze the AdoMet dependent methylation of tRNA molecules at G37, a modification that prevents frame shifting (22). Whereas most missense errors do not eliminate the activity and stability of
proteins, frameshift errors which are the result of an inactive \textit{trmD} gene are detrimental for cellular viability (27). It is likely that maintenance of proper reading frames would be a strongly conserved property of all cellular forms, and a prerequisite for the emergence of the three domains of life, namely Archaea, Bacteria and Eukaryotes (7). Thus, this enzyme is part of the minimal set of gene products required for the evolution of all cellular forms (25). Mutational analysis performed in two bacterial species confirmed that a defect in the protein will cause the bacterium to grow more slowly (\textit{E. coli}) or be lethal to the organism (\textit{S. pneumonia}) (21). Deficiency of TrmD appears to have a global effect on cell physiology. Bjork et. al. observed changes in carbon source metabolism and resistance or sensitivity to amino acid analogs (26). In particular, lack of enzyme affects metabolism of thiamine and pantothenate (27, 28).

This project included cloning, expression, characterization and crystallization of \textit{Mycobacterium tuberculosis} \textit{trmD}. In the characterization experiments, kinetic assays and some inhibitor work were performed to compare this enzyme with that of previous data for other \textit{trmD} orthologs determined by Holmes’s laboratory. We have not yet shown that TrmD is an essential protein in the Mycobacteria, and this should be done if we are to move ahead in the development of a new antimicrobial agent active against this organism. Other issues that are important to deal with will be permeability problems of any drug developed since the cell wall of Mycobacteria is very hydrophobic and relatively impermeable. Other barriers will involve defeating pump mechanisms that might exist.

The enzyme was relatively easy to crystallize, and crystals were seen within one week under certain conditions. Sodium formate crystals formed relatively quickly and
were the largest seen. Considerable time was spent optimizing conditions with this precipitant. It was observed that increasing protein concentration more than 22 mg/ml produced showers of micro-crystals. Decreasing the protein concentration less than 12 mg/ml yielded no crystals. It was determined that a range of 12 to 20 mg/ml was optimal for obtaining large crystals under these conditions. Other techniques employed (Dialysis buttons, macro and micro-seeding) did not yield larger crystals. Magnesium was explored as a factor for obtaining better crystals because it is required for optimal catalytic activity for this enzyme. Interestingly, magnesium actually prevented crystal formation. In previous TrmD crystal structures no magnesium was ever seen. Perhaps its role is in post tRNA interaction events such as base flipping in tRNA. Most previous TrmD proteins were crystallized with AdoMet bound to enzyme, but we found that the *M. tuberculosis* TrmD did not crystallize when AdoMet was added to protein samples at least under the methods used here. Attempts in adding AdoMet directly in protein mixture or adding AdoMet to protein and buffer mixture did not yield any crystals. Further work needs to be performed on producing crystals with ligand bound to enzyme. It will be important to obtain these co-crystals if inhibitors which bind this region are to be developed in order to gain insight into overall enzyme mechanisms.

Other conditions for optimizing crystallization were explored to include temperature and pH. It appears that pH’s near neutrality is important for the crystallization of this protein. A reduced temperature of 10 °C did not improve crystallization. If fact, no crystals were formed after a month of incubation under this condition.
Other precipitants were observed to crystallize the protein in the initial Hampton screen. Crystals were seen under the following conditions: 1.2M Sodium Formate, 0.1M Sodium Acetate pH= 4.6 (Figure 28e); 30% Polyethylene Glycol (PEG) 4000 0.1M Tris HCl pH=8.5 .2M Lithium Sulfate (Figure 28 a,b); 1.0 M Ammonium Phosphate 0.1 M Sodium Citrate pH=5.6 (Figure 28 c,d), 25% PEG 6000 .1M HEPES pH= 7.5; 20% PEG 3350 0.2M Calcium Acetate (Figure 28h); 20% PEG 3350 0.2M di-Ammonium Citrate; 40% PEG 200 0.1M MES pH=6.5 and 20% PEG 3350 0.2M di-Ammonium tartrate; 0.8M Ammonium Phosphate, .1M Sodium Citrate pH=5.6 (Figure 28f); and .9M Ammonium Phosphate, 0.1M Sodium Citrate pH=5.6 (Figure 28g). The following crystals were selected for subsequent X-ray analysis: 1.2 M Sodium Formate, 0.1M Na Acetate pH=4.6; 1.0M Ammonium Phosphate, 0.1M Na Citrate pH=5.6; 20% Polyethylene Glycol (PEG) 3350 0.2M Calcium Acetate.

In the 1.0M Ammonium Phosphate 0.1M Na Citrate pH 5.6 (Figure 28c) buffer one single crystal formed after three weeks. The resolution was 4.9 Å for this crystal form. The spot intensity dropped after 7.8 Å and the shape was hexagonal. The unit cell dimensions were a=b=119.765 Å, c= 172.29. After repeated optimization attempts larger crystals were formed, however the larger crystals did not diffract.

In the 1.2 M Sodium Formate .1 M Sodium Acetate pH=4.6 the unit cells were a=b=207.88, c=172.1269. Several crystals were analysed, and the final attempt with this condition yeilded a resolution of 2.9 Å which was the best resolution seen in these studies. The Programs Phoenix® and CCP4® were used to integrate the data. Using Matthew’s coefficient, 12 residues were found per asymmetric unit and the solvent content was about
Matthew’s calculation estimates of the number of molecules per unit cell (Z). Because of this, the structure proved to be very difficult to solve. This is part of the symmetric object from which the whole is built up by repeats. During the last effort of crystallography another protein condition yielded large crystals, 20% PEG 3350 0.2M di-Ammonium tartrate. The unit cell was again a=b=207.88, c=172.1269. This is similar to data for the sodium formate crystals.

After obtaining similar results for two different buffer conditions it can be inferred that the enzyme itself does not pack properly. The number of molecules in the asymmetric unit helps define the crystal packing as non-Centro symmetric. This is a space group which lacks an inversion center. Center of Symmetry (or Center of Inversion) is a point through which an inversion operation is performed, converting an object into its mirror image. As a result of the high number of molecules per asymmetric unit the cell packing is affected. A crystal is a three-dimensional periodic arrangement of molecules. When the material precipitates from a solution, its molecules attempt to reach the lowest free energy state. The search for a minimum free energy, and as a consequence the regular packing of molecules in a crystal lattice, often leads to a symmetric relationship between molecules. Due to the number of residues per asymmetric unit “loose” binding occurs between the units. When the crystal does not have tight binding between units, packing issues arise and resolution of the crystal is also affected.

Another issue to consider is the cryoprotectant Glycerol was the only cryoprotectant used for crystals obtained. Initially, 20% glycerol was used to check diffraction of the sodium formate crystals. However, it was noticed that as the amount of
glycerol decreased, better resolution was observed. When collecting data for the last crystal, 10% glycerol was used in 20% PEG 3350 .2M Calcium Acetate. Other options to consider in the future involve trying different cryoprotectant conditions. A CryoPro® kit, which includes 36 conditions available, produced by Hampton Research Company, was considered for usage in the future.

An alternative we contemplated for future work involved protein modification. One modification would be to remove the 6x His-tag from the protein. There are a number of kits and protocols to assist in removal of the His-tag. Another mutation would involve changing the amino acid sequence of the protein.

Why then, does the mycobacterial trmD enzyme pose such problems in packing? One major difference exists from most other trmD genes. The TrmD protein is substantially shorter than the E. coli and most other TrmD proteins. It has 231 amino acid residues whereas E. coli has 255 residues. Therefore the final alpha helix seen with the E. coli enzyme is absent from the Mycobacterial TrmD protein. If for example the C terminus is a key stable contact point in crystals then other contact points may occur in the Mycobacterial TrmD creating packing problems. For example, now the mobile his tag sequences might create problems with the resulting heterogeneous packing that might ensue. If this is true it will be important to remove the His tag sequence by proteolysis and again attempt crystallization.

The pdb files of the following trmD enzymes were all viewed and compared: Streptococcus pneumonia, Bartonella henselae, and Eschericha coli. After close observation of the residues within the asymmetrical unit, it was found that the missing
amino acids from the *Mycobacterium tuberculosis* organism greatly impacted the stability of the structure. The residues at the C-terminus played an important role in interacting between the residues of different lattices. Thus, packing of the crystal would be impacted if those important interactions did not occur. This may account for the problems of low resolution, diffraction and large numbers of residues per asymmetric unit.

One possible approach for improving crystal packing could involve the removal of the His-tag which might introduce heterogenous packing. Alternatively, since many other trmDs have been crystallized with the His-tag it seems reasonable the C-terminus contact points were important. Therefore, if a terminal alpha helix were added to the TB protein then better packing might be achieved.

In summary, we have initiated an extensive effort to obtain the crystal structure of an important drug target from a very important pathogen. Considerable progress has been made which point the way towards a successful solution of the structure.
Literature Cited
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Zohal Hamidi was born on June 9, 1984, in Kabul, Afghanistan. She immigrated to the U.S. in 1990 and became a citizen in 2003. She graduated from West Springfield High School, Springfield, Virginia in 2002. She received her Bachelor of Science degree in Nutrition and minor in Chemistry from Virginia Polytechnic Institute & State University, Blacksburg, Virginia in 2006 and subsequently worked for a research firm in Washington D.C for a period of one year. She later enrolled into the Post- Baccalaureate program at Virginia Commonwealth University in 2007, transitioning into the Master’s Program in the department of Physiology in 2008.