Characterization of bacterial peptidyl-tRNA hydrolase 1 from different phylogenetic clades

Daniel Joseph Scott Strange

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CHARACTERIZATION OF BACTERIAL PEPTIDYL-tRNA HYDROLASE 1
FROM DIFFERENT PHYLOGENETIC CLADES

by

DANIEL JOSEPH SCOTT STRANGE

A DISSERTATION

Submitted in partial fulfillment of the requirements
For the degree of Doctor of Philosophy
in
The Biotechnology Science & Engineering Program
to
The School of Graduate Studies
of
The University of Alabama in Huntsville
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Submitted by Daniel Joseph Scott Strange in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biotechnology Science and Engineering and accepted on behalf of the Faculty of the School of Graduate Studies by the dissertation committee.

We, the undersigned members of the Graduate Faculty of The University of Alabama in Huntsville, certify that we have advised and/or supervised the candidate on the work described in this dissertation. We further certify that we have reviewed the dissertation manuscript and approve it in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biotechnology Science and Engineering.
ABSTRACT
The School of Graduate Studies
The University of Alabama in Huntsville

Degree ____Doctor of Philosophy____ College/Dept. Biotechnology Science and Engineering

Name of Candidate Daniel J. Scott Strange

Title Characterization of bacterial peptidyl-tRNA hydrolase 1 from different phylogenetic clades

Antibiotic resistant bacterial infections have become increasingly difficult to treat resulting in approximately 23,000 deaths per year in the U.S. alone. Some bacterial infections, like *P. aeruginosa* and *M. tuberculosis*, have become pan- and multi-drug resistance further limiting treatment options. This has placed an increased demand on the medical community to find novel antibiotics and novel antibiotic drug targets that can be used for narrow spectrum inhibition of bacteria. One such target is bacterial peptidyl-tRNA hydrolase 1 (Pth1). Pth1 recycles tRNA by hydrolyzing the bond between peptidyl-tRNA and the growing polypeptide chain during abortive translation, thus, allowing free tRNA to be recycled for future protein biosynthesis. Past studies have shown Pth1 to be essential to bacterial, but not eukaryotic, life. Where bacteria possess a single copy of Pth1 in their genome, eukaryotes have a multicomponent tRNA recycling system that is predicted to compensate for loss of Pth1 function. Likewise, several previous studies have provided valuable insight into Pth1 structure and dynamics. However, these studies were limited to single bacterial Pth1 homologs. In contrast, this study used multiple homologs across phylogenetic space to characterize the in vitro biochemical properties, kinetic properties, and inhibition profiles from different phylogenetic clades. This study established three distinct phylogenetic clades with their own unique characteristics. Importantly, this study also found that both narrow spectrum activity and small molecule inhibition was possible across phylogenetic space. Thus, this data further established the attractiveness of bacterial Pth1 as a novel drug target.
ACKNOWLEDGEMENTS

The work described in this dissertation would not been possible without the assistance of several people who deserve special mention. First, I would like to thank Dr. Robert McFeeters for his suggestion of the research and for his patient guidance throughout all the stages of the work. Second, the other members of my committee have been very helpful with guidance, comments, and suggestions throughout my time here at The University of Alabama in Huntsville. Third, I would like to thank my lab mates in the McFeeters research group for all the help and amazing memories throughout the years.

I would like to thank my parents for their constant support and encouragement throughout life and graduate school. Likewise, I would like to thank my family and friends for the constant encouragement and understanding throughout the course of this work. I’d specifically like to acknowledge my friends from Judson who have always been there.
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<td>tRNA</td>
<td>Transfer RNA</td>
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<tr>
<td>Pth</td>
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<tr>
<td>NPPE</td>
<td>Natural product plant extract</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
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<tr>
<td>SDM</td>
<td>Site-directed mutagenesis</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>GDP</td>
<td>Guanine disphosphate</td>
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<tr>
<td>IC(_{50})</td>
<td>Inhibitory concentration at 50%</td>
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<td>MIC</td>
<td>Minimum inhibitory concentration</td>
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DEDICATION

To Dan Strange, Brenda Strange, Jennifer Ingram, Karrington Jude Oliver and Cira Nerino-Davis.
CHAPTER 1

INTRODUCTION

1.1 Current treatments ineffective against antibiotic resistant infections

Antibiotic resistances have emerged to become a major obstacle to treating bacterial infections. Current treatments have started to become ineffective leading to septicemia and death. Diseases, like Methicillin-resistant staphylococcal infections, remain a concern with a minimal number of antibiotics like vancomycin or daptomycin available. However, several bacteria, like *P. aeruginosa*, have been shown to be naturally pandrug- (PDR) or extensively drug resistant (XDR, resistant to all but two or fewer antibiotics) (Walker, 2019). Elimination of infections associated with multidrug (MDR), XDR and PDR proved difficult to treat (Magiorakos, 2012). Recently, XDR *M. tuberculosis*, the MDR *Enterobacteriaceae* species *S. typhi* and *E. coli*, and PDR *P. aeruginosa* were named four of the “scariest superbugs” (McCaughey, 2018). The CDC estimated over 2 million new antibiotic resistant bacterial infections occur yearly resulting in 37,000 deaths in the U.S. alone (CDC, 2015). The 20-year stagnation in discovering new classes of antibiotics or novel drug targets have placed an extra impetus on addressing this growing concern.
1.2 Pth1 is a novel drug target

One emerging new target is bacterial peptidyl-tRNA hydrolase (Pth1). Pth1 is a 21-25 kDa enzyme that hydrolyzes the ester bond between peptidyl-tRNA and N-acetylated aminoacyl-tRNA and polypeptide chain, and is responsible for recycling tRNA following abortive translation (Goodall, 2004). Pth1 knockout studies found this to be a vital enzyme in bacteria, but not eukaryotes (Menez, 2002; Rosas-Sandoval, 2002). Knockout studies in yeast noticed the non-essentiality in both haploid and diploid cell lines (Das, 2006). Bacteria have only one copy of Pth1; whereas, eukaryotes have multiple Pth1-containing domains, Pth1, and a structurally and mechanistically different Pth2 (Menez, 2002). Likewise, bacterial Pth1s had much lower amino acid consensus with Homo sapiens Pth1 than seen between different bacterial homologs. Therefore, it may be possible to selectively inhibit bacterial Pth1s, further lessening impacts of eukaryotic cells. Given the essential nature of Pth1 in bacteria and the multicomponent Pth system in eukaryotes, inhibiting bacterial Pth1 is a viable option for antibiotic development.

1.2.1 Pth1 essential in bacteria

Pth1 was determined to be vital to bacterial life. Loss of Pth1 resulted in tRNA starvation and cell death (Kabra, 2017). During normal protein biosynthesis, mRNA bind to the 3’ of the 16S rRNA at the Shine-Dalgarno sequence. Initiation factor 3 (IF-3) bound to the aminoacyl, or the A-site of the ribosome, recognizes and binds the initiation codon following the Shine-Dalgarno sequence. This recruits initiation factor 2 (IF-2)
complexed with tRNA^{fMet} that complements the AUG codon of the initiation codon.

GDP hydrolysis catalyzes the assembly of the large and small ribosomal subunits, thus expelling IF-3 and IF-2. GTP hydrolysis of elongation factors (EF) Tu-GTP complex allows aminoacyl-tRNA to bind to the A site of the ribosome. EF-Ts facilitates the ribosomal release of EF-Tu-GDP complex and recycles EF-Tu by converting GDP to GTP. Following this, a peptidyl transferase moves the growing nascent strand to the tRNA occupying the a site. EF-G:GTP hydrolysis translocates the tRNA in the A-site to the P-site and moves down one codon on the mRNA in the 3’ direction. The free tRNA having already transferred its amino acid leaves the ribosome through the exit or E-site. This continues in a GTP-hydrolysis dependent fashion until a termination codon is reached. Recognition of a termination codon, UGA, UAA, or UAG, activates release factor 3 to release the polypeptide chain from the peptidyl-tRNA, or the tRNA occupying the P-site. The ribosome will then disassociate in a GTP-hydrolysis dependent fashion. (Clancy and Brown, 2008).

During abortive translation, translation stalls. If this cannot be rescued, the ribosome will be disassembled, ejecting peptidyl-tRNA, or the tRNA in the P-site still attached to the nascent polypeptide chain, into the cytosol of the cell. Peptidyl-tRNA must be cleaved from the bound polypeptides to be recycled for future protein biosynthesis. In bacteria, Pth1 is the enzyme responsible for hydrolyzing the nascent polypeptide chain from the peptidyl-tRNA in the cytosol. Without this tRNA recycling mechanism, bacteria will die due to tRNA starvation, with tRNA tied up and unable to be used in further protein biosynthesis (Das, 2006).
Figure 1.1 Pth1 prevents tRNA starvation: During abortive translation, the ribosome stalls, disassociates and releases peptidyl-tRNA into the cytosol. Loss of Pth1 function results in tRNA starvation and eventual cell death.

1.2.2 Inhibiting protein biosynthesis is a proven strategy

Historically, disruption of protein biosynthesis has been demonstrated to be a proven strategy for antibiotics. Several currently available antibiotics have targeted the ribosome. These antibiotics achieved bactericidal or bacteriostatic effects through several mechanisms such as: inhibiting tRNA from entering the A site (tetracyclines), blocking the peptidyl transferase center (macrolides) (Wilson, 2014). Targeting the ribosome also has a stoichiometric disadvantage compared to Pth1. It has been determined there are 1,300 Pth1/cell compared to 10,000 ribosomes in the bacterial cell (Cruz-Vera, 2000). Therefore, targeting Pth1 also has a stoichiometric advantage compared to ribosome. Overall, Pth1 inhibition could be used by itself as an avenue for
antibiotic development but also shows considerable promise for use synergistically with known antibiotics that target the ribosome.

1.2.3 Different Pth enzyme families

Based on differences in structure, mechanism and amino acid sequence two families of Pth enzymes were found: the cytosolic Pth1 and mitochondrial Pth2 families of enzymes. X-ray structures of Pth1 and Pth2 were demonstrably different with Pth1 retaining the monomeric α/β hydrolase folds. The *H. sapiens* Pth2 structure revealed a very different four-stranded antiparallel β-sheet core with two α-helices on either end that was dimeric in solution (De Pereda, 2004). Mechanistically, Pth1 enzymes differed from Pth2 in enzymatic reaction and product. Pth1 was determined to cleave the ester bond between the 3’ OH of the terminal tRNA adenosine and the polypeptide chain, leaving behind free tRNA and a polypeptide chain. The catalytic triad of Asn10, His20, and Asp93 as noted in Figure 1.1A differed from *H. sapiens* Pth2 (Schmitt, 1997). Prior to discovery of Pth2, a phosphodiesterase was shown to cleave the adenosine from the CCA acceptor stem of peptidyl-tRNA in rabbit reticulocytes. This enzymatic reaction recognizes and cleaves tRNA^{fMet} similar to that of *S. sulfataricus* Pth2, the first Pth2 discovered (Fromant, 2003). This enzymatic reaction hydrolyzes the bond between the 3’-adenosine and 3’-cytosine in the 3’-CCA acceptor stem and created CC-tRNA and AMP-polypeptide chain reaction products (De Pereda, 2004; Rosas-Sandoval, 2002). The catalytic triad for *H. sapiens* Pth2 was determined to be Lys 81, Asp93, and Ser 155
shown in Figure 1.1B (De Pereda, 2004). As such, loss of Pth1 function in eukaryotes could be compensated for via structurally and mechanistically different Pth orthologs.

Figure 1.2 Differences in Pth structure and catalytic residues: A.: *E. coli* Pth1 (PDB: 2PTH), shown in blue, has a α/β hydrolase structure of six α helices surrounding a mixed β sheet core with the catalytic triad of Asn 10, His 20, and Asp93. B.: *H. sapiens* Pth1 (PDB: 1Q7S), shown in grey, has an α/β fold with two helices on either end surrounding four antiparallel β sheets. The catalytic triad of Lys 88, Asp 145, Ser 155 was found in *H. sapiens* Pth2.

1.2.4 Elevated production of most tRNAs cannot compensate for loss of Pth1 function

Bacterial tRNA production and maturation are energetically costly, but required to ensure proper translation (Toh, 2001; Altman, 1975). Multistep maturation, post-transcriptional modifications of tRNA nucleosides, and methylation of nucleotides throughout the tRNA must be completed prior to protein biosynthesis for tRNA to be translationally competent (Roh, 2009). Therefore, the bacterial tRNA recycling system is requisite on Pth1 function. Mature tRNAs are routinely recycled for future protein
biosynthesis during normal translation or via Pth1 activity in the case of abortive translation to avoid tRNA starvation. Lengthy and energetically expensive tRNA syntheses would be required to compensate for loss of Pth1 function during abortive translation.

\[ \text{tRNA}^{\text{Lys}} \] has been noted to help maintain low levels of Pth1 in C600 E. coli cells possessing the Pth(TS) mutant (Cruz-Vera, 2000). As such, tRNA\(^{\text{Lys}}\) production can compensate for loss of Pth1 function. However, deletion of the Pth gene in E. coli determined tRNA\(^{\text{Lys}}\) with other rescue mechanisms, such as tmRNA and RelA, was not enough to prevent cell death (Singh and Varshney, 2004). Therefore, loss of Pth1 has been shown to cause cell death suggesting that synthesis of new tRNAs alone was not be able to prevent tRNA starvation and cell death (Cruz-Vera, 2000). This further demonstrates the essentiality of Pth1 in bacteria but not in eukaryotes. Eukaryotes can compensate for the loss of Pth1 and energetic cost of syntheses and maturation of new tRNAs with Pth1 orthologs and multiple Pth1-containing domains.

1.3 Pth1 structure and dynamics

Pth1’s structure and functionality remain conserved despite variability in their primary sequences between bacterial and eukaryotic homologs. Several X-ray crystallography structures have been deposited in the protein database (PDB). Much attention has been paid to *E. coli* Pth1 (EcPth1) as this has been the most studied structurally. However, several other homologs have been studied from such species as *V. cholerae*, mycobacterial species *M. tuberculosis* and *M. smegmatis*, and *P. aeruginosa*. 
Most of the structures have been elucidated from gram-negative or mycobacterial species, but a handful of Pth1 structures from gram-positive have been determined.

1.3.1 Pth1 structure

Pth1 structure Pth1 has the recognized α/β hydrolase structural motif. Pth1 consisted of a seven-stranded twisted β-sheet core surrounded by six α-helices (Schmitt, 1997; Giorgi, 2011). Although Pth1 retained the fold of other hydrolases, the catalytic residues were not maintained. Typically, a hydrolase contains a nucleophilic residue, usually a serine or cysteine, in its catalytic triad (Bischoff, 2012; de Pereda, 2004). The catalytic cleft, shown in Figure 1.3, was found buried within the β-sheet core consisting of Asn10, His20, and Asp93 numbered via EcPth1 residues (Schmitt, 1997). The catalytic triad of Pth1 was conserved across bacterial homologs. Schmitt et al (Schmitt, 1997) discovered His20 was essential for catalytic activity via site-directed mutagenesis of the catalytic triad in EcPth1 (shown in Figure 1.2). This finding was later confirmed by two other independent studies (Goodall, 2004; Fromant, 1999).
Figure 1.3: Pth1 structure: Pth1 maintains the α/β hydrolase fold with six α helices surrounding a mixed β sheet core. Pth1 has a catalytic triad of Asn10, His 20, and Asp93 (in black) with putative RNA-binding residues in helix 3, or the base loop (purple) and helix 4, or the lid loop (yellow). Helix 3 binds the 3′-CCA acceptor stem of peptidyl-tRNA, while helix 4 is hypothesized to act as a clamp. The gate loop (in blue) has a conserved structural fold.

The proposed mechanism put forth in the literature has been a nucleophilic attack on the ester bond between the 3′ OH of the ribose and the 3′-adenosine of tRNA by His20. This cleavage of the ester bond, therefore, allows free tRNA to be recycled for future protein biosynthesis by cleavage of the ester bond (Figure 1.3).
Figure 1.4: Proposed mechanism of peptidyl-tRNA hydrolysis by Pth1: 1. His20 protonation by water. 2. Hydroxyl anion attack of the ester bond forms an unstable intermediate. 3. The unstable intermediate dissociates into free tRNA and polypeptide chain thus recycling peptidyl-tRNA for future protein biosynthesis.

Moreover, the conservation of the α/β hydrolase structural motif and these catalytic residues across orthologs were found amongst orthologous hydrolase enzymes despite genetic diversity and slight variations in the catalytic pocket itself (Figure 1.4).
Figure 1.5 Conserved structure of Pth1: This superimposed structure of EcPth1 in blue (PDB 2PTH), SaPth1 in white (PDB 4YLY), MtuPth1 in purple (PDB 2JRC) and PaPth1 in yellow (PDB 4FYJ) reveal conserved α/β hydrolase fold and gate loop confirmation circled and highlighted in red.

Putative residues involved in RNA-binding and recognition were further described (Giorgi, 2011). Conserved Asn21 near helix-1 and Asn68 near helix-2 demonstrably interacted with the 3’-CCA acceptor stem mimic, 3-(L-[N,N-diacetyl-lysiny]l)amino-3-deoxyadenosine (Giorgi, 2011; Kabra, 2017). Residues important in RNA recognition of the 3’-CCA acceptor stem were found to include Asp96, Gly111, Asn113, Leu115, Lys116 in or near helix-3. Therefore, the residues near and in helix-3 (Fig 1.2 shown in purple) has been called the binding cleft or the “base loop” (Kumar, 2012; Kabra, 2017). Likewise, the residues Phe146 and Val149 in or near helix-4, or the “lid loop”, have been revealed to be of putative importance in Pth1:peptidyl-tRNA interactions using chemical shift perturbation nuclear magnetic resonance spectroscopy (NMR) with modified tRNA substrate (Giorgi, 2011, Kabra, 2017).
The Pth1 mutant, Pth(rap), possesses an Arg133His mutation on β7-strand within the catalytic pocket that makes the mutant temperature sensitive. However, unlike the Pth(TS), Pth(rap) was shown to have very low specific activity when mutated to a histidine (Garcia Villegas, 1991; Schmitt, 1997, Cruz-Vera, 2000). This mutation only recognized specific peptide moieties and was thought to be involved in substrate recognition. Furthermore, when Pth(rap) mutation was combined with K105A (on β6-strand) mutation, Pth1 lost the ability to recognize the 5’-phosphate of peptidyl-tRNA on the acceptor stem (Fromant, 1999). As such, both Arg133 and Lys105 are thought to be involved in RNA binding or substrate recognition within the catalytic pocket.

Residues His142 – Leu154 in helix 4 (shown in yellow in Figure 1.2) have been previously defined as the lid loop (Schmitt, 1997). Similarly, 5’-phosphate binding residues Lys107, Lys109 and Arg137 have been shown to aid in the proposed clamping mechanism attributed to helix-4 lid loop (Schmitt, 1997).

The gate loop region of Pth1 has a conserved fold regardless of amino acid sequence. Residues Leu97 – Val or Lys105 have been defined as the “gate loop” (Selvaraj, 2007). The Gly101Ala mutation induces temperature sensitivity, causing Pth1 to be inactivated at 42 °C (Menninger 1979; Cruz-Vera, 2000).

1.3.2 Enzymatic dynamics

Previous studies determined Pth1 acts like a clamping mechanism with the lid loop open in its apo form and binding the 3’-phosphate of adenosine as well as part of the 3’-CCA acceptor stem in its closed form (Kabra, 2017; Giorgi, 2011). These previous
studies performed NMR chemical shift perturbations and relaxation to determine putative dynamics in both *V. cholerae* and *E. coli* Pth1, respectively, to map putative residues involved in dynamics. Prior to these studies, most comparisons used computational *in silico* molecular dynamics (MD) studies to hypothesize the dynamics constraints. The prevailing hypothesis was the gate loop and the lid loop were inversely correlated to the dynamics between apo and closed. However, $^1$H-$^{15}$N nuclear Overhausen effect (NOE) NMR relaxation studies performed by Kabra et al. (Kabra, 2017) disproved this hypothesis. Moreover, these studies revealed the highly flexible $\alpha$-helices 3 and 4 were largely responsible for global protein dynamics (Kabra, 2017; Giorgi, 2011; Goodall, 2004). Despite having several residues and highly flexible regions with fast time scale motion, global protein dynamics were reported to be intermediate time scale motions in the 2–10 ns range (Kabra, 2017).

Global Pth1 dynamics has presented the unique challenge as the 3’-CCA acceptor stem of peptidyl-tRNA has high flexibility. As such, high resolution Pth1:peptidyl-tRNA complexed structures are not available currently. Hames et al. (2014) provided a low-resolution small angle neutron scattering complexed structure that confirmed Pth1 recognized and bound the 3’-CCA acceptor stem at the final adenosine. This further confirmed the previous chemical shift perturbation NMR findings (Giorgi, 2011). Without a high-resolution complexed structure, global dynamics of Pth1 have proven difficult to elucidate. However, several studies have provided insight into local protein regions and individual residues.

Mutational studies within *V. cholerae* Pth1 showed key conserved residues were involved in both discernment of peptidyl-tRNA from aminoacylated-tRNA and protein
dynamics. Giorgi et al. (Giorgi, 2011) determined Asn10 was putatively responsible for substrate recognition via site-directed mutagenesis of Asn10Asp. Pth1 recognized N-aminoacyl-tRNA and peptidyl-tRNA only. Importantly, fMethionyl-tRNA^{fMet} has a nucleotide mismatch of nucleotide 1 and 72 thus preventing hydrolysis of the initiator peptidyl-tRNA by bacterial Pth1.

Site-directed mutagenesis of His20 to alanine or asparagine does not alter protein dynamics significantly. However, H20A and H20N rendered Pth1 catalytically inactive (Schmitt, 1997; Kabra, 2017, Giorgi, 2011; Goodall, 2004). These previous studies identified slower mobility in the base loop upon binding of peptidyl-tRNA. Specifically, Gly111 and Asn113 – Lys116 in or near the α-helix 3 and some α-helix 2 residues were shown to have slower mobility based on chemical shift perturbations and backbone NOE relaxation studies (Giorgi, 2011; Kabra, 2017, Selvaraj, 2012). Similarly, residues near the catalytic His20 residue showed evidence of slower motion (Kabra, 2017).

Alternatively, His 145 and Asp 147 in the lid loop exhibited faster time scale motion based on NOE relaxation studies. The N- and C- terminus, β3-β4 sheets, and Asn72 in α-helix 2 also had faster time scale mobility and greater flexibility than the rest of the regions of Pth1 (Kabra, 2017; Giorgi, 2011). The slower mobility and lowered flexibility of residues near His20. In silico MD studies and NMR relaxation studies of flexibility in α-helix 5 and 6 showed similarity in both the computational and experimental data (Kabra, 2017).

In silico MD simulations have suggested the high degree of lid and base loop flexibility could be responsible for the mobility of the largely inflexible gate loop and the
widening of catalytic pocket (Kabra, 2017). Based on slower chemical exchange and relaxation times, the positioning of the residues near and in base loop have been hypothesized to bind peptidyl-tRNA as well help to position the ester bonds and nascent peptide chain for hydrolysis. The faster closing of the lid loop has been proposed to encapsulate the nascent peptide chain allowing for cleavage of the ester bond between the 3’-adenosine and polypeptides (Giorgi, 2011; Kabra, 2017; Schmitt, 1997). Based on the in silico MD, chemical shift perturbation NMR mapping, and $^1$H-$^{15}$N NOE NMR relaxation studies performed, the proposed residues involved have been largely identified and found to be conserved.

It should be noted that most of the Pth1 dynamics studies until now have been mainly comprised of in silico MD simulations. Only a small number of $^1$H-$^{15}$N NOE NMR relaxation studies exist in the literature. More research will be needed to confirm the proposed dynamics. However, thus far, global and local in silico MD simulations and $^1$H-$^{15}$N NOE NMR relaxation studies have been qualitatively similar (Kabra, 2017; Giorgi, 2011).

1.4 Pth1 Kinetics

Pth1 kinetics previously was determined in several watershed Pth1 studies. Three studies found striking similarities in the kinetics of EcPth1 (Shilock, 1979; Schmitt, 1997; Goodall, 2004). Every study determined non-native (e.g. cycloaminated or 3’-CCA mimics such as 3-(L-[N,N-diacetyl-lysiny]l)amino-3-deoxyadenosine) had a $K_m$ ranging from 5.5 – 6.0 μM for wildtype EcPth1. Various mutational studies of residues showed
variations as well, such EcPth1 N68A and N114A. The new $K_m$ for these mutations were 12.5 and 3.9 μM, respectively (Fromant, 1999). Interestingly, Goodall et al. (Goodall, 2004) reported significant changes in the $K_m$ when catalytic residues were mutated. Asn10Asp and Asp93Asn mutations resulted in 7.9 and 3.8 μM, respectively. Both Schmitt et al. (Schmitt, 1997) and Goodall et al. (Goodall, 2004) demonstrated His20Ala or His20Arg mutations made the enzyme catalytically inactive. As such, kinetic parameters were unattainable. Further evidence of Asn10Asp using a 3’-CCA mimic to obtain kinetic parameters resulted in a $K_m$ of 0.8 μM for EcPth1 Asn10Asp (Giorgi, 2011). This prompted the hypothesis that N10 was the residue responsible for discerning N-protected-aminoacyl-tRNA and peptidyl-tRNA from free floating aminoacyl-tRNA. As these residues are highly conserved, the role in Asn10 in tRNA recognition can be extrapolated to Pth1 homologs.

Peptidyl-tRNA has been shown to not bind aminoacyl-tRNA unless it is N-protected. Furthermore, when a monopeptide or monopeptide bound to a 3’-CCA mimic was used as a substrate, peptidyl-tRNA binding and recognition, as well as kinetic parameters and hydrolysis, were shown to be greatly reduced (Giorgi, 2011; Schmitt, 1997; Shiloch, 1975). Therefore, Pth1 was determined to show a greater preference for $N,N$-diacetyl or dipeptide nascent chain lengths compared to N-protected-monopeptide bound tRNA. Using acylmono- to hexapeptide nascent lengths, Pth1 kinetics becomes faster after dipeptide and increased until a tetrapeptide length. $K_m$ values decreased from 72 μM for N-block acetylated Val-tRNA (AcVal-tRNA) and Gly-Val-tRNA to 17 μM for (Gly)$_3$-Val-tRNA (Shiloch, 1975). Vmax was also found to increase from 3.5 pmol/min to 42 pmol/min for AcVal-tRNA and (Gly)$_3$-Val-tRNA respectively (Shiloch, 1975).
Polypeptide chain length equal to or greater than a tetrapeptide were determined to have consistent kinetic parameters. As it can be ascertained from EcPth1, kinetic parameters are dependent on nascent polypeptide length up to a tetrapeptide.

1.5 Summary of current study

Past studies probed the potential of Pth1 as a novel antibiotic and its structural characteristics in a single homolog. While the aim of this research was also to expand upon the antimicrobial potential of Pth1, the scope of this research included characterizing the similarities and differences between bacterial homologs. The current study explored three areas: characterization of phylogenetic clades and their respective inhibition profiles, characterization of kinetic parameters across phylogenetic space, and characterization of nucleotide specificity in relations to phylogenetic clades.

1.5.1 Phylogenetic characterization

Bacterial Pth1 homologs have a wide genetic distribution and consensus between homologs were demonstrably low. These characteristics have not previously been explored and could greatly impact the ability for Pth1 to be a novel drug target. Multiple X-ray crystallography and solution NMR structures were derived largely from gram-positive or mycobacterial species. Similarly, molecular docking studies both differed in results and binding strength depending on which docking program was used. Also, the docking studies were computational without any in vitro validation. In this current
research, bacterial homologs from across phylogenetic clades were accessed to ascertain whether the large genetic distribution would greatly alter the characteristics of each homolog and their respect clades. These characteristics were comprised of phylogeny, expression, gram-specificity, and solubility. An inhibition screen against EcPth1, *P. aeruginosa* Pth1 (PaPth1), *S. typhimurium* Pth1 (StPth1), *M. tuberculosis* Pth1 (MtuPth1), *S. aureus* Pth1 (Sa Pth1) and *B. cereus* Pth1 (BcPth1). The results for both can be found in chapter 3.

1.5.2 Characterization of kinetic parameters across phylogenetic space

Despite structural conservation and high conservation within clades, kinetics analysis frequently only addressed EcPth1 kinetic parameters. Several mutational studies assayed differences between wildtype and mutant EcPth1, but little to no data on different homologs have been published. This project obtained and analyzed the kinetic parameters of five wild-type homologs and one mutant homolog: EcPth1, PaPth1, MtuPth1, BcPth1, SaPth1, and SaPth1 E99P. These five homologs were representative of at least one homolog per clade with two clades having two different homologs. The results have this investigation will be presented in chapter 4.
1.5.3 Nucleotide inhibition

During this current project, it was discovered EcPth1 was weakly inhibited by adenosine triphosphate (ATP) using *in vitro* assays. ATP was selected due to the 3’-CCA acceptor stem of tRNA. Likewise, adenosine was previously reported to be the only thing needed for Pth1 to bind a 3’-CCA mimic. Sixteen nucleotides were screened for inhibition against EcPth1, StPth1, and SaPth1. While further in vitro screening against *Homo sapiens* Pth1 (HsPth1) and *Homo sapiens* Pth2 (HsPth2) were required, the results of nucleotide inhibition screen against bacterial Pth1s can be found in chapter 5.
CHAPTER 2

METHODS

2.1 Background

Described in this chapter is the phylogenetic characterization between bacterial Pth1 clades as well as general methodology for Pth1 purification and natural product plant extract (NPPE) inhibition screens used to verify inhibition characteristics between orthologs of bacterial Pth1. Although several X-ray crystal structures exist of different homologs of Pth1, little is known about similarities and differences between individual homologs and phylogenetic clades.

2.2 Methods

Described below are the methods used to characterize similarities and differences between phylogenetic clades by using NPPE inhibition screens of Pth1 homologs, Michaelis-Menten kinetics studies, and nucleotide inhibition.
2.2.1 Phylogenetic determination of Pth1

Several hundred bacterial Pth1 sequences of medical significance was aligned using the multiple alignment sequencing tool MUSCLE using EcPth1 as a reference, and using four *H. sapiens* Pth1 sequences, *S. solfataricus*, *H. sapiens*, and *A. fulgidus* Pth2 as outgroup members. Sequences were empirically scored for conservation and formatted into a phylogenetic tree using freely available EMBL tools. Any conservation score under 0.25 was not considered. The phylogenetic tree was constructed using neighbor-joining without distance corrections algorithm found on the EMBL website. Any duplicate sequences were removed prior to determination of phylogeny.

2.2.2 Pth1 growth and expression optimization

Apart from *M. tuberculosis* Pth1 (MtuPth1), all Pth1 homologs were cloned into a N-terminus his-tagged pKQV4 vectors with carbenicillin resistance. MtuPth1 was in pRESTB vector with carbenicillin resistance (Selvaraj, 2006). Pth1 vectors were transformed into BL21 *E. coli* competent cells on LB plates with 20 μg/mL carbenicillin. A 3 mL starter culture was grown overnight in a shaking incubator at 180 rpm at 37 °C. The starter culture was transferred to 30 mL with same media and under same conditions until an OD$_{600}$ of ~0.6 was reached. The culture was transferred to 1 L of LB with 0.2 μg/mL carbenicillin. The culture reached an optimal OD$_{600}$ for each homolog and was induced with appropriate concentration of IPTG as noted in Table 2.1 below. The induced culture was placed in a shaking incubator at the appropriate temperature for the
optimal time and aeration rate for each Pth1 homolog. Since no induction temperature for MtuPth1 was included in the literature, MtuPth1 was induced at the temperature for its genetically closest homolog \textit{M. smegmatis} Pth1 (Selvaraj, 2006). Cells were harvested using centrifugation at 20,000 xg rpm for 15 minutes and stored at -80 °C until ready to use. Samples were taken prior to and after induction to confirm expression on 12% SDS-PAGE gel.

<table>
<thead>
<tr>
<th>Pth1 Homolog</th>
<th>Vector/Antibiotic</th>
<th>Growth Media</th>
<th>Aeration</th>
<th>[IPTG]</th>
<th>Induction OD$_{600}$</th>
<th>Induction Time (hr)</th>
<th>Induction Temperature (°C)</th>
<th>Antibiotic Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{B. cereus}</td>
<td>pKQV4/carbenicillin</td>
<td>2X M9</td>
<td>1:4</td>
<td>1 mM</td>
<td>0.7</td>
<td>16</td>
<td>37</td>
<td>1:1,000</td>
</tr>
<tr>
<td>\textit{E. coli}</td>
<td>pKQV4/carbenicillin</td>
<td>LB</td>
<td>1:4</td>
<td>1 mM</td>
<td>0.6</td>
<td>6</td>
<td>37</td>
<td>1:1,000</td>
</tr>
<tr>
<td>\textit{M. tuberculosis}</td>
<td>pRESTB/carbenicillin</td>
<td>LB</td>
<td>1:4</td>
<td>0.5 mM</td>
<td>0.6</td>
<td>16</td>
<td>27</td>
<td>1:1,000</td>
</tr>
<tr>
<td>\textit{P. aeruginosa}</td>
<td>pKQV4/carbenicillin</td>
<td>LB</td>
<td>1:4</td>
<td>1 mM</td>
<td>0.6</td>
<td>16</td>
<td>18</td>
<td>1:1,000</td>
</tr>
<tr>
<td>\textit{S. aureus}</td>
<td>pKQV4/carbenicillin</td>
<td>LB</td>
<td>1:8</td>
<td>0.5 mM</td>
<td>0.9</td>
<td>16</td>
<td>14</td>
<td>1:2,000</td>
</tr>
<tr>
<td>\textit{S. typhi}</td>
<td>pKQV4/carbenicillin</td>
<td>LB</td>
<td>1:4</td>
<td>1 mM</td>
<td>0.8</td>
<td>3</td>
<td>37</td>
<td>1:1,000</td>
</tr>
</tbody>
</table>

Table 2.1 Pth1 growth and induction conditions

2.2.3 Pth1 purification

Frozen, pelleted cells, transformed were thawed and resuspended in a 20 mL Buffer A, see Table 2.2 (typically in 50 mM NaHPO$_4$, pH 7.4, 300 mM NaCl buffer) per original liter of cell culture. Cells were incubated with 15 mg lysozyme for 30 mins at room temperature, followed by sonication (5 secs on, 5 secs off for 5 mins). The lysate was separated into soluble supernatant and insoluble pellet using centrifugation 20,000 xg
for 30 mins Samples of total, supernatant and pellet were taken, heated for 15 mins, and run on 12% SDS-PAGE gel to determine the solubility.

Supernatant, containing soluble protein, was loaded onto a freshly charged His-trap nickel column for metal chelation affinity chromatography. Purification buffers used were: Buffer A (50 mM NaHPO₄, pH 7.4, 300 mM NaCl) and Buffer B (50 mM NaHPO₄, pH 7.4, 300 mM NaCl, 150 mM Imidazole) unless otherwise specified in Table 2.2. His-tagged Pth1 was bound to His-Trap Nickel column and washed with 21 mM imidazole and 33 mM imidazole. His-tagged Pth1 was eluted off the column with 150 mM imidazole. MtuPth1 did not fully elute with 150 mM imidazole and required 200 – 250 mM imidazole for elution. Selvaraj et al (2006) used 500 mM imidazole for eluting MtuPth1. The purification buffers were: Buffer A (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10% glycerol) and Buffer B (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10% glycerol, 500 mM imidazole) (Selvaraj, 2006). The elution buffer was modified to have only 250 mM imidazole. Fractions were analyzed on 12% SDS-PAGE gel. Those fractions with His-tagged Pth1 were pooled and buffer exchanged against buffer needed for each Pth1 homolog as seen in Table 2.2. Purified his-tagged Pth1 was concentrated using ultracentrifugation and stored at 4 °C until ready to access activity using an acid urea-minigel (Holloway, 2015).
### Table 2.2: Pth1 homolog purification and buffer exchange conditions

<table>
<thead>
<tr>
<th>Pth1 Homolog</th>
<th>Buffer A</th>
<th>Buffer B</th>
<th>[Imidazole] required for Elution</th>
<th>Buffer Exchange Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. cereus</em></td>
<td>50 mM NaHPO$_4$, pH 7.4, 300 mM NaCl</td>
<td>50 mM NaHPO$_4$, pH 7.4, 300 mM NaCl, 150 mM Imidazole</td>
<td>150 mM</td>
<td>25 mM NaHPO$_4$, pH 6.0, 250 mM NaCl, 2 mM DTT at 4 °C</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>50 mM NaHPO$_4$, pH 7.4, 300 mM NaCl</td>
<td>50 mM NaHPO$_4$, pH 7.4, 300 mM NaCl, 150 mM Imidazole</td>
<td>150 mM</td>
<td>10 mM Tris-Acetate, pH 8.0</td>
</tr>
<tr>
<td><em>M. tuberculosis</em></td>
<td>20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10% Glycerol</td>
<td>20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10% Glycerol, 500 mM Imidazole</td>
<td>≥ 150 mM</td>
<td>20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM β-mercaptoethanol</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>50 mM NaHPO$_4$, pH 7.4, 300 mM NaCl</td>
<td>50 mM NaHPO$_4$, pH 7.4, 300 mM NaCl, 150 mM Imidazole</td>
<td>150 mM</td>
<td>20 mM Bis-Tris, pH 6.6,</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>50 mM NaHPO$_4$, pH 7.4, 300 mM NaCl</td>
<td>50 mM NaHPO$_4$, pH 7.4, 300 mM NaCl, 150 mM Imidazole</td>
<td>150 mM</td>
<td>25 mM NaHPO$_4$, pH 6.0, 250 mM NaCl, 2 mM DTT</td>
</tr>
<tr>
<td><em>S. typhi</em></td>
<td>50 mM NaHPO$_4$, pH 7.4, 300 mM NaCl</td>
<td>50 mM NaHPO$_4$, pH 7.4, 300 mM NaCl, 150 mM Imidazole</td>
<td>150 mM</td>
<td>10 mM Tris-Acetate, pH 8.0</td>
</tr>
</tbody>
</table>

#### 2.2.4 Peptidyl-tRNA extraction

*C600 TS* *E. coli* cells were grown to isolate nascent peptide peptidyl-tRNA. At 30 °C, Pth1 in the C600 TS *E. coli* cells is active; however, at 42 °C, Pth1 is inactive allowing for accumulation of peptidyl-tRNA (Varshney, 1991). A 5 mL starter culture of C600 TS *E. coli* cells in LB were grown at 30 °C with no antibiotics until an OD$_{600}$ of 0.6 at 180 rpm in a shaking incubator. The culture was transferred to 30 mL LB culture
under the same conditions. The 30 mL culture was transferred to 100 mL LB and grown to an OD$_{600}$ of 0.4. C600 TS *E. coli* culture was induced at 42 °C for 70 mins, rapidly cooled and harvested at 20,000 xg for 15 mins. Cell pellets were stored at -80 °C for a minimum of 24 hours for optimal peptidyl-tRNA extraction.

Following a published protocol, C600 TS *E. coli* cell pellets were resuspended in 0.3 mM NaOAc, pH 5.2, 10 mM EDTA. Phenol extractions with resuspended cells in 1:1 ratio was carried out, and sonicated for 5 secs, 5 secs off for 5 mins (Varshney, 1991). Lysate was separated from insoluble organic layer via centrifugation at 1,100 xg for 5 mins. The aqueous layer was taken, and this phenol extraction was repeated. Following the final phenol extraction, lysate was chloroform extracted, and separated with centrifugation at 1,100 xg for 5 mins. The aqueous layer was removed, and ethanol precipitated with absolute ethanol in 1:2.5 ratio. Ethanol precipitation was repeated three times prior to aliquoting 500 μL of bulk peptidyl-tRNA into Eppendorf tubes. Centrifugation at 26,000 xg and sterile air drying were used to remove an excess ethanol. Peptidyl-tRNA was stored at -80 °C. Each batch was tested for consistency in intensity on urea-PAGE assay. Bulk peptidyl-tRNA was quantified using the UV spectrophotometer absorbance at A$_{260}$, and an A$_{260}$/A$_{280}$ ratio was assessed for purity of the peptidyl-tRNA extraction. A typical peptidyl-tRNA extraction yielded a concentration of ~28 – 30 μM per aliquot. Uniform gel intensity and peptidyl-tRNA concentration were important for an accurate analysis of Pth1 activity and inhibition when using urea-PAGE.
2.2.5 Pth1 Activity

Reactions varied only in the amount of Pth1 present to determine the concentration needed for each assay. The concentration needed was defined as the concentration that had equivalent activity to 3 μL of 30 μL EcPth1. Reaction volumes were 20 μL and contained varying concentrations (0.5 – 4 μL of 30 μL Pth1). To this was added, 3 μL of 30 μM bulk peptidyl-tRNA, 5 μL of 4X Buffer B (40 mM Tris-Acetate, pH 8.0, 40 mM magnesium acetate, 80 mM ammonium acetate) and DEPC-treated water to reach a total reaction volume. The positive control using 30 μL EcPth1 was used to standardize all assays. The negative control with no Pth1 was also used to aid in quantification during urea-PAGE analysis. The reactions were quenched after 30 minutes.

Likewise, Pth1 activity was analyzed for kinetics using time course assays. The reaction volume remained the same; however, 3 μL of 30 μL mg Pth1 was used without any changes with respect to the rest of the reaction set-up. Each reaction was quenched every 5 minutes until 30 minutes was reached using with a urea loading dye (100 mM sodium acetate, 35 mM urea, 0.0025 g bromophenol blue, DEPC-treated water to 5 mL total volume). Reactions were run on 8 M urea-PAGE and analyzed for differences in migration of peptidyl-tRNA between controls and samples.
2.2.6 Urea-PAGE

Following the protocol of Holloway et al. (Holloway, 2015), 6 M urea-PAGE gels were run for ~2.5 hours at 100 V with 100 mM NaOAc, pH 5.2 running buffer. All lanes were pre-run and rinsed to remove any residual urea from the wells. For better resolution, gel electrophoresis box was packed in ice to help maintain a running temperature of 6 – 10 °C. Urea gels were stained with 0.3 g methylene blue in 0.5 M NaOAc for 3 hours. After 3 hours, urea gels were destained with deionized water for approximately 30 mins to remove any excess stain. Destained urea gels were imaged allowing for densitometric analysis.

2.2.7 Urea-PAGE Analysis

Analysis of the acid urea-minigels was performed using the published protocol (Holloway, 2015). The distance cleaved peptidyl-tRNA (positive control) and uncleaved peptidyl-tRNA (negative control) migrated was compared to the migration of the peptidyl-tRNA in the sample (Holloway, 2015). This comparison allowed a quick, inexpensive, and less toxic way to analyze the percentage of cleaved peptidyl-tRNA in NPPE samples by setting positive control to equal 100% cleavage and negative control to equal 0% cleavage. Therefore, the following equation can be used:

$$\frac{(\text{Positive control} - \text{Sample})}{(\text{Positive control} - \text{Negative control})} \times 100\%$$

Percent inhibition was obtained from 100% – percent cleaved.
2.2.8 Site-directed mutagenesis

The Agilent QuickChange Lightning kit protocol was to mutate residues in the SaPth1 gate loop. The following primers for each homolog in their respective pKQV4 vectors:

**SaPth1 E99P Fwd:**

5’TTAAGCGAACCTTGCTCTTGTGGTAAATCTAAATCATCATATAAGACAATTAAATCT
TCTGG 3’

**SaPth1 E99P Rev:**

5’CCAGAAGATTTAATTGTCTTTATATGATGATTATTTACCAACAAGGACAAGTTCG
CTTAA 3’

**SaPth1 Q100P Fwd:**

5’ TTTTTGTCTTAAAGCGAACCTTGCTCTGTTCTAAATCTAAATCATCATATAAG 3’

**SaPth1 Q100P Rev:**

5’ CTTATATGATGATTAGATTAGAGACCCAGGAAAGTTCGCTTAAAGACAAAAA 3’

**SaPth1 Q102V Fwd:**

5’ CGCACTTCTTTTTGTCTTAAAGCGAACAACCTCCTGTCTTAAATCTAAATCATCATATA
3’

**SaPth1 Q102V Rev:**

5’TATGATGATTTAGATTAGACCAAAGGAGTTTGCTTAAAGACAAAAAAGGAAGTGCG
CG 3’

PCR products were restricted with DpnI and ligated using T4 DNA Ligase provided with the Agilent kit. Ligations were transformed into DH5α *E. coli* competent cells and plated on LB-carbenicillin plates. Resulting colonies were grown overnight in a 5 mL LB culture (5 μL carbenicillin). Transformants were harvested using centrifugation at 20,000
xg, and plasmid DNA was extracted using GeneJET plasmid miniprep kit. Plasmids were sequenced to confirm site-directed mutagenesis.

Transformations of SaPth1 mutants were repeated in BL21(DE3) *E. coli* competent cells. Transformants for each mutation were grown overnight and transferred to 30 mL cultures as previously described. Following this, the SaPth1 mutants were transferred into equal 500 mL volumes under optimized wild-type SaPth1 expression conditions and EcPth1 expression conditions as noted in Table 2.1. However, induction OD$(_{600}$ of 0.9 and temperature was maintained. The cells were harvested and placed in -80 °C until purification. Purification conditions were unchanged from Table 2.2. Those purifications that yielded soluble protein were quantified using UV spectrophotometry at 280 nm, and activity was confirmed using 8M acid urea-minigels.

2.2.9 Natural product plant extract (NPPE) inhibition screen

56 NPPE obtained from North Queensland, Australia, Monteverde, Costa Rica, Matabeleland, Zimbabwe; and Abaco Island, Bahamas were graciously provided by Dr. William Setzer’s research group (Listed in Appendix A). Lyophilized fractions were resuspended in DMSO and 1% w/v solvent. These extracts were then accessed for inhibition against EcPth1, PaPth1, StPth1, MtuPth1, SaPth1, and BcPth1. 20 μL reactions were prepared using positive and negative controls as previously described. The reaction set up for NPPE screen was: the concentration needed to equal that of 0.63 mg EcPth1 (usually 3 – 4 μL of 0.63 mg Pth1), 5 μL 4X buffer B, 4 μL of NPPE, 3 μL of 30 μM bulk peptidyl-tRNA, and DEPC-treated H$_2$O for a total reaction volume of 20 μL.
Each NPPE was incubated with Pth1 for 5 minutes prior to addition of peptidyl-tRNA. Reactions were quenched after 30 minutes and run on a 6 M acid urea-minigel. Urea-PAGE analysis was carried out.

2.2.10 Pth1 kinetics

Pth1 was diluted to a concentration of 30 μM with DEPC-treated water. Peptidyl-tRNA was quantified using A$_{260}$/A$_{280}$ ratio to be 30 μM. Reactions as described above were carried out at 1:0.1.3, 1:0.25, 1:0.5, 1:1, 1:2, 1:3, and 1:4 Pth1:peptidyl-tRNA molar ratio. These reactions were quenched at 5 - 10 minute intervals ending at 30-50 minutes dependent on substrate concentration. Reactions were run on 8 M urea gels and analyzed as stated above. Percent cleaved multiplied by the initial substrate concentration was equal to product concentration. Michaelis-Menten product versus time curves were plotted in Excel. The slope of the tangent line of the curve at time zero was equivalent to the $V_o$ at that substrate concentration. This assay was repeated in triplicate for all time intervals and peptidyl-tRNA concentrations used. $V_o$ was averaged and plotted with their respective standard deviations represented as the error bars. Following this, a Wilkinson non-linear regression was performed using Excel to determine the least squares to best fit the data (Wilkinson, 1961; Duggleby, 1995). Provisional kinetic parameters were estimated using sum of least squares for determined $V_o$ and initial substrate concentration ($S_o$). The parameters were further refined using calculations to minimize the least squares of the Michaelis-Menten equation and the first derivative with respect to $K_m$ using the provisional kinetic parameters. The Michaelis-Menten equation was then used
to determine expected values using the refined parameters from the nonlinear regression analysis. These values were fitted to the observed velocities for each substrate concentration, and Solver function was used to further minimize the summation of the squared error. Pearson correlation analysis of the observed and expected velocities for each $S_0$ to find coefficient of determination. This was to ensure the quality of the fit between expected and observed velocities.

2.2.11 Nucleotide inhibition screen

16 nucleotides were screened against EcPth1, StPth1, and SaPth1. The assay followed the same protocol as 2.2.4, however 4 μL of 100 mg/mL nucleotide concentration (final reaction volume of 20 mg/mL) was incubated with Pth1 for 5 minutes prior to addition of peptidyl-tRNA for initial quantitative screen. Assays were quenched at 30 minutes followed by urea-PAGE, and urea-PAGE analysis. Any nucleotides yielding inhibitory results were tested quantitatively to determine the concentration at which 50% inhibition (IC$_{50}$) was observed. Decreasing concentrations of nucleotides were achieved by using a 1:1 dilution. 4 μL of each concentration (100 mg/mL, 50 mg/mL, 25 mg/mL, 12.5 mg/mL, 6.25 mg/mL, 3.13 mg/mL) was incubated with 30 μM Pth1 prior to addition of 30 μM peptidyl-tRNA to a total 20 μL final reaction volume. The final nucleotide concentrations in the reaction were 20 mg/mL, 10 mg/mL, 5 mg/mL, 2.5 mg/mL, 1.3 mg/mL, and 0.63 mg/mL, respectively. Each reaction was quenched after 30 minutes. Urea-PAGE analysis was as in section 2.2.8 to determine the
percent cleaved and percent inhibition for each inhibitory nucleotide concentration. Each nucleotide concentration was run in triplicate.
CHAPTER 3

PHYLOGENETIC CHARACTERIZATION OF PTH1 CLADES

3.1 Background

With the emergence of antibiotic-resistant bacterial infections being of great concern, new drug targets to combat these diseases are of utmost importance. One such target is Pth1. Pth1 functions to cleave the nascent chain of peptides from peptidyl-tRNA during abortive translation (Goodall, 2004). Previous studies have shown that loss of function of Pth1 leads to tRNA accumulation and cell death in bacteria (Kabra, 2017). Many current antibiotics target protein biosynthesis, and as such, has been shown to be an effective antibiotic strategy.

However, very little is known about how these vital enzymes differ between bacterial species. To address this gap in knowledge, Pth1 sequences from pathogenic bacterial species were aligned allowing for phylogenetic characterization. Subsequently, Pth1 enzymes from each clade were recombinantly expressed and had purification conditions optimized and characterized. These same Pth1 enzymes were assayed for inhibition using a common set of 56 NPPE. In addition and augmenting the phylogenetic
characterization, described in this chapter are the growth conditions and the patterns of inhibition observed from these NPPE inhibition screens.

3.2 Results and Discussion

Three distinct clades emerged from phylogeny of bacterial Pth1 with their own biochemical characteristics. These three clades were accessed for the similarities and differences in levels of expression, purification yields, and the possibility of achieving broad spectrum, narrow spectrum, or clade specific inhibition. The three clades are defined as: the *E. coli*-like clade, herein referred to as Clade 1; the *M. tuberculosis*-like clade, referred to as Clade 2; and the *S. aureus*-like clade, referred to as Clade 3.

3.2.1 Pth1 Phylogeny

To date, only a handful of Pth1 homologs have been extensively studied. Most findings have been limited to EcPth1 or MtuPth1. It was hypothesized based on these results most Pth1 homologs behaved similarly due to the conserved structure (Kabra, 2016). However, this current project confirmed that Pth1 homologs were not the same with regards to phylogeny or biochemical characteristics. Phylogenetic analysis allowed for homologs across phylogenetic space to be identified and studied.

Clade 1 homologs were highly conserved. EcPth1 showed high similarity with PaPth1 and StPth1, respectively. These enzymes only differ in 9 and 13 residues out of 190-194, respectively, when compared to EcPth1. It was of note that these residue changes were usually synonymous to one another, such as an aromatic phenylalanine for an aromatic tyrosine. Clade 2 Pth1 homologs similarly showed high sequence
conservation (80% identity) within the clade. In comparison, Clade 3 showed less conservation within its respective clade, with only ~ 40% conservation of sequence identity. Clade differences were apparent when conservation, any substitution that preserves the physiochemical properties of the residue, which was approximately 60% conservation between clades. Likewise, the identity between clades was 35 – 40 % identity. The conservation between bacterial homologs, even between clades, was much higher than when bacterial homologs were compared to human sequences. Only 30 – 40 % sequence homology exists between bacterial and human Pth1 homologs. HsPth1 was more closely related to the structurally and mechanistically different HsPth2 than to any bacterial homologs.
Figure 3.1 Phylogenetic tree of Pth1: Pth1 is divided into three distinct clades based on gram specificity. Clade 1 consisting of Pth1 homologs from gram negative bacteria. Clade 2 largely consists of mycobacterial Pth1 homologs; while Clade 3 Pth1 are made up of Pth1 homologs from gram positive bacteria.

3.2.1.1 Gram specificity

As Figure 3.1 shows, Pth1 phylogeny was found to separate according to the bacterial species gram-status. Pth1 homologs from gram-negative bacteria like *P. aeruginosa*, *S. typhi*, and *E. coli* were shown to make up the content of Clade 1. Clade 3 Pth1s were found to be from gram-positive bacteria, such as *S. pneumoniae*, *S. aureus*,
and *S. pyogenes*. Pth1 from mycobacterial species, which have both gram-negative and gram-positive characteristics, formed Clade 2 (Fu and Fu-Liu, 2002).

One possible explanation for the gram-specificity of Pth1 homologs could be due to the differences in tRNA, 16S rRNA and 23S rRNA methylation patterns for gram-negative, and gram-positive species (Klagsbrun, 1973). Both *E. coli* and *P. aeruginosa* tRNA contained 1-methylguanine, 7-methylguanine, ribothymidine, and 2-methyladenine (Klagsbrun, 1973). In contrast, gram-positive species were found to also have 1-methyladenine in their tRNA. Based on this, Pth1 could have diverged with respect to gram-status to accommodate for the differences in methylation of tRNA. Clearly, Clade 3 homologs recognized and cleaved peptidyl-tRNA extracted from *E. coli* C600 TS cells. As such, Clade 3 homologs do not require 1-methyladenine to recycle peptidyl-tRNA *in vitro*, and the chemistry of the reaction does not change. The phylogenetic gram-specificity could potentially explain why Clade 3 homologs had more favorable binding affinity for bulk peptidyl-tRNA than EcPth in terms of their kinetics (discussed in further detail in chapter 4). Pth1 has been shown to tightly interact with the TψC arm of peptidyl-tRNA where 1-methyladenine is found at A58 (Hames, 2014). Other enzymes, such as aminoacyl-tRNA synthetases, are known to need the appropriate tRNA methylation for enzymatic activity or substrate recognition (Guenther, 1994). It has been hypothesized that Pth1’s tight interaction could potentially be involved in substrate recognition (Hames, 2014). The gram-specificity of Pth1, tRNA methylation and modifications, and rRNA modifications lends credence to this hypothesis.

In comparison, gram-specificity did not play a significant role in small molecule or nucleotide inhibition. The residues in the catalytic cleft of Pth1 were highly
conserved. Likewise, structure and predicted mechanism were conserved across phylogenetic space evidenced by the Vmax parameters (discussed further in chapter 4). Any tRNA modifications would likely not affect catalytic residues in the catalytic cleft. Past studies found that alterations in these catalytic residues affected activity, but not substrate binding (Giorgi, 2011; Goodall, 2004). As such, small molecule inhibition would likely involve interaction with these residues (discussed in chapter 5). Future work is required to determine if Clade 1 homologs would have reduced or no activity in the presence of 1-methyladenine tRNA. Likewise, Clade 3 homologs would also need to be screened for differences in activity when peptidyl-tRNA, or N-acetylated tRNA, from a gram positive species were used.

3.2.2 Biochemical characterization of Pth1 clades

Several X-ray crystal and NMR structures of Pth1 have helped to determine key residues in Pth1, such as the catalytic triad of Asn10, His20, and Asp93 or putative RNA recognition and binding sites (Schmitt, 1997; Goodall, 2001, Giorgi, 2011). His20 was demonstrated to be the catalytic residue (Schmitt, 1997). Kinetic and structural studies of EcPth1 and V. cholerae Pth1 have shown helix 3 acts as a receiving lip pushing peptides into the catalytic pocket. Helix 4 is thought to act as a clamp that closes about the adenosine in the 3’-CCA acceptor stem and nascent polypeptide chain. Low resolution structures exist helping to form an understanding of the peptidyl-tRNA orientation with respect to Pth1 when complexed (Hames, 2014). These key residues, shown in Figure 3.2, are well conserved between bacterial homologs.
Although Pth1 homologs all function to cleave peptide from peptidyl-tRNA during abortive translation, differences in expression and solubility make purifying and crystallizing homologs very difficult. The PDB database shows most of Pth1 crystal structures come from either the *E. coli*-like or *M. tuberculosis*-like clades, therefore most of the current knowledge in structure and enzymatic actions are derived from these two clades.

3.2.2.1 Pth1 growth and general trends associated with phylogenetic clades

It should be noted that as recombinant Pth1 growth was performed using different amino acid sequences or plasmids that a direct comparison could not be made. These general growth trends were considered more correlative, and could have resulted from any number of cellular, metabolic, or plasmid constraints placed on *E. coli* BL21 competent cells. Clade 1 Pth1 homologs had greater expression than that of Clade 2 and
Clade 3 Pth1s. MtuPth1, a clade homolog, had the least expression of all homologs tested under optimized conditions. The Clade 3 homologs, SaPth1 and BcPth1, showed more expression when grown under their optimized conditions.

![Figure 3.3 Expression differences between clades: The blue arrow (noting Pth1) shows the differences between clades after induction. The Clade 2 homolog, MtuPth1 (in blue) showed the least amount of expression. Clade 1 homologs (in black) had the most expression of all three clades.](image_url)

As can be seen in Figure 3.3, most Clade 1 Pth1 homologs produced greater yields of soluble protein with standard growth, and induction conditions with respect to aeration rate, IPTG and antibiotic concentrations (as previously shown in Table 2.1). Of note, PaPth1 has a C-terminus truncation of the last 10 residues resulting in the noTable molecular weight difference. This truncation does not affect activity. Similarly, Clade 2
Pth1s required 1:4 aeration rate, 1 mM carbenicillin, 500 μM IPTG, overnight incubations, and 27 °C induction temperature. As stated previously, growth conditions for MtuPth1 were ascertained from Selvaraj et al (Selvaraj, 2006) with induction temperature obtained from the closely related M. smegmatis Pth1 (Kumar, 2012). It was noteworthy that Kumar et al. (Kumar, 2012) used 300 μM IPTG to induce transformed bacterial cultures. The general trend from this and previous studies was that Clade 2 homologs required more optimization of induction conditions than Clade 1 homologs, but significantly less optimization of growth, expression and purification conditions than Clade 3 Pth1s. As such, Clade 2 homologs were intermediate with regards to optimization of growth conditions. However, it should be noted that Clade 2 homologs were cloned into a different plasmid. As such, this was a general correlative trend. In stark contrast, Clade 3 Pth1s had significantly different growth conditions even between homologs within its own clade. BcPth1 growth was not aeration rate dependent but 2X M9 minimal media was required for optimum expression (Taylor-Creel, 2014). However, antibiotic and IPTG concentrations were identical to Clade 1 Pth1s. The growth conditions for SaPth1 presented in Table 2.1 deviated from previously published sources (Bonin, 2002). SaPth1 required an aeration rate two times greater, and longer, slower induction at lower temperatures. Any deviation from these conditions resulted in very little to no soluble protein following sonication. Growth conditions of other Clade 3 homologs like S. pyogenes Pth1 (SpPth1) with X-ray crystal structures were known, but not used in this current study (Singh, 2014). Although aeration rates for SpPth1 was not noted in Singh et al. (Singh, 2014), their growth and expression conditions were correlated with slower incubation times at lower temperatures. Therefore, based on
growth conditions, Clade 2 was found to be an intermediate between Clade 1 and Clade 3. Although Clade 2 shared similarities with Clade 3 Pth1s, homologs in Clade 3 were discovered to produce a far lower yield with little or no allowance for deviation in these conditions. Clade 3 homologs also needed individualized optimization. Each homologous enzyme tested often necessitated considerable optimization. Although these were correlative, this could provide insight to future optimization of expression and purification for novel bacterial Pth1 homologs in the future.

These findings also illustrated why very few Clade 3 Pth1 crystal or solution NMR structures exist in the PDB to this point as well further illustrated the differences both between and within clades. Optimization of growth, expression, and purification of Clade 3 enzymes proved cumbersome. Unlike Clade 1 or Clade 2 homologs, Clade 3 did not have similar conditions between the homologs tested so far for optimal yields of soluble protein (Taylor-Creel, 2014). Stark differences were seen in these conditions in the literature with some requiring specialized media like terrific broth that increased solubility (Bonin, 2002). Even under optimized conditions, Clade 3 Pth1 purification yields were relatively low in comparison to Clade 1 and Clade 2 Pth1 with significantly less time as both soluble and active. SaPth1 had the lowest yields of soluble protein and required the greatest amount of optimization.

3.2.2.2 Shared motifs within clades

To improve SaPth1’s solubility and purification yields, amino acid sequences for Pth1 homologs from across phylogenetic space were aligned to determine any potential
regions or residues that might account for the difficulties in obtaining soluble recombinant protein. One notable difference was the residues in the gate loop region surrounding G101. Clade 1 Pth1’s had a characteristic Pro-Pro-Gly-Val sequence, as seen in Figure 3.4A. Clade 2 Pth1’s, likewise, shared a Glu/Asp-Phe-Gly-Arg motif around the G101 residue. Clade 3 Pth1 homologs had a mostly conserved Glu/Asp-Gln-Gly-Gln motif around the G101 residue. The gate loop region housing the G101A mutation responsible for temperature sensitivity in EcPth1. Temperature sensitivity was hypothesized to cause aggregation thereby affecting its solubility (Garcia-Vilegas, 1991, Cruz-Vera, 2000). Similarly, the gate loop structure was highly conserved despite differences in amino acid sequence (Figure 3.4B). As such, the gate loop region was assessed to determine its effect on Pth1 solubility in SaPth1.

Figure 3.4: Gate loop region of bacterial Pth1: A: Multi alignment sequencing of the gate loop region revealed a conserved motif within phylogenetic clades. B: The gate loop structure maintained a highly conserved fold following superimposition of crystal structures.
3.2.2.3 Site directed mutagenesis of SaPth1 gate loop affects solubility

Site directed mutagenesis of SaPth1 gate loop was performed to increase the soluble yields of recombinant Pth1 obtainable from homologs that required significant optimization to express and purify nominal yields as this would be both time-saving and cost-effective. For an easier comparison, EcPth1 residue numbering was used with regards to the SaPth1 gate loop mutations. Gate loop mutations affected solubility both positively and negatively. For instance, SaPth1 E99P significantly increased solubility in both WT SaPth1 and EcPth1 expression conditions. Mutating glutamate to proline proved to overcome previous aeration rate and IPTG rate dependence seen in wildtype SaPth1 expression. Purified yield was 4.8 ± 0.2 mg for WT SaPth1 expression conditions and 1.6 ± 0.1 mg for EcPth1-like growth conditions (as noted in Table 3.1).

Table 3.1: SaPth1 gate loop mutations soluble protein yield

<table>
<thead>
<tr>
<th>Mutations</th>
<th>Soluble protein concentration: 1:8 expression conditions (mg)</th>
<th>Soluble protein concentration: 1:4 expression conditions (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SaPth1 E99P</td>
<td>4.8 ± 0.2</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>SaPth1 Q100P</td>
<td>Not Soluble</td>
<td>Not Soluble</td>
</tr>
<tr>
<td>SaPth1 Q102V</td>
<td>1.5 ± 0.1</td>
<td>Not Soluble</td>
</tr>
</tbody>
</table>
Alternatively, SaPth1 Q101V was soluble only at the WT SaPth1 expression conditions. SaPth1-like growth conditions yielded 1.5 ± 0.1 mg following purification and concentration of protein. The Q101V mutation did not have an affect solubility or increase the yields obtained (Figure 3.5). In stark contrast, SaPth1 Q100P was largely insoluble for both growth conditions.

![Image of gel electrophoresis results](image)

Fig 3.5 Site directed mutagenesis of SaPth1 gate loop affects solubility: SaPth1 E99P yielded the most soluble protein and was no longer aeration rate or IPTG-concentration dependent. SaPth1 Q102V had some soluble when grown and expressed at wildtype SaPth1 conditions (labeled 1:8 in figure). SaPth1 Q100P was not soluble under wildtype SaPth1 or wildtype EcPth1 (labeled 1:4 in figure).

Firstly, this demonstrated insolubility in SaPth1 and possibly other Pth1s could be resolved via mutating negatively charged Glu or Asp residues to Pro in the gate loop. This was consistent with phylogeny as the homologs easier to work with contained at least one Pro in its gate loop (Fig 3.4). The gate loop of MtuPth1 and other Clade 2 homologs was either Asp or Glu-Phe-Gly-Arg. Likewise, most Clade 3 homologs also had an Asp or Glu as the first residue within the gate loop followed typically by a Val-Gly-Lys. The gate loop was the only area not as stochastic in residue differences.
between clades or even individual homologs. Likewise, as can be gleaned from Fig 3.4, the gate loop structure was highly conserved. The Pro-Pro-Gly-Val motif appears to be more energetically favorable in this confirmation as Pro is dihedrally more constrained than other amino acids. However, this would be less ideal for Glu-Phe-Gly-Arg or Glu-Val-Gly-Lys as this was energetically unfavorable for both glutamate, phenylalanine and glycine bonds being forced into a static fold most suitable for prolines. Previous dynamics data reinforces that the gate loop was not a highly dynamic region (Kabra, 2016).

Figure 3.6 SaPth1 Q100P disrupts salt bridge: A: EcPth1’s gate loop showed no interactions with helix 5. B: A salt bridge between SaPth1 Glu 154 in helix 5 and Gln 100 in the gate loop was shown in the X-ray crystal structure (PDB: 4YLY). Disruption of the salt bridge between Glu 154 and Gln 100 (based on EcPth1 residue numbering) likely led to little solubility when SaPth1 Q100P was grown.

E. coli Pth1 (2PTH):
Dark blue residues: Gate loop residues Pro 98, Pro 99, and Val 101.
Pink: No interaction with gate loop

S. aureus Pth1 (4YLY):
Dark blue residues: Gate loop residues Glu 99, Gln 100, and Gln 102. Light blue: Glu 154 (Helix 5) interacts with Gln 100 (Gate loop)

Loss of this salt bridge yields no soluble protein
A comparison of crystal structures between EcPth1 and WT SaPth1 showed the Gln100 residue to undergo hydrogen bonding with Glu154 in helix 5 (Figure 3.6). This salt bridge was unique to SaPth1 based on the available crystal structures.

SaPth1 Q100P likely failed to produce soluble protein due to the loss of this stabilizing salt bridge between Q100 and E153 in helix 5. This salt bridge was unique to SaPth1 of the superimposed structures of Pth1 (Fig 3.6b).

3.2.3 NPPE inhibition screen

Natural products have proven a robust source for antibiotics. Similar strategies have been employed with drug discovery and bioactive fractionation studies to varying levels of success (Nothias, 2018). This present study did not seek to identify the compound, or compounds, responsible for inhibition, but to further highlight the similarities and differences that exist between the clades. As such, these NPPE were heterogenous mixtures extracted in different solvents. Importantly, the assay described in Holloway et al (2015) could successfully screen for inhibition regardless of solvent system used. While only screened against six Pth1 enzymes, narrow spectrum and clade specific inhibition emerged among the six enzymes tested.

3.2.3.1 Pth1 inhibition

Using an array of NPPE, inhibition was screened for Pth1 inhibition across phylogenetic space. In particular, Pth1 homologs from *E. coli*, *S. typhimurium*, *P.*
*P. aeruginosa* (Clade 1), *M. tuberculosis* (Clade 2) *B. cereus*, and *S. aureus* (Clade 3) were included. Figures 3.7 shows a tabulation of the inhibitory profiles for each homolog.

![Figure 3.7 Patterns of Pth1 inhibition for the top 25 inhibitory NPPE: Clade 1 Pth1 species showed a pattern of broad spectrum and clade specific inhibition. *P. aeruginosa* Pth1 showed some species specific inhibition but tended to follow the emerging trend of mostly broad spectrum and clade specific inhibition. Clade 2 was inhibited by both Clade 1 and Clade 3 natural product plant extracts as well as those not seen in Clade 1 or Clade 3.](image)

3.2.3.1.1 Narrow spectrum inhibition possible across phylogenetic space

With some NPPE, Pth1 inhibition strictly followed the clade boundary as for *

*Ardisia compressa, Mandevilla veraguasenis, Exothea paniulata*, and *Inga sierra* (extract numbers 3, 4, 5, and 6, respectively in Figure 3.7 and Appendix A) which was only inhibitory within Clade 1 enzymes and the intermediate Clade 2 homolog, MtuPth1. Extracts from extracts 15. *Acacia aulacocarpa*, 16. *Ocotea floribunda*, and 18. *Urera caracasma* (extract numbers 15, 16, and 18, respectively in Figure 3.7 and Appendix A)
demonstrate narrow spectrum inhibitory activity against just PaPth1 within Clade 1. Similarly, *Albizia adenocephala* inhibits BcPth1 and MtuPth1 (extract number 14 in Figure 3.7). Likewise, SaPth1 showed narrow spectrum inhibition, but at a much greater occurrence than BcPth1. Where BcPth1 was inhibited by one narrow spectrum extract, SaPth1 was inhibited in a narrow spectrum fashion by ten other NPPE (Figure 3.7 extract numbers: 19. *Psychotria parvifolora* 25. *Grevilla lilliana*, 21. *Cinnamomum tonduzii*, 22. *Syzygium johnsonii*, 23. *Ocotea species from Los llama*, 25. *Grevilla lilliana*, 26. *Neolitsea dealbata* (listed in Appendix A), 40. *Cryptocarya corrugate* (listed in Appendix A), 55. *Bursera* (listed in Appendix A), and 56. *Bocconia frutescens* (listed in Appendix A)). This provided the first indication that narrow spectrum inhibition was possible. Again, it must be kept in mind that the inhibitory activity may not be due to a single component, but the potential for small molecule inhibition is clear. Even with the high degree of sequence similarity, differences in the active site exist and lead to differential small molecule inhibition based on findings from nucleotide inhibition (discussed further in chapter 5).

Interestingly, Pth1 from *P. aeruginosa* and *M. tuberculosis* were inhibited by the most extracts yet bacterial infections of *P. aeruginosa* in humans are extremely difficult to treat due to natural pan-drug resistances. Both *P. aeruginosa* and *M. tuberculosis* have commonly been found in soil. Past studies have shown selective pressure from natural and antimicrobial sources to drive pan- and multi-drug resistances in both species. (Lopez-Causape, 2018; Palomino and Martin, 2014). This present study has identified several novel narrow spectrum inhibitors of PaPth1 and MtuPth1 that could potentially
inhibit P. aeruginosa and M. tuberculosis, given the compound(s) responsible for inhibition can be discovered.

With the understanding that natural product extracts are heterogeneous mixtures, several distinct patterns of inhibition were observed. In some cases, broad spectrum inhibition of all Pth1 tested was observed. Thus, broad spectrum inhibition was possible. In recent years, the pharmaceutical and medical industries have started to move away from broad spectrum antibiotics. Broad spectrum inhibition has been noted for the increased colonization of and infection by C. difficile as well as increases in antibiotic resistances (Schäffler, 2018). For this reason, pharmaceutical companies have overlooked Pth1 as a viable drug target. It was assumed due to the vital function of Pth1 that Pth1 would only cause broad spectrum inhibition. However, this current project confirmed narrow spectrum or clade specific inhibition of Pth1 were possible in all homologs tested. Therefore, this brought greater promise to bacterial Pth1 being a novel antibiotic drug target because narrow spectrum inhibition was observed in all the homologs tested.

3.2.3.2 Patterns of Inhibition throughout clades

The patterns of inhibition that emerged between Clade 1, Clade 2, and Clade 3 fits the phylogenetic data. Specifically, Clade 2 Pth1s were inhibited by inhibitors narrow spectrum or clade specific inhibitors of Clade 1 and narrow spectrum inhibitors of Clade 3. However, outside of the Clade 2 Pth1 enzyme tested, these inhibitors were limited to their respective clades. Like mycobacteria sharing traits of both gram-negative and gram-positive bacteria, mycobacterial Pth1 was inhibited by both gram-negative Pth1only
(Clade 1) and gram-positive only (Clade 3) inhibitors of Pth1. This further illustrated Clade 2 being an intermediate clade mixed with, but closer related to Clade 1 Pth1s. For example, *Inga sierra*, *Ocotea floribunda* (Clade 1), *Syzygium johnsonii* and *Cinnamomum tonduzii* (Clade 3) had observed inhibition in MtuPth1.

Clade 1 had the most susceptibility to NPPE inhibition. Broad spectrum, narrow spectrum and some clade specific inhibition was observed in Clade 1 homologs. Clade 3 homologs showed the least susceptibility. These homologs demonstrated little broad spectrum inhibition in the presence of NPPE. As such, Clade 3 homologs had characteristically more narrow spectrum inhibition observed. These findings demonstrate the potential not only for broad spectrum inhibition, but also narrow spectrum. It seemed probable the lowered substrate affinity discussed in chapter 4 played a large role in the inhibition profiles of each phylogenetic clade.

3.2.3.3 Further qualitative clade characterizations

To further characterize the clades, it was noted that Clade 1 homologs typically had far greater yields than homologs from Clades 2 or 3. This was due to the solubility and ease of purifying Clades 1. Clade 1 homologs typically yielded 3.5 – 5 mg per liter of cell culture. However, PaPth1 did produce 33 mg from a 4 L cell culture. Clade 2 homologs yielded 1.5 – 2 mg of soluble enzyme per liter of cell culture. While approximately 1.3 – 1.5 mg of soluble protein could be purified from a 1 L culture of Clade 3 homologs. Clade 3 homologs required the most optimization and were significantly harder to express and purify than clades 1 or 2. Again, as there were
differences in plasmids or plasmid DNA sequences, these were also seen as largely correlative.

3.3 Conclusions

This study discovered three distinct phylogenetic clades which could be exploited in the development of antibiotics that selectively inhibit an individual Pth1 homolog. The distinct clades based on sequence alignment showed gram-specificity. Pth1 showed divergence and diversity in its phylogeny. Three distinct clades, defined above, emerged with stochastic residue changes throughout their sequence that do not cause any significant changes in overall charge interactions apart from the gate loop region. These residues were mainly aromatic residues, such as phenylalanine and tyrosine, to aliphatic residues, such as leucine, glycine, and alanine. Despite this, key catalytic and putative RNA-binding residues in the catalytic pocket and helices 3 and 4 were highly conserved among all homologs screened. As such, the chemistry of the reaction was not altered as established by the kinetic parameters and small nucleotide inhibition (in chapter 4 and 5).

In contrast to their chemistry, biochemical and phylogenetic characteristics were quite different. Phylogeny fell along on gram-specific lines. This trend was supported by previous results regarding gram-specific tRNA and rRNA modifications (Fu and Fu-Liu, 2002; Klagbrun, 1973; Guenther, 1994). Clade 1 homologs derived from gram-negative bacteria were generally more soluble and susceptible to inhibition than Clade 2 or Clade 3 homologs. The ease of which Clade 1 homologs were expressed and purified also explained why Clade 1 Pth1 are overrepresented in protein databank. MtuPth1, a Clade 2
homolog, was an intermediate clade of Clade 1 and Clade 3 homologs with respect to gram-status, solubility, inhibition, and kinetics, but not expression. This was indeed an emerging theme throughout this project. Conversely, Clade 3 homologs required the most optimization to produce minimal yields of soluble protein and was the least susceptible to NPPE inhibition. This clade was mostly inhibited in a narrow spectrum fashion.

Importantly, all homologs were susceptible to narrow spectrum inhibition. As more antibiotic-resistant infections have emerged, the use of broad spectrum antibiotics has been discouraged. Broad spectrum antibiotics were known to hasten the rate of antibiotic-resistance in bacteria. Therefore, antibiotic drug targets capable of narrow spectrum inhibition have a greater clinical importance and a better chance of development compared to those drug targets only capable of broad spectrum inhibition. As such, the narrow spectrum inhibition observed provided further evidence that targeting bacterial Pth1 would be a viable means of potentially treating bacterial infections. Furthermore, this could potentially be used synergistically with other antibiotics that also inhibit protein biosynthesis or alone as there is a stoichiometric advantage of targeting Pth1 over the ribosome.

Likewise, HsPth1 was shown to be phylogenetically closer to HsPth2 than any bacterial Pth1 homolog. Therefore, it seemed likely that HsPth1 would retain the structure and chemistry of Pth1, but not the same biochemical characteristics, such as expression and solubility and inhibition. Thus, inhibition of a bacterial homolog might not inhibit HsPth1 as evidenced by the diversity in inhibition profiles through phylogenetic space.
While most of the data from biochemical and phylogenetic characterization sought to further the attractiveness of Pth1 as a novel antibiotic drug target, site directed mutagenesis of the gate loop region determined mutating aspartate/glutamate to proline could rectify solubility issues within certain homologs. This knowledge could pave the way to structurally study or identify inhibitors to less soluble bacterial Pth1 homologs in the future.

In conclusion, these findings further demonstrated the attractiveness of Pth1 as a novel drug target. Pth1 had three distinct, gram-specific phylogenetic clades. These clades behaved biochemically different with regards to expression, solubility, and inhibition with Clade 2 being intermediate between the very different Clade 1 and Clade 3 homologs. It was previously believed Pth1 inhibitors would only produce broad spectrum inhibition. However, this project showed that narrow spectrum inhibition was possible, and selective targeting of individual Pth1 homologs was plausible in the future. These two findings were of the greatest significance for antibiotic development because broad spectrum antibiotics are known to increase the rate of antibiotic resistance clinically. As a novel antibiotic drug target, Pth1 showed more promise because individual Pth1 homologs could be accomplished with narrow spectrum inhibition. As well, biochemical and kinetic differences can be exploited to selectively target individual Pth1 homologs. Furthermore, this project established that more difficult homologs could have their solubility increased via glutamate to proline mutation in the gate loop of Pth1. This would allow for future structural or kinetic studies and inhibition screens on difficult Pth1 homologs from medically relevant bacteria to occur more easily.
Chapter 4

Pth1 Kinetics

4.1 Background

Pth1 kinetics has been well researched in Clade 1 Pth1s, specifically EcPth1. Previous experiments have shown variation in polypeptide chain length alters kinetic parameters up to tetrapeptide length (Shiloch, 1975). As the nascent polypeptide chain length increases, Vmax decreases while Km increases (Shiloch, 1975). After tetrapeptide length, kinetics parameters of 17 μM and 42 pmol/min for Km and Vmax, respectively, remained relatively consistent (Shiloch, 1975).

Whereas previous studies focused on a single homolog this project’s scope allowed for a broader interpretation of Pth1 kinetics. This current project was the first to compare kinetic parameters of multiple Pth1 homologs spanning phylogenetic space. However, interpretations of this data must be bounded by the limited number of homologs from each clade that were tested: one Clade 1 Pth1 (EcPth1), one Clade 2 Pth1 (MtuPth1) and two Clade 3 Pth1s (BcPth1 and SaPth1). The inclusion of EcPth1, MtuPth1 and SaPth1 have the additional benefit of previous existing kinetic literature for
comparison. BcPth1 kinetic analysis using bulk peptidyl-tRNA, performed by a previous graduate student in this research group, was also included to provide a more robust characterization of Clade 3 Pth1 kinetics (Taylor-Creel, 2014). Similarly, previous unpublished values of EcPth1 were used solely for the purposes to verify the accuracy of the Wilkinson least squares non-linear regression employed in this project prior to use in the analyses of MtuPth1 and SaPth1 (Wilkinson, 1961).

The vast majority of the previously reported studies used diacetyl-lysyl-tRNA\textsuperscript{Lys} as Pth1 was found to have the greatest affinity for this partial substrate to all other peptidyl-tRNA species (Menninger, 1973). Bulk peptidyl-tRNA was used for each Pth1 homolog for this project allowing for a more complete understanding of Pth1 kinetics in its native state. Naturally, only a relative comparison can be made between this study and previous studies using diacetyl-lysyl-tRNA\textsuperscript{Lys}.

Similarly, bulk peptidyl-tRNA extracted from C600(TS) \textit{E. coli} cells would have polypeptide chain lengths that were not limited to just $N$-acetylated-dipeptidyl-tRNAs. Hence, one could reasonably assume a stochastic distribution between $N$-acetylated-peptidyl-tRNA to tetrapeptide chains or greater where V\textsubscript{max} has been empirically determined to remain consistent (Shiloch, 1975). Substrate affinity was also determined to increase as peptide length increased. Thus, Pth1 would have a greater likelihood of hydrolyzing peptidyl-tRNA with longer polypeptide chains at a constant rate. For this reason, steady state assumptions were used in determination of kinetic parameters.
4.2 Results and Discussion

Kinetics parameters were identified using bulk peptidyl-tRNA for Pth1 homologs representative of all three clades. Well studied homologs were selected as comparisons could be made to results in the published literature. When possible, previous data using full substrate were included into this current project as is the case with BcPth1 (Taylor-Creel, 2014). The results obtained were found to be consistent with those studies previously published using bulk peptidyl-tRNA but differed from those using radiolabeled isotopes or 3’-CCA mimics in absolute terms, but consistent in relative terms.

4.2.1 Verification of nonlinear regression analysis

Percentage of cleaved peptidyl-tRNA from the in vitro activity assay (section 2.2.10) was multiplied by the peptidyl-tRNA concentration to determine the amount of cleaved peptidyl-tRNA for each time interval. Data were then averaged for the triplicate measurement, and a standard deviation was determined. The error bars in Figure 4.1 were representative of the standard deviation of the averaged cleaved peptidyl-tRNA concentration. An initial estimate of $V_o$ was obtained by finding the slope of the tangent line at time zero for each peptidyl-tRNA concentration. An example of the cleaved peptidyl-tRNA versus time plot used for $V_o$ determination can be found in Figure 4.1. Unlike previous figures from this current project, black, green, and blue are used to indicate different initial bulk peptidyl-tRNA concentrations, $S_o$, rather than to differentiate between clades. Red, black and blue represented a $S_o$ of 28 μM, 56 μM, and
112 μM peptidyl-tRNA, respectively. Initial Vo for EcPth1 were 1.2 μmol/min, 2.7 μmol/min, and 3.5 μmol/min for a So of 28 μM, 56 μM, and 112 μM peptidyl-tRNA, respectively.

![E. coli Pth1 V₀ Determination](image)

Figure 4.1 V₀ determination of EcPth1: Initial V₀ for EcPth1 was determined at each bulk peptidyl-tRNA concentration. The slope of the tangent line at time zero was equal to V₀ for that substrate concentration.

Nonlinear regression of kinetic parameters has been demonstrated to be more accurate than linearized analyses. For this reason, experimentally determined V₀ for each substrate concentration were analyzed using a least squares nonlinear regression first described by Wilkinson et al (Wilkinson, 1961). Velocity and substrate concentrations were the only data needed to obtain provisional kinetic parameters using Wilkinson least squares nonlinear analysis. This analysis used can be seen in Tables 4.1. Wilkinson
least squares nonlinear analysis was chosen as this was the standard kinetic analysis in the literature, and what many more labor intensive, computational kinetic approximations have sought to improve.

Table 4.1: Determination of Provisional *E. coli* Pth1 Kinetic Parameters

<p>| Provisional <em>E. coli</em> Pth1 Kinetic Parameters: Least Squares Nonlinear Regression Analysis |
|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|</p>
<table>
<thead>
<tr>
<th>$S_o$</th>
<th>$V_{max _obs}$</th>
<th>$x = V^2$</th>
<th>$y = V^2/S$</th>
<th>$\alpha_i \Sigma (v^ix)$</th>
<th>$\beta_i \Sigma (x^2)$</th>
<th>$\gamma_i \Sigma (v^2y)$</th>
<th>$\delta_i \Sigma (v^2y)$</th>
<th>$\epsilon_i \Sigma (y^3)$</th>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3.5</td>
<td>0.30</td>
<td>0.09</td>
<td>0.03</td>
<td>0.03</td>
<td>0.01</td>
<td>0.01</td>
<td>0.002</td>
<td>0.1 x 10^{-2}</td>
</tr>
<tr>
<td>7</td>
<td>0.60</td>
<td>0.36</td>
<td>0.05</td>
<td>0.21</td>
<td>0.13</td>
<td>0.03</td>
<td>0.02</td>
<td>0.3 x 10^{-2}</td>
</tr>
<tr>
<td>14</td>
<td>1.20</td>
<td>1.44</td>
<td>0.10</td>
<td>1.73</td>
<td>2.07</td>
<td>0.13</td>
<td>0.15</td>
<td>1.2 x 10^{-2}</td>
</tr>
<tr>
<td>28</td>
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<td>2.72</td>
<td>0.09</td>
<td>4.49</td>
<td>7.40</td>
<td>0.16</td>
<td>0.26</td>
<td>1.0 x 10^{-2}</td>
</tr>
<tr>
<td>56</td>
<td>2.70</td>
<td>7.29</td>
<td>0.13</td>
<td>19.68</td>
<td>53.13</td>
<td>0.35</td>
<td>0.95</td>
<td>2.0 x 10^{-2}</td>
</tr>
<tr>
<td>84</td>
<td>3.10</td>
<td>9.61</td>
<td>0.11</td>
<td>29.79</td>
<td>92.34</td>
<td>0.35</td>
<td>1.10</td>
<td>1.3 x 10^{-2}</td>
</tr>
<tr>
<td>112</td>
<td>3.50</td>
<td>12.25</td>
<td>0.10</td>
<td>42.88</td>
<td>150.10</td>
<td>0.38</td>
<td>1.34</td>
<td>1.1 x 10^{-2}</td>
</tr>
<tr>
<td>Sums</td>
<td>98.81</td>
<td>305.18</td>
<td>1.41</td>
<td>38.2</td>
<td>7.0 x 10^{-2}</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$\Delta_i = a_i \epsilon_i - y_i \delta_i = (98.81*0.07)-(1.41*3.82) = 1.06$

$V_{max}^0 = (\beta_i \epsilon_i - \delta_i)^2/\Delta_i = (305.18*0.07-3.82^2)/1.06 = 5.02$

$K_{m}^0 = (\beta_i \epsilon_i - \delta_i) / \Delta_i = ((305.18*1.41) - (98.81*3.82))/1.06 = 49.98$

$V_{max}^0$ - provisional $V_{max}$  \hspace{0.5cm} $K_{m}^0$ - provisional $K_{m}$

Provisional kinetic parameters were refined using least squares of the Michaelis-Menten equation and the first derivative with respect to $K_m$ as seen in Table 4.2. This analysis method was verified against previously unpublished EcPth1 kinetic data using bulk peptidyl-tRNA performed by a previous graduate student in this research group prior to expanding this method to other homologs. Previous results were determined to be $V_{max}$ of 4.5 ± 0.1 μmol/min and $K_m$ of 40.0 ± 3.0 μM. Representative analyses of each clade (MtuPth1 and SaPth1) can be found in Appendix B.
Following nonlinear regression analyses, the $V_0$ for each $S_0$ concentration were plotted. Further refinement of kinetic parameters was carried out using the substrate $V_{max}$ and $K_m$ determined from the Wilkinson nonlinear regression to estimate expected values to fit a curve to experimentally derived values (seen in blue in Figures 4.2, 4.3, and 4.4). These expected values were plotted using the Michaelis-Menten equation, and refinement using the solver function on Excel was performed to further minimize the least squares. The slower rate seen in the curves at later time intervals were due to the lack of substrate as free tRNA was greater than that of bulk peptidyl-tRNA. As more peptidyl-tRNA was cleaved, less substrate was present for catalysis as Pth1 will only hydrolyze peptidyl-tRNA or N-protected acyl-aminoacyl-tRNA. This trend was seen throughout all kinetic analysis presented in this chapter.
4.2.2 Kinetic parameters across clades

This current project sought to characterize kinetic parameters across phylogenetic space. As previously mentioned, the homologs selected were the most studied from each phylogenetic clade. The Wilkinson nonlinear regression analysis was used on EcPth1, MtuPth1, and SaPth1 to estimate kinetic parameters. Previous results from BcPth1 were included in this current project allowing for a slightly more expanded interpretation of Clade 3 homologs as this study also used bulk peptidyl-tRNA (Taylor-Creel, 2014).

4.2.2.1 Clade 1 Pth1 kinetic parameters

EcPth1, a Clade 1 homolog, was found to have a Vmax of 5.1 μmol/min ± 0.1 μmol/min and a Kᵐ of 51.0 μM ± 3.0 μM. Kcat was approximated to be 3.0 x 10⁻³ s⁻¹. This resulted in a catalytic efficiency (Kcat/Km) of 6.3 x 10⁻⁵ μmol⁻¹ s⁻¹. The R² value of 0.99 from the nonlinear regression fit was significantly higher than the linearized R² value of 0.83 from the original fit. This was indicative that the nonlinear regression utilized provided a better fit and kinetic approximations compared to the previous linearized model using an integrated Michaelis-Menten equation.

EcPth1 had the highest Kᵐ of all homologs tested even when the partial substrate diacetyl-lysyl-tRNA_Lys was used. Previous Kᵐ estimates remained consistent between 5.5 – 6.0 μM for wildtype EcPth1 in vitro (Giorgi, 2011; Kabra, 2017; Goodall, 2004; Schmitt, 1997). Higher Kᵐ values indicated a weaker affinity to form an enzyme-
substrate complex (Robinson, 2015). No matter if partial or full substrate were used in kinetic analysis, $K_m$ values for EcPth1 were consistently weaker than others reported (Giorgi, 2011; Shiloch, 1975; Goodall, 2004; Bal, 2007; Bonin, 2002). Thus, decreased affinity for partial or bulk peptidyl-tRNA was seen as a trend, and served as further validation of EcPth1’s kinetics parameters in this project. Evidence of a higher $K_m$ value could also be established from the less defined parabolic fit of the data seen in Figure 4.2 and Figure 4.3 compared to MtuPth1’s fit (Figure 4.4) and SaPth1’s fit (Figure 4.5).

Figure 4.2 Nonlinear regression fit of EcPth1 kinetics: EcPth1 rate data (in red) was fit with the expected values (in blue) using $V_{max}$ and $K_m$ obtained from the nonlinear regression and $S_0$ concentrations. These yielded an $R^2$ value of 0.99 between the actual and expected data.
While EcPth1 had the lowest substrate affinity, it did not have the lowest catalytic efficiency. Kinetic analysis of PaPth1, the other Clade 1 homolog tested, yielded similar results to EcPth1 with a Vmax of 5.0 ± 0.9 μmol/min, Km of 47 ± 0.9 μM, a Kcat of 3.0 x 10^-3 s^-1, and a catalytic efficiency of 6.3 x 10^-5 μmol^{-1}s^{-1} (Figure 4.3 and Table 4.3). PaPth1 was the least efficient enzyme kinetically out of all the homologs tested. Both a general trend that substrate affinity and catalytic efficiency were decreased in both homologs in comparison to Clade 2 or Clade 3 homologs.

Figure 4.3 Nonlinear regression fit of PaPth1 kinetics: PaPth1 rate data (in red) was fit with values (in blue) using Vmax and Km obtained from the nonlinear regression and S0 concentrations.
4.2.2.2 Clade 2 Pth1 kinetic parameters

The Clade 2 homolog, MtuPth1, was found to have a $K_m$ of $17.0 \, \mu M \pm 1.3 \, \mu M$, a $V_{\text{max}}$ of $5.3 \, \mu mol/min \pm 0.2 \, \mu mol/min$ (Figure 4.3). $K_{\text{cat}}$ and $K_{\text{cat}}/K_m$ for MtuPth1 were $3.2 \times 10^{-3}$ and $1.9 \times 10^{-4}$, respectively (Table 4.3). The nonlinear regression fit had a $R^2$ value of 0.96. While the $V_{\text{max}}$ was similar to that of EcPth1, its $K_m$ was three times less than that of EcPth1. This meant that the overall biochemical reaction was consistent between EcPth1 and MtuPth1. This was expected due to the high conservation of the structural folds and catalytic residues. No previous $V_{\text{max}}$ data existed in the literature. Thus, $V_{\text{max}}$ results from this project could not be compared to past studies.

![Figure 4.4 Nonlinear regression fit of MtuPth1 kinetics: MtuPth1 data (in red) was fit with the expected values (in blue) using $V_{\text{max}}$ and $K_m$ obtained from the nonlinear regression and $S_0$ concentrations. This fit produced an $R^2$ value of 0.96.](image-url)
A $K_m$ of 0.7 μM was found by measuring the hydrolysis of tritium labeled diacetyl-[3H]-lysyl formtRNA$^{Lys}$ (Bal, 2007). The difference in results likely stemmed from different the methodologies in obtaining kinetic parameters and nascent polypeptide length. Residues radiolabeled with tritium or other radioactive isotopes could provide much greater sensitivity, and therefore could easily account for the differences in the data between this project and the previous project (Randerath, 1970). Despite this, both $K_m$ values were within micromolar range.

MtuPth1, showed similar results to previously published kinetics of Clade 3 homologs using full substrate (Taylor-Creel, 2014). MtuPth1 kinetic parameters, specifically its $K_m$ value, was intermediate of Clade 1 homologs and Clade 3 homologs. While the chemistry of each homolog tested was similar, the substrate affinity differed across phylogenetic space. Thus, MtuPth1 had 33% greater substrate affinity than EcPth1.

### 4.2.2.3 Clade 3 Pth1 kinetic parameters

Kinetic parameters for Clade 3 homologs have been sparse in the literature to date. Past studies were limited to fluorophore labeled partial substrate using a different assay in the case of SaPth1. However, results previously published from this research group were used for BcPth1 (Taylor-Creel, 2014). There were no differences between the assay or substrate used allowing for direct comparisons to be made. Parameters for BcPth1 were $5.5 \pm 0.3 \, \mu$mol/min and $8.0 \pm 1.0 \, \mu$M for $V_{max}$ and $K_m$, respectively.
SaPth1 kinetic parameters were $6.0 \pm 0.1 \, \mu\text{mol/min}$ and $10.0 \pm 1.0 \, \mu\text{M}$ for $V_{\text{max}}$ and $K_m$, respectively. $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$ were $3.6 \times 10^{-3} \, \text{s}^{-1}$ and $3.6 \times 10^{-4} \, \mu\text{mol}^{-1}\cdot\text{s}^{-1}$ (Table 4.3) indicating greater substrate affinity, turnover rate, and catalytic efficiency than Clade 1 and Clade 2 homologs.

Figure 4.5 Nonlinear regression fit of SaPth1 kinetics: SaPth1 rate data (in red) was fit with the expected values (in blue) using $V_{\text{max}}$ and $K_m$ obtained from the nonlinear regression and $S_o$ concentrations. This fit produced an $R^2$ value of 0.99.

The fit for SaPth1 kinetics was found to be 99% correlated to the expected values (as noted in Figure 4.5). $V_{\text{max}}$ for SaPth1 was 0.5 $\mu\text{mol/min}$ faster than BcPth1. However, BcPth1 had slightly more affinity to peptidyl-tRNA than SaPth1. $K_m$ values were with 2.0 $\mu\text{M}$ of one another (Table 4.3).
Previous studies of Clade 3 Pth1 homologs have been limited in their scope. Previous work in our research group demonstrated the kinetic parameters of BcPth1 to be a Vmax of 5.5 μmol/min and a Kᵡ of 8 μM using bulk peptidyl-tRNA (Holloway, 2015; Taylor-Creel, 2014). Other studies on SaPth1 using diacetyl-[³H]-lysyl-tRNA_Lys found kinetic parameters in the micromolar range however significantly smaller values of Vmax of 0.13 μmol/min and Kᵡ of 2.8 μM were reported (Bonin, 2002).

Kinetic analysis was also carried out on the SaPth1 E99P mutant to determine the gate loop mutations’s impact on SaPth1 kinetics. Kinetic analysis was not carried out on SaPth1 Q100P due to the loss of solubility. Likewise, kinetic analysis of SaPth1 Q102V as this mutation did not yield any difference in solubility from WT SaPth1. SaPth1 E99P Vmax and turnover values were both very similar (Figure 4.6). Therefore, site directed mutagenesis of the gate loop region did not affect the overall chemical reaction. However, Kᵡ and Kcat/Kᵡ of SaPth1 E99P decreased by half to 20.0 ± 1.7 μM and 1.8 x 10⁻⁴ μmol⁻¹·s⁻¹, respectively. As such, mutating aspartate to proline in the gate loop region of SaPth1 resulting in half the substrate affinity and catalytic efficiency of WT SaPth1, and was more similar to the intermediate Clade 2 homologs kinetic characteristics with respect to Kᵡ and Kcat/Kᵡ.
Figure 4.6 Gate loop mutation affects Km but not Vmax: WT SaPth1 (red circles) was compared against SaPth1 E99P. Vmax was consistent between WT and the mutant, however the Km for SaPth1 E99P was double that of WT SaPth1.
Table 4.3 Kinetic Parameters between clades

<table>
<thead>
<tr>
<th>Pth1 Homolog</th>
<th>Vmax (μM*min⁻¹)</th>
<th>Km (μM)</th>
<th>Kcat (s⁻¹)</th>
<th>Kcat/Km (μmol⁻¹*s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>5.1 ± 0.1</td>
<td>51.0 ± 3.0</td>
<td>3.0 x 10⁻³</td>
<td>5.9 x 10⁻⁵</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>5.0 ± 0.1</td>
<td>47.0 ± 0.9</td>
<td>3.0 x 10⁻³</td>
<td>6.3 x 10⁻⁵</td>
</tr>
<tr>
<td>M. tuberculosis</td>
<td>5.3 ± 0.2</td>
<td>17.0 ± 2.2</td>
<td>3.2 x 10⁻³</td>
<td>1.9 x 10⁻⁴</td>
</tr>
<tr>
<td>S. aureus</td>
<td>6.0 ± 0.1</td>
<td>10.0 ± 0.4</td>
<td>3.6 x 10⁻³</td>
<td>3.6 x 10⁻⁴</td>
</tr>
<tr>
<td>B. cereus*</td>
<td>5.5 ± 0.3</td>
<td>8.0 ± 1.0</td>
<td>3.3 x 10⁻³</td>
<td>4.1 x 10⁻⁴</td>
</tr>
</tbody>
</table>

*Denotes values previously published using full substrate

As, such Km values from this and previous studies were consistently in micromolar range despite the differences in substrate. Any decrease in substrate affinity between partial and full peptidyl-tRNA substrate was likely due to the differences in assays and increased length in the nascent peptide chain as previously noted.

Clade 3 homologs hydrolyzed peptidyl-tRNA faster, but the Vmax and Kcat were consistent with those from Clade 1 and Clade 2. As such, the chemistry across phylogenetic space was similar. This was an expected result given the highly conserved structure and catalytic residues across phylogenetic space. Clade 3 homologs had the greatest substrate affinity among the three clades using bulk peptidyl-tRNA.
4.2.3 Comparison of Pth1 clade kinetics

As can be ascertained from Table 4.3, Vmax only varied slightly between the clades. Vmax is defined as the maximum velocity an enzymatic reaction can attain when the enzyme is fully saturated with a substrate. The Clade 1 homologs, EcPth1 and PaPth1 had the lowest Vmax throughout the clades (Figure 4.7). Thus, at saturation, EcPth1 and PaPth1 had the slowest rate of reaction. SaPth1 was found to have the highest Vmax. However, these values were within a 1.0 μmol/min range. Larger variations were seen in Vmax when compared to past results, such as the increase of MtuPth1 (Bal, 2007).

Figure 4.7 Comparison of kinetics from different phylogenetic clades: SaPth1 (in black) representative of Clade 3 homologs had a greater Vmax, Kcat, and Kcat/Km than the intermediate Clade 2 homolog, MtuPth1 (in green), and Clade 1 homologs, EcPth1 (navy blue) and PaPth1 (blue).

However, this likely arose from differences in assay type and use of partial substrate (Figure 4.7). The Michaelis constant, Km, represents the concentration (in
molarity) when rate is half of the Vmax. As such, a higher $K_m$ has a less favorable binding with its substrate and requires greater substrate concentration to reach its Vmax. As such, the enzyme has different rates dependent on substrate concentration due to different levels of saturation. Alternatively, a lower $K_m$ means the enzyme has more favorable binding to its substrate. This would also require lower substrate to achieve saturation. For this reason, enzymes possessing a lower $K_m$ typically have a more consistent rate due to their being saturated. As such, $K_m$ has also been described as its substrate affinity, or how favorably an enzyme will bind to its substrate (Robinson, 2015). In this project, EcPth1 $K_m$ value was found to be 15% greater than BcPth1, 20% greater than SaPth1, and 33% greater than that of MtuPth1. Therefore, EcPth1 had less favorable binding to peptidyl-tRNA than Clade 2 and Clade 3 homologs. This was evident from the Similarly, MtuPth1’s $K_m$ was 47% and 58% greater than the $K_m$ for BcPth1 and SaPth1, respectively.
Figure 4.8 Comparison of $K_m$ from different phylogenetic clades: Substrate affinity and catalytic efficiency was greater in Clade 3 homologs with clade 1 homologs having the lowest substrate affinity and catalytic efficiency. Clade 2 was intermediate of Clade 1 and Clade 3 with respect to $K_{cat}$, $K_m$, $K_{cat}/K_m$, and $V_{max}$.

Based on these findings, the homologs tested from Clade 2 and Clade 3 had an increased for affinity for peptidyl-tRNA compared to EcPth1. This was consistent with other findings in the literature. Previous studies using partial substrate found EcPth1 had consistently lower substrate affinity (Giorgi, 2011; Schmitt, 1997; Goodall, 2004). Likewise, MtuPth1 and SaPth1 were found to have greater affinity for peptidyl-tRNA even when a partial peptidyl-tRNA substrate was used (Bal, 2007; Bonin, 2002). Homologs from Clade 2 and Clade 3 have consistently shown greater affinity towards peptidyl-tRNA than EcPth1 both in the literature and this current project. It is of note that the SaPth1 E99P mutant determined perturbations in the gate loop would, at least in part, substantially decrease substrate affinity.
This also further demonstrated that MtuPth1 was intermediate of the Clade 1 and Clade 3 homologs. MtuPth1 had less affinity towards peptidyl-tRNA than Clade 3 homologs, BcPth1 and SaPth1. However, MtuPth1 had greater affinity towards peptidyl-tRNA than the Clade 1 homolog, EcPth1. Likewise, the Vmax of MtuPth1 and EcPth1 were closer to each other than the Clade 3 homologs.

4.3 Conclusions

This was the first study to compare the kinetic parameters of the different clades using full substrate. Prior to this knowledge of Pth1 kinetics was derived largely from EcPth1. These results showed that phylogenetic clades behave differently from one another while maintaining structural and mechanistic conservation, just like the trends observed in gram-specificity, expression and solubility, and inhibition profiles.

One such insight was Vmax for each homolog tested were similar. The hydrolytic residues and structural folds of bacterial Pth1 being highly conserved. Similarly, homologs from all three clades hydrolyze the bond between the 3′-adenosine and the growing peptide chain. Therefore, the conservation in activity, structure, and important residues could explain why the Vmax were similar across the clades. The chemistry of the reaction does not change.

In contrast, the large differences in $K_m$ and $K_{cat}/K_m$ values were observed. $K_m$ values have been related to the affinity of Pth1:bulk peptidyl-tRNA complex formation while $K_{cat}$ refers to the turnover rate (Robinson, 2015). $K_{cat}/K_m$ is related to the catalytic efficiency of an enzyme. EcPth1 had the highest $K_m$ value out of all the homologs tested,
while Clade 2 and Clade 3 homologs had lower $K_m$ values. This relative trend agreed with previous studies (Giorgi, 2011; Schmitt, 1997; Goodall, 2004). Analyses with partial substrate showed EcPth1 had a $K_m$ consistently between 5 – 6 μM. However, previous kinetics studies using N,N-diacetyl-lysyl-tRNA$^{\text{Lys}}$ had greater substrate affinity and better turnover numbers and catalytic efficient than with bulk peptidyl-tRNA. Previous experiments using partial substrate found EcPth1 to have a $K_{\text{cat}}$ of 1.0 – 3.6 s$^{-1}$ and $K_{\text{cat}}/K_m$ to be $\sim$0.5 μmol$^{-1}$*s$^{-1}$. As only a relative comparison can be made, it could be inferred the lower substrate affinity and catalytic efficiencies could arise for a greater specificity to specific tRNA species. Previous studies determined that peptidyl-tRNAs do not accumulate at the same rate with tRNA$^{\text{Lys}}$ having 75% of its cellular concentrations accumulating as peptidyl-tRNA rapidly (Menninger, 1978). The family of tRNAs consisting of aspartate, histidine, phenylalanine, threonine and valine accepting tRNAs accumulated the fastest (Menninger, 1978). Not inconsequentially, many of these residues are common at the start of many E. coli protein amino acid sequences (Cruz-Vera, 2000). The fact that so many ribosome stalling residues are at the beginning of many E. coli protein sequences has led to the suggestion that ribosome stalling could be a means to ensure enough tRNA are present for translation to occur (Cruz-Vera, 2000). Likewise, peptidyl-tRNA accumulation and starvation would be noticed much more quickly with more commonly used tRNAs. Therefore, it seems likely there would be a greater affinity for the more rapidly accumulating peptidyl-tRNAs and ribosome stalling is a way to check and maintain translational health (Menninger, 1978; Cruz-Vera, 2002). For example, while EcPth1 will hydrolyze prolyl-tRNA$^{\text{Pro}}$, it does so much more slowly and less efficiently than dipeptide - hexapeptide tRNA$^{\text{Lys}}$ until a concentration threshold
of Pth1 was met (Janssen, 2009). As such, this reduction in $K_m$, $K_{cat}$, and $K_{cat}/K_m$ could also be a reflection that at higher in vitro substrate concentrations, Pth1 would take longer to hydrolyze peptidyl-tRNA which Pth1 has lower affinity or Pth1-concentration dependent peptidyl-tRNA species such as prolyl-tRNA$_{Pro}$.

While very few kinetics studies of any Clade 2 or 3 homologs exist outside this current project, MtuPth1 and SaPth1 were previously shown to have a $K_m$ of 0.7 μM and 2.8 μM, respectively (Bal, 2007; Bonin, 2002). $K_m$ values from this current project and previous studies clearly showed the Clade 1 homolog, EcPth1, had less affinity to peptidyl-tRNA than Clade 2 and Clade 3 homologs. Conversely, Clade 3 homologs had the lowest $K_m$ values. As such, Clade 3 homologs consistently showed more favorable binding to bulk peptidyl-tRNA than Clade 1 and Clade 2.

Clade 2 being an intermediate clade of Clade 1 and Clade 3 was increasingly observed throughout this project. MtuPth1 had greater substrate affinity than Clade 1, but lower substrate affinity than Clade 3 homologs. This trend was also seen in mixed or indeterminate gram status, solubility yields, and inhibition profiles. MtuPth1’s substrate affinity was more similar to Clade 3 than to Clade 1.

Likely, differences in substrate affinity and clade specific gate loop motifs caused the differences seen in inhibition profiles across phylogenetic space. Substrate affinity could also account for the increased incidence of broad spectrum and clade-specific inhibition seen in Clade 1 homologs. Based on this data, EcPth1 would have less favorable binding to peptidyl-tRNA. This could potentially allow for greater inhibition as EcPth1 would require greater peptidyl-tRNA concentrations to reach saturation. As
such, EcPth1 would have more enzyme available to bind more favorable substrates. As such, greater levels of inhibition. It seemed likely that other Clade 1 homologs would also have lowered substrate affinity like EcPth1 based on the high degree of conservation and shared inhibition characteristics. Moreover, narrow spectrum inhibition was seen as substrate affinity increased in Clade 2 and Clade 3. Based on this data, Clade 3 homologs were more saturated and had greater substrate affinity than Clade 2 and Clade 3. As such, Clade 3 homologs were less susceptible to inhibition, and any inhibitory compound(s) would likely have to provide more favorable Pth1 binding than peptidyl-tRNA. Similarly, SaPth1 E99P possessing the gate loop mutation had half the substrate affinity of wildtype SaPth1 suggesting, at least in part, the gate loops involvement in substrate affinity. The most likely explanation for this would be the exchange of a flexible aspartate residue to a rigid proline residue not allowing for wider lid opening or widening of the catalytic pocket.

One possible explanation for differences in substrate affinities could be due to the phylogenetic gram-specificity and gram-specific tRNA modifications. tRNA methylation, 1-methyladenine, and modifications were seen in Clade 2 and Clade 3, but not present in Clade 1. In addition, some mycobacterial species, such as *M. smegmatis*, are known to lack ribothymidine in their TψC arm (Klagbrun, 1972; Vani, 1979). Previous models have suggested Pth1 tightly interacts with the TψC arm and plays a role in substrate recognition (Hames, 2014). Outside of the TψC arm and 3’-CCA acceptor stem, Pth1 interaction with peptidyl-tRNA has not been noted (Hames, 2014). As such, tRNA methylation in the TψC arm of peptidyl-tRNA could aide in peptidyl-tRNA recognition by Clade 2 and Clade 3 Pth1s similar to other tRNA modifying enzymes.
Past results found that tRNA mimics missing the TψC arm showed EcPth1 had 10-fold less substrate affinity, but retained the ability to hydrolyze peptidyl-tRNA (Giorgi, 2011). Furthermore, Pth(rap) mutant harboring R133A mutation had significantly lowered specific activity. Mutants possessing both R133A and K105A lacked both recognition of the 5’-phosphate of peptidyl-tRNA and loss of specific activity suggesting both were involved in substrate recognition. While 1-methyladenine is only found at A22 and A58 of tRNA in bacteria, 1-methylguanine or 2’-O-methyluridine modifications are present in or near the 5’-tRNA acceptor stem from some gram positive not present in gram-negative species (Boccaletto, 2017). Because peptidyl-tRNA is bound at the 5’- and 3’-acceptor stem, wider lid loop and catalytic pocket opening could be required to accommodate for the presence of these tRNA modifications as the significant loss of substrate affinity caused by a gate loop mutation might indicate (Fromant, 1999). Based on the available data, the current hypothesis is that methylation in both the TψC arm and the 5’-acceptor stem affect substrate affinity in Clade 2 and Clade 3 homologs. However, without a high-resolution bound Pth1:peptidyl-tRNA structure, this remained highly speculative. Further work would be needed to resolve tRNA methylations role, if any, in Pth1 recognition or substrate affinity.

In conclusion, the chemistry of the reaction does not change across phylogenetic space. In contrast, K_m values were different. EcPth1, the Clade 1 homolog, had the lowest affinity for peptidyl-tRNA based on results from current and previous projects. MtuPth1 was kinetically intermediate of the Clades 1 and 3 homologs once again. This further supported previous evidence found in the inhibition screen and biochemical characterization that MtuPth1 was the intermediate clade. The Clade 3 homologs had the
greatest affinity for substrate and the fastest Vmax of the enzymes tested so far when bulk peptidyl-tRNA was used. These differences could account for the phylogenetic characteristics seen during the NPPE inhibition screen. Furthermore, the evidence from phylogenetic analysis and kinetic characterization that tRNA methylation in the TψC arm of peptidyl-tRNA could play a role in substrate affinity or recognition as previous models have suggested. However, these results should be expanded to more Pth1 homologs across phylogenetic space and peptidyl-tRNA substrate from a gram-positive species to confirm the emerging kinetic trends due to the limited number of homologs tested.
Chapter 5

Nucleotide inhibition of Pth1

5.1 Background

Nucleotide and nucleoside analogs were previously found to bind to helices 5 and 6 of MtuPth1 (Kabra, 2016; Giorgi, 2011). No activity assays were done in conjunction with these studies. Therefore, it remained unknown whether nucleotides or nucleotide analogs would actually have an inhibitory effect on any Pth1 homolog.

Pth1 was shown to complex with peptidyl-tRNA at the 3’-CCA acceptor stem based on low-resolution small angle neutron scattering (Hames, 2014). Virtual docking screens also determined inhibition likely occurred by interaction with residues within the catalytic pocket (Harris, 2011). This study was conceived and undertaken to address the question if nucleotide species themselves were inhibitory based on the structural data from the previous SANS bound complex by testing nucleotides and nucleosides for inhibition against Pth1 homologs.
5.2 Results and Discussion

This current project initially screened EcPth1 inhibition against adenosine and adenosine phosphates. Of the adenosine species, only adenosine triphosphate was found to be weakly inhibitory *in vitro*. However, EcPth1 was found to be inhibited by ATP at eukaryotic concentrations. Thus, nucleotide inhibition against homologs from Clade 1 and Clade 3 were extended to include other nucleotides and nucleosides. Sixteen nucleotide species were tested for inhibitory activity against EcPth1, StPth1, and SaPth1. The sixteen nucleotides tested were: adenosine, adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), cytidine, cytosine monophosphate (CMP), cytosine diphosphate (CDP), cytosine triphosphate (CTP), guanine, guanine monophosphate (GMP), guanine diphosphate (GDP), guanine triphosphate (GTP), uridine, uridine monophosphate (UMP), uridine diphosphate (UDP), uridine triphosphate (UTP). Following initial qualitative screen against all sixteen nucleotides, those nucleotides which exhibited inhibition against EcPth1, StPth1 and SaPth1 were furthered screened to determine an IC$_{50}$ value for that homolog, as seen in Figure 5.1.
Figure 5.1 Nucleotide inhibition of bacterial Pth1 homologs: Both EcPth1 and SaPth1 were inhibited by both ATP and GDP. Each had IC$_{50}$ values of 5 mg/mL and 10 mg/mL for ATP and GDP, respectively. StPth1 was only inhibited by GDP with an IC$_{50}$ value of 2.5 mg/mL.

5.2.1 ATP inhibition

*In vitro* reactions containing 30 μM Pth1, 30 μM peptidyl-tRNA, and 4 μL ATP. ATP concentrations were varied from 20 mg/mL to 0.63 mg/mL utilizing a 1:1 serial dilution prior to the 4 μL ATP addition. EcPth1 and SaPth1 were both inhibited by ATP *in vitro*. Interestingly, StPth1 was not inhibited by ATP.
Figure 5.2 ATP Inhibition of EcPth1: A. Urea-PAGE of EcPth1 with ATP at decreasing concentrations. B. Bar graph determination of EcPth1: EcPth1 was inhibited by ATP with an IC\textsubscript{50} of 5 mg/ml ± 0.95 mg/mL ATP. These results were similar to those of SaPth1.

5.2.1.1 EcPth1 ATP inhibition

Inhibition screens to determine an IC\textsubscript{50} value were determined for EcPth1. As can be seen in Fig 5.2, EcPth1 had an IC\textsubscript{50} value of 5.0 ± 1.0 mg/mL. Pth1 activity did not fully return even at the lowest ATP concentrations tested. This was likely due to lower concentrations of ATP needed to achieve the return to activity. This seemed likely as SaPth1 was not inhibited by 0.6 mg/mL ATP and all the GDP inhibition curves approached zero. In the future, concentrations of 0.3 mg/mL or lower should be tested against EcPth1 to verify this hypothesis. Importantly, 0.6 mg/mL ATP (1.2 mM ATP) and above was within the eukaryotic physiological concentrations range of 1 mM to 10
mM ATP (Traut, 1996). This was also within known bacterial concentration of ATP. Previous quantitative ATP fluorescent based experiments established a median concentration of $1.54 \text{ mM} \pm 1.22 \text{ mM}$ (or $0.78 \text{ mg/mL} \pm 0.62 \text{ mg/mL}$) in *E. coli* (Yaginuma, 2014). Using the median value of $1.54 \text{ mM}$, the IC$_{50}$ value of $5.0 \pm 1.0 \text{ mg/mL}$ was 6.4 times higher than intracellular ATP concentrations meaning EcPth1 was minorly inhibited by ATP. Therefore, at physiological eukaryotic concentrations of ATP, EcPth1 will not be fully active.

Figure 5. 3 ATP inhibition of *S. aureus* Pth1: A. Urea-PAGE of SaPth1 with ATP at decreasing concentrations. B. SaPth1 had an IC$_{50}$ value of $5.0 \text{ mg/mL} \pm 1.4 \text{ mg/mL}$ ATP.

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5.2.1.2 SaPth1 ATP inhibition

SaPth1 was found to have an ATP IC$_{50}$ equal to that for EcPth1 (e.g. 5 mg/mL ATP). These results were performed until three matching IC$_{50}$ values were determined. This was to account for the standard deviation in obtaining the IC$_{50}$ values seen in Fig 5.3. Therefore, ATP’s IC$_{50}$ for SaPth1 was 5.0 ± 1.4 mg/mL. This was similar to EcPth1, which also had an IC$_{50}$ value of 5.0 ± 1.0 mg/mL ATP. Both SaPth1 and EcPth1 were inhibited *in vitro* by eukaryotic physiological concentrations of ATP. Therefore, at physiological eukaryotic concentrations of ATP, SaPth1 was not fully active like EcPth1.

5.2.1.3 StPth1 not inhibited by ATP

StPth1 was screened against ATP. However, there was no ATP inhibition against StPth1 (Fig. 5.1). Previous data revealed high intracellular ATP concentration to be a virulence factor for *S. typhimurium* infections. *S. typhimurium* was found to evade host immune response by hiding in macrophages (Lee, 2012). *S. typhimurium* have evolved to use macrophagic signals such as ATP and acidic pH to upregulate *Salmonella* pathogenicity island-2 (SPI-2) responsible for the creation of a *Salmonella*-containing vesicle (Anderson, 2017). This allowed *S. typhi* to escape autophagic and phagocytotic processes within macrophages and served to increase the virulence of the bacteria. Therefore, the lack of inhibition by StPth1 was potentially explained given that this is a way for *S. typhimurium* to evade immune response. Importantly, *S. typhimurium* and *S. typhi* have identical Pth1 amino acid sequences. Therefore, both *S. typhimurium* and *S. typhi* Pth1 were not susceptible to ATP inhibition.
5.2.2 GDP inhibition

Following the experimental method used for ATP inhibition, each homolog was screened for GDP inhibition. All Pth1 homologs tested were inhibited by GDP at or near eukaryotic physiological concentrations. Like ATP, there was larger deviation with regards to StPth1, but little deviation between EcPth1 and SaPth1 in terms of their respective IC₅₀.

![Figure 5.4 GDP Inhibition of EcPth1](image)

Figure 5.4 GDP Inhibition of EcPth1: A. Urea-PAGE of EcPth1 with GDP at decreasing concentrations. B. EcPth1 had an IC₅₀ of 10 mg/mL ± 2 mg/mL GDP. In contrast to EcPth1 IC₅₀ for ATP, only 5% activity was seen at 0.6 mg/mL GDP.
5.2.2.1 EcPth1 GDP inhibition

EcPth1 had an IC₅₀ of 10.0 ± 2.0 mg/mL GDP. Standard deviation was determined by the three in vitro inhibition screens and is reported in the error bars for Figure 5.4. Unlike the IC₅₀ curve for ATP, EcPth1 GDP screen did not show stagnation from 0.6 mg/mL GDP – 1.3 mg/mL GDP with 5 % and 23 % inhibition, respectively. As such, this inhibition curve did not require the fitting as with ATP. However, GDP concentrations in both eukaryotes and bacteria were 10 – 100 times lower than ATP (Traut, 1997; Buckstein, 2008). Therefore, this was not necessarily indicative that GDP would be inhibit EcPth1 at physiological eukaryotic concentrations but showed that GDP was weakly inhibitory against EcPth1.
Figure 5.5 GDP Inhibition of SaPth1: A. Urea-PAGE of SaPth1 with GDP at decreasing concentrations. B. SaPth1 had an IC\textsubscript{50} of 10 mg/mL ± 1.6 mg/mL \textit{in vitro}. The inhibition was 0.2 % inhibited at 0.63 mg/mL. Inhibition was almost complete at 20 mg/mL.

5.2.2.2 SaPth1 GDP inhibition

Similarly, SaPth1 also had an IC\textsubscript{50} of 10.0 mg/mL ± 1.6 mg/mL against GDP. These results were performed in triplicate and the standard deviation for each was represented by the error bars in Figure 5.4. Like EcPth1, SaPth1 IC\textsubscript{50} for GDP was two times that of its IC\textsubscript{50} value for ATP. At 20 mg/mL GDP, SaPth1 was 98 % inhibited. Alternatively, SaPth1 was only 0.2 % inhibited at 0.6 mg/mL. Like EcPth1, SaPth1 was not fully active with both ATP and GDP.

Figure 5.6 GDP inhibition of StPth1: A. Urea-PAGE of StPth1 with GDP at decreasing concentrations. B. StPth1 had an IC\textsubscript{50} of 2.5 ± 0.4 mg/mL. StPth1 was only inhibited by GDP.
5.2.2.3 StPth1 GDP inhibition

StPth1 was inhibited by GDP at an IC$_{50}$ value four times lower than both EcPth1 and SaPth1. The IC$_{50}$ value for StPth1 against GDP was 2.5 mg/mL ± 0.4 mg/mL. This meant StPth1 showed more susceptibility to GDP than other homologs with regards to the IC$_{50}$, but still outside the physiological concentrations of *S. typhimurium* and eukaryotes. StPth1 was 89% inhibited at 20 mg/mL GDP. At 0.6 mg/mL, StPth1 was fully active.

5.2.3 ATP and GDP competitively inhibit Pth1

$K_i$ was determined for ATP and GDP against both EcPth1 and SaPth1 in order to determine inhibition type. Based on this analysis, which utilized the technique previously described in chapter 4 to find a $K_m$ apparent ($K_m$, app). From this $K_m$, app, $K_i$ could be found by using the formula $K_m$, app = $K_m$ ($1 + [I]/K_i$). Due to the increase in $K_m$ and $V_{max}$ being unaffected by the presence of inhibitor, both ATP and GDP competitively inhibited EcPth1 and SaPth1. As no kinetic data existed at this time for StPth1, $K_i$ determination was not carried out, but GDP likely is competitively inhibiting StPth1 given both SaPth1 and EcPth1 were also competitively inhibited by GDP.
Figure 5.7 ATP and GDP $K_i$ determination against E. coli Pth1 and S. aureus Pth1: Both ATP and GDP do not affect the Vmax of the enzymes without inhibition (shown in black), but does reduce the $K_m$.

5.3 Conclusions

Small molecules, such as ATP and GTP, inhibited bacterial Pth1 homologs in vitro. There were two schools of thought as to how Pth1 inhibition could occur in the literature. It was previously predicted that peptidyl-tRNA binding occurred along residues in helices 5 and 6 of Pth1 (Giorgi, 2011). The crux of the argument that Pth1 bound peptidyl-tRNA about helices 5 and 6 relied on the gate loop relied heavily on a highly dynamic gate loop region. The evidence pointed to incorrect assumptions being made about Pth1’s global and local dynamics (Kabra, 2017).
In contrast, results published by Hames et al (Hames, 2014) showed Pth1 complexed with peptidyl-tRNA at the 3’-CCA acceptor stem. This low-resolution SANS structure could only give the overall shape of a bound Pth1:peptidyl-tRNA complex, but demonstrated binding occurred only about the 3’-CCA acceptor stem of peptidyl-tRNA.

Previous NMR structural studies in EcPth1 also supported Pth1 binding peptidyl-tRNA at the 3’-CCA acceptor stem (Giorgi, 2011). Moreover, the putative RNA-binding residues in helices 3 and 4 were shown to specifically bind to the 3’-adenosine (Giorgi, 2011).

As such, any small molecule bound to helices 5 and 6 would be unlikely inhibitors based on findings of the complexed SANS structure, NMR structural studies and NMR dynamics studies. Likewise, virtual docking screens have shown that inhibition of Pth1 would most likely occur in the catalytic pocket rather than helices 5 and 6. These virtual screens found small molecules that directly interact with or indirectly block access to the catalytic residues would be the most likely inhibitory candidates (Harris, 2011). The most likely scenario remained inhibition occurred via disrupting the interaction between peptidyl-tRNA and putative RNA-binding residues near helices 3 and 4 and/or catalytic residues in the catalytic cleft. For this reason, inhibitory small molecules in this project, such as nucleotides or nucleosides, were predicted to prevent peptidyl-tRNA hydrolysis via this mechanism rather than interacting with helices 5 or 6.

It was discovered bacterial Pth1 was not fully active at eukaryotic concentrations of ATP. While GDP inhibition was interesting, this finding was not nearly as significant as ATP inhibition of the Pth1 homologs tested since GDP concentrations in both bacteria
and eukaryotes were 200 μM or lower (Traut, 1997; Buckstein, 2008). Furthermore, bacterial GDP concentrations were typically higher than for eukaryotic cells barring those concentrations from malignant carcinomas (Traut, 1997). Human cells in particular had concentrations usually less than 100 μM GDP (Traut, 1997). On average, eukaryotic physiological ATP concentration was two or more orders of magnitude greater than physiological concentrations of GDP. As such, while GDP inhibits Pth1, these are not within the physiological range of bacteria or eukaryotes.

Unlike GDP inhibition of Pth1 homologs, ATP inhibition against EcPth1 and SaPth1 were in physiological range. Importantly, bacterial Pth1 was not fully active at eukaryotic ATP concentrations. This result provided possible clues towards why higher-ordered eukaryotes, such as Homo sapiens, possessed multiple Pths and Pth1-like domains within their genomes. Some credence for this hypothesis was garnered from the phylogeny of HsPth1 in comparison to bacterial homologs. HsPth1 was more closely related to HsPth2 than any bacterial homolog. Likewise, amino acid conservation was only showed 40% similarity, at its best, with homologs from across phylogenetic space. The characteristic α/β hydrolase fold and catalytic residues of HsPth1 were conserved, but little else was shared between bacterial Pth1 homologs and HsPth1. Given this new evidence, the current hypothesis was the complexity of the eukaryotic tRNA recycling system would be able to compensate for HsPth1 inhibition. Further work was needed to determine if nucleotides are inhibitory against HsPth1 or HsPth2. Based on the available data, HsPth2 was hypothesized to not be inhibited by nucleotides, specifically ATP.

StPth1 was only inhibited by GDP. S. typhimurium and S. typhi had the same copy of Pth1. Therefore, both S. typhimurium Pth1 and S. typhi Pth1 were not inhibited
by ATP. Several studies have determined high ATP concentrations to be virulence factors for *S. typhimurium* infections *in vivo*. ATP upregulated *Salmonella* pathogenicity island II which helped *S. typhimurium* escape the macrophage in a vesicle. As such, ATP being a virulence factor for the escape and survival of *S. typhimurium* could provide a potential explanation as to why StPth1 was not inhibited by ATP as Pth1 activity is required for cell survival.

In conclusion, this current project established that bacterial Pth1 was inhibited by GDP. Likewise, Pth1 was not fully active at physiological ATP concentrations except for StPth1. This evidence pointed to a possible evolutionary reason why eukaryotes have multiple Pth1-like domains and orthologous Pth enzymes. Pth1 inhibition caused by increasing nucleotides concentration would create a need for an orthologous enzymes to perform the same vital task of recycling peptidyl-tRNA.
Chapter 6

Conclusions and Future Work

6.1 Conclusions

Past studies established Pth1 as a promising drug target to combat antibiotic resistant bacterial infections. Without Pth1, bacterial cells would undergo tRNA starvation and die. Loss of Pth1 function caused cell death in *E. coli*, *B. subtilis*, and *M. tuberculosis*. Therefore, Pth1 was found to be vital to bacterial survival, making it a viable drug target. Eukaryotes, unlike bacteria, possessed multiple Pths and Pth-like domains. Alternatively, both Pth1 and Pth2 were found to be non-essential in yeast. Importantly, HsPth1 was much more closely related to HsPth2 than any bacterial homolog. Thus potential for selective inhibition of bacterial Pth1 may be possible, supported by the findings hereing. Further, several current antibiotics have targeted the ribosome and protein biosynthesis. As such, this has been a proven strategy in combatting bacterial infections. Pth1 would have a distinct stoichiometric advantage over targeting the ribosome alone since there were 1,3000 Pth1/cell compared to 10,000 ribosomes/cell in bacterial cells (Cruz-Vera).
2000). Thus Pth1 is a promising new antibiotic target and the finding herein advance Pth1 as a novel antibiotic target.

While much of the previous literature focused on EcPth1, to ascertain the similarities and differences between homologs, this study sought to uncover the impact phylogeny had on enzymatic behavior. The characteristics of six bacterial homologs within their respective phylogenetic clades and across phylogenetic space were investigated. This was the first project to explore these differences in a systematic way. Based on these findings, it can be concluded that bacterial Pth1 homologs did not behave the same with regards to qualitative biochemical characteristics. Moreover, quantitative differences were observed in kinetics and natural product inhibition.

6.1.1 Phylogenetic clades

Three distinct phylogenetic clades of Pth1 emerged from multi-alignment sequencing that behave differently from one another as noted in Figure 3.1. Importantly, the phylogenetic clades did not behave similarly. Clade 1 homologs were qualitatively easier to express and purify than Clade 2 or Clade 3 (Figure 3.3). Clade 3 homologs required considerable optimization with their respective growth conditions to yield a minimal amount of soluble purified protein. Clade 3 homologs were aeration rate and IPTG-concentration dependent in their growth. While the Clade 2 homolog MtuPth1 did not express as well as Clade 1 or Clade 3 homologs, the conditions for this homolog did not require optimization. As such, MtuPth1 was qualitatively easier to purify producing more soluble protein than Clade 3 Pth1 homologs. This also explained why most of the
crystal structures within the PDB were elucidated using Clade 1 Pth1 homologs, and why Clade 2 and Clade 3 had considerably fewer crystal structures in the PDB.

This study also demonstrated the differences between, and sometimes within, clades manifested in differences in their respective inhibition profiles (Figure 3.7), small molecule/nucleotide inhibition (Figures 5.1, 5.2 and 5.3) and their kinetics (Figures 4.7 and 4.8). This dispelled the previous assumption that all Pth1s would behave the same. The differences between phylogenetic clades could be potentially exploited as an antibiotic drug target to provide selective inhibition of individual Pth1 homologs in the future.

The phylogenetic clades fell across gram-specific lines (Figure 3.1). Clade 1 Pths were derived from gram-negative bacteria, such as *E. coli*, *P. aeruginosa*, and *S. typhimurium*. Clade 3 Pth1 homologs came from gram-positive species such as *S. aureus*, *B. cereus*, and *S. pyogenes*. Clade 2 Pth1s came from bacterial species that had mixed or were impervious to gram stain. Gram-positive and mycobacterial species have tRNA and rRNA methylation not observed in gram-negative bacteria (Klagbrun, 1972; Vani, 1979; Bocchetta, 1998; Liu and Douthewaite, 2002). Specifically, tRNAs from gram-positive and mycobacterial possessed 1-methyladenine at A58 in the TψC arm of tRNA (Klagbrun, 1972; Vani, 1979). Recognition of tRNA modifications along the TψC and 5’-acceptor stem of peptidyl-tRNA could have played a role in the differences in their respect $K_{cat}$, $K_m$ and $K_{cat}/K_m$ (Table 4.3). However, some mycobacterial species, such as *M. smegmatis*, are known to lack ribothymidine at T54 (Vani, 1979). Therefore, Clade 2 Pth1 homologs lacking in ribothymidine but possess 1-methyladenine appear to not only be intermediate of Clade1 and Clade 3 in their gram status, kinetics and
inhibition profiles (Figure 3.7), but also the methylation or modifications of tRNA. Furthermore, other translational systems also show gram-specificity when processing, transferring or charging tRNAs. For instance was also found in 23S rRNA, which is vital for peptidyl-tRNA binding to the P-site in the ribosome (Bocchetta, 1998). Gram-negative bacteria possessed guanine methylation at G745 in 23S rRNA; while gram-positive bacteria were methylated at G748 in 23S rRNA (Liu and Douthewaite, 2002). Therefore, it seemed probable that tRNA methylation also was responsible for the gram-specificity seen in this project.

Importantly, when the gate loop regions had more flexible residues as seen in Clade 2 and Clade 3 homologs (Figure 3.4), substrate affinity and catalytic efficiency was better. When one of these residues was mutated to a rigid proline, catalytic efficiency and substrate affinity of SaPth1 decreased by half, but solubility and ease of purification increased. As such, the gate loop region of Pth1 played a much larger role in being determinate of clade characteristics than previously reported.

6.1.2 Narrow spectrum inhibition possible

Narrow spectrum inhibition was observed in the NPPE inhibition screen. Broad spectrum and clade-specific inhibition was also present to varying degrees, but broad spectrum antibiotics hasten antibiotic resistances (Fair and Tor, 2014). Clinicians have been moving away from broad spectrum antibiotics due to the increased incidence of antibiotic resistant when broad spectrum antibiotics are used (Fair and Tor, 2014). As such, any new antibiotics and antibiotic drug targets should be narrow spectrum. The
possibility of narrow spectrum inhibition seen in this study makes Pth1 a promising drug target. Similarly, the differences between the clades further highlighted the differences and the potential for selective inhibition of individual Pth1 homologs. Clade 1 Pth1s were more susceptible to NPPE inhibition and had significantly more broad spectrum or clade specific inhibition compared to Clade 3 homologs (Figure 3.7). Clade 3 homologs were largely narrow spectrum and showed that individual Pth1s could be targeted \textit{in vitro}. As with yield of soluble protein and tRNA modifications, the Clade 2 homolog, MtuPth1, was intermediate in terms of its inhibition profiles (Figure 3.7). Clade 2 (MtuPth1) inhibition profile contained broad spectrum or clade-specific inhibition only seen in Clade 1 and Clade 2. Conversely, MtuPth1 shared narrow spectrum inhibition with Clade 3 homologs. These finding reinforced that each homolog behaved differently. It would be expected that the same NPPE would cause inhibition in all homologs if they behaved the same or similarly, but there was only a few that produced broad spectrum inhibition. Likewise, while clade-specific inhibition was observed in some clades, this was not observed in Clade 3 homologs. This showed even further promise for the possibility of narrow spectrum inhibition of an individual homolog if the component(s) of the NPPE causing inhibition were inhibited. As such, the NPPE displaying the most potent inhibition against Pth1 homologs \textit{in vitro} should be screened against their respective bacteria to determine the minimum inhibitory concentration (MIC) and any \textit{in vivo} Pth1 inhibition.
6.1.3 Nucleotide inhibition demonstrates small molecule inhibition possible

These results showed that small molecule inhibition against bacterial Pth1 homologs was possible. Apart from StPth1 and ATP, all three homologs were inhibited by the small molecules, ATP and GDP (Figure 5.1). These findings gave even further credence to the well-established role of ATP in *S. typhimurium* pathogenicity. More importantly, the insusceptibility of StPth1 to ATP demonstrated that even within phylogenetic clades each homolog can behave slightly different while still maintaining much of the respective clade’s characteristics.

This further adds to the potential that even within clades selective inhibition of individual Pth1 homologs could eventually be possible when combined with the narrow spectrum inhibition seen in the NPPE inhibition screen. This new knowledge could be used in conjunction with the inhibition profiles to target individual Pth1 homologs, thus limiting the role broad spectrum antibiotics play in the development of antibiotic resistances (Fair and Tor, 2014). Thus, this study established that small molecule inhibition was possible across phylogenetic space.

At eukaryotic ATP concentrations, bacterial Pth1 was not fully active apart from StPth1 (Figure 5.1). This finding lent further support that inhibition of HsPth1 could potentially be compensated for by orthologs, such as HsPth2, within the eukaryotic tRNA recycling system. Previous studies also showed the non-essentiality of Pth1 in lower ordered eukaryotes. This could potentially explain why the multicomponent tRNA recycling system seen in eukaryotes was more complex than their bacterial counterparts. Therefore, it has been hypothesized that HsPth2 would not be inhibited by ATP as both
the structure and mechanism are different than Pth1 homologs. Further testing would be required to confirm this hypothesis.

6.1.4 Kinetic differences

Homologs across phylogenetic space showed very little deviation in their V\textsubscript{max}. As structure and key catalytic residues were highly conserved across phylogenetic space, V\textsubscript{max} being within less than 1 μmol/min of each other was indicative that their chemistry was unchanged. Likewise, K\textsubscript{cat} was similar for each homolog. However, the K\textsubscript{m} and K\textsubscript{cat}/K\textsubscript{m} parameters for each homolog across phylogenetic space, which measured Pth1’s affinity for peptidyl-tRNA and catalytic efficiency, respectively, revealed stark differences between Clade 1 and Clade 3 homologs. As seen with soluble protein yield, gram status, and the NPPE inhibition screens, MtuPth1’s kinetic parameters were intermediate of Clades 1 and 3. EcPth1, the Clade 1 homolog, had the slowest V\textsubscript{max} and highest K\textsubscript{m}. As such, EcPth1 had the least affinity for bulk peptidyl-tRNA. Clade 3 homologs had the fastest V\textsubscript{max} and K\textsubscript{m} values approximately 20% lower. Therefore, the Clade 3 homologs, SaPth1 and BcPth1 had 5.1 times and 6.3 times greater affinity for bulk peptidyl-tRNA than EcPth1. MtuPth1, like Clade 3 homologs, had 3 times greater affinity for peptidyl-tRNA than EcPth1.

While kinetic parameters were not directly comparable to previous results, the overall trend observed with respective K\textsubscript{m} was consistent with past studies. The differences in K\textsubscript{m} values could potentially be explained by gram-specific tRNA methylation. This and past studies used modified, partial, or full peptidyl-tRNA substrate
largely derived from *E. coli*, lacking 1-methyladenine. Despite this, SaPth1 and MtuPth1 had consistently greater affinity for peptidyl-tRNA than EcPth1. Lack of differentially methylated peptidyl-tRNA likely contributed to the faster $K_{\text{cat}}$ and greater catalytic efficiency in both Clade 2 and Clade 3 homologs seen in this project. Likewise, more flexible residues in the gate loop region of these clades meant wider opening of the lid loops and catalytic pocket required to accommodate for tRNA modifications. In comparison, EcPth1 or PaPth1 which has a more inflexible Pro-Pro-Gly-Val motif (Figure 3.4). This could very well account for the greater velocities, turnover rate, and substrate affinity found in Clade 2 and Clade 3 homologs. Site-directed mutagenesis of SaPth1’s gate loop provided evidence that this might alter the dynamics of the lid loop opening and catalytic pocket widening. It is hypothesized at this time that both gate loop residue accommodation for modified peptidyl-tRNAs and recognition of the TψC arm could aid in substrate recognition, cleavage and specificity. However, at this time, it remains uncertain whether substrate specificity would be affected by tRNA methylation. As such, further work would be required to determine what role, if any, tRNA methylation played in Pth1 substrate affinity.

Alternatively, Pth1’s substrate affinity could potentially explain other phylogenetic characteristics, such as the inhibition profiles of the phylogenetic clades. Clade 1 homologs were the most conserved of the clades and most susceptible against NPPE inhibition, but Clade 1 had the least affinity for peptidyl-tRNA. Therefore, it seemed probable that the decreased affinity for peptidyl-tRNA allowed for greater broad spectrum and clade specific inhibition against NPPE. Conversely, Clade 3 homologs had the most diversity in amino acid sequences, least susceptibility against NPPE inhibition,
and greatest affinity for peptidyl-tRNA. This could account also account for the largely narrow spectrum inhibition seen with this clade as Clade 3 homologs were more likely to form Pth1:peptidyl-tRNA complexes than Clades 1 or 2. Likewise, MtuPth1 shared commonality with both clades in its inhibition profile. While further kinetic analyses across phylogenetic space would certainly be required to confirm these trends, these findings further demonstrate that each clade behaves differently in terms of ease of purification, inhibition profiles, or kinetic parameters. Importantly, these differences in kinetic parameters and inhibition profiles could be exploited to provide small molecule narrow spectrum inhibition in the future.

6.1.5 Gate loop mutations affect solubility and substrate affinity

Pth1 homologs required significant optimization to obtain soluble protein outside of Clade 1 homologs. The Clade 3 homolog, SaPth1 required the most optimization to obtain minimal yields of soluble protein. To improve solubility and purified yields issues in SaPth1, site directed mutagenesis of the three residues in its gate loop motif was performed individually to mirror the Pro-Pro-Gly-Val motif found in Clade 1 homologs. SaPth1 E99P, Q100P and Q102V was mutated to match the P99, P100, and V102 gate loop of Clade 1 homologs. Gly101 was not mutated in SaPth1 as this residue is known to create temperature dependent Pth1s (Menninger, 1973). Past studies hypothesized that the G101A mutation responsible for an EcPth1 temperature sensitive mutant caused it to aggregate and become insoluble. Likewise, previous dynamics study showed the gate loop of Pth1 to be static even with the highly flexible and dynamic Asp-Phe-Gly-Lys
motifs found in MtuPth1 and *M. smegmatis* Pth1 (Kabra, 2017). Prior to the study by Kabra et al (Kabra, 2017), it was hypothesized that the gate loop was highly dynamic. However, the conservation of this structure forced normally dynamic residues like Phe-Gly into energetically unfavorable folds. This current study found the E99P mutation to increase solubility and soluble protein yield. This mutation also abolished aeration rate and IPTG-concentration dependence normally seen in SaPth1. Importantly, the E99P mutation decreased substrate affinity 2-fold (Fig 4.5). Proline is a more rigid residue that doesn’t allow the dynamic opening of an aspartate in that position. It was hypothesized this loss of substrate affinity was due to limited mobility in the lid loop and catalytic pocket caused by the presence of a proline residue.

SaPth1 Q100P was not predicted to improve solubility. Q100 in the gate loop forms a salt bridge with N153 in helix 5 of SaPth1. As expected, disruption of the salt bridge yielded very little solubility. Q100 likely was structurally important and loss of this residue could have caused SaPth1 to not fold properly.

SaPth1 Q102V did not negatively affect solubility. Under normal wildtype SaPth1 growth conditions, this mutation was soluble. Yet, this mutation did not resolve the aeration and IPTG concentration dependence when grown under normal EcPth1 conditions.

Based on this data, site directed mutagenesis of Asp/Glu to Pro in the gate loop of Clades 2 and 3 should improve solubility like that of SaPth1. Therefore, Asp/Glu to Pro in the first residue of the gate loop motifs of MtuPth1 could also be used to ease the
difficulty in expressing and purifying these homologs for future kinetic and structural studies and inhibition screens that could aid in drug discovery.

6.2 Future Work

This current study established three, distinct, gram-specific phylogenetic clades with qualitative differences with respect to expression, solubility, and purification yields. Mutating glutamate to proline in the gate loop increased solubility, abolished aeration and IPTG concentration dependent growth of SaPth1. Inhibition profiles established Clade 1 was more susceptible to NPPE, but also arrow-spectrum and broad spectrum inhibition of Pth1 was possible in vitro. The Clade 2 homolog, MtuPth1, was intermediate of Clade 1 and Clade 3 in terms of kinetics and inhibition. This study also discovered Clades 2 and 3 homologs had greater affinity to bulk peptidyl-tRNA than the Clade 1 homolog, EcPth1. Finally, physiological concentrations of ATP were inhibitory to bacterial Pth1s. This could possibly be an evolutionary consequence of increased intercellular concentrations of ATP in eukaryotes. Based on these conclusions, the following future work studies were proposed.

6.2.1 Activity and affinity using *B. subtilis* peptidyl-tRNA possessing 1-methyladenine

Current evidence showed improved catalytic substrate affinity, turnover rate, and catalytic efficiency was likely due to gate loop motifs causing greater local and/or global dynamic movement and potential recognition of tRNA modifications. To determine
whether the Clade 3 homologs had better catalytic efficiency and substrate affinity due to tRNA modifications, repetition of the kinetics experiments on bulk peptidyl-tRNA from a gram-positive species will be done. *B. subtilis* would be ideal to directly compared the bulk peptidyl-tRNA from a gram-negative and gram-positive species as this is a non-pathogenic gram-positive bacteria. Importantly, *B. subtilis* tRNA are methylated with 1-methyladenine at A58, and much is known about its Pth1 homolog, SpoVC, that was first identified for its role in regulating sporulation (Young, 1976). However, SpoVC knockout was also found to be vital to cell growth as well (Menez, 2002). A temperature sensitive SpoVC mutant cell line, *B. subtilis* Spo285 (TS), was found to be complimentary to *E. coli* C600 Pth TS cells (Menez, 2002). This cell line still possesses vegetative and sporulation growth 30 °C, but sporulation was arrested at the non-permissible temperature of 40 °C (Young, 1976). Likewise, the mechanism of temperature sensitivity in *B. subtilis* Spo285 (TS) appeared to be like the peptidyl-tRNA drop off seen in *E. coli* C600 Pth TS cells (Menez, 2002). Bulk peptidyl-tRNA extraction from vegetative *B. subtilis* could be accomplished using the phenol:chloroform extraction as previously described for *E. coli* C600 Pth TS cells with an additional phenol extraction (Bishop, 1969). Furthermore, bulk peptidyl-tRNA had the additional benefit of being recoverable from lyophilized *B. subtilis* spores (Bishop, 1969). This would require optimization of growth and induction conditions, but rich media, such as LB broth, can be used. If this cell line proved unattainable, previously established *N*-diacetyl-dilysyl-tRNA<sup>Lys</sup> could be purified as described by Schmitt et al. (Schmitt, 1997). Prior to aminoacylation, half of the purified *N*-diacetyl-dilysyl-tRNA<sup>Lys</sup> would be methylated with *P. abyssi* TrmI known to methylate A58 at the carbon 1 of adenosine. However, this
would be laborious and time consuming. Extraction of bulk peptidyl-tRNA from *B. subtilis* Spo-285 TS is preferred. In both cases, activity and kinetics against peptidyl-tRNA from gram-negative and gram-positive bacteria would be carried out as described in this study to determine the effects of tRNA methylation.

6.2.2 SDM of gate loop in MtuPth1 and *R. rickettsia* Pth1 to increase solubility

Following the protocol set forth for SaPth1 E99P, Q100P, and Q102V, site-directed mutagenesis of MtuPth1 and *R. rickettsia* Pth1 (RrPth1) gate loop to improve both expression and soluble protein yields. *M. tuberculosis*, responsible for tuberculosis, and *R. rickettsia*, responsible for Rocky Mounted Spotted Fever, continued to be medically relevant infections. Yet, MtuPth1 and RrPth1 have been historically difficult to express and purify in laboratory conditions. Both the gate loop for MtuPth1 and RrPth1 contain a negatively charged Asp or Glu in the first position of their respective gate loop motifs. Mutation of MtuPth1 E101P and RrPth1 E97P would be beneficial in not only future structural studies but also inhibition screens and kinetic analysis. This would be cost saving in terms of both time and money to produce greater yields of protein from a single batch.

6.2.3 MIC of NPPE *in vivo*

To expand upon the *in vitro* NPPE screen, macrobroth dilution minimum inhibitory concentration (MIC) will be assayed to determine if the best 20 NPPE
demonstrate *in vivo* inhibition in the bacteria from which the Pth1 homologs were derived. Bacterial growth conditions will be assessed with growth curves and appropriate rich media for each bacteria tested prior to MIC assays. 1:2 serial dilutions of the NPPE will be placed in a culture with a final inoculum size of $5 \times 10^5$ cfu/mL, grown in Mueller-Hinton Broth at 37 °C, and visually quantified for lowest concentration with no visible bacterial growth (Balouiri, 2016). Each MIC will have a positive control of cells and broth only, a negative control with cells, broth and an antibiotic, and a solvent control with cells, broth and the solvent the NPPE was dissolved to assure inhibition was not from the solvent used. Any positive results will be followed up with a solvent control, cells only control, and cells transformed with their respective Pth1 homolog to determine if the NPPE inhibition was due to Pth1 inhibition.

6.2.4 Determine kinetic parameters for Pth1s across the clades

Due to the limited number of homologs tested, expanding the homologs tested to occupy greater phylogenetic space would be required to obtain a fuller understanding of any differences in kinetics already observed. Yet, with only two Clade 1 and one Clade 2 homologs, it was difficult to ascertain if the kinetic parameters experimentally obtained were outliers or truly representative of clade differences. Therefore, performing kinetic analysis across phylogenetic space would be of benefit. This would be performed as was with the five homologs and one mutant tested in this current study.
6.2.5 Screen HsPth1 and HsPth2 for nucleotide inhibition

To test the hypothesis that eukaryotes possess multiple Pths due to rising nucleotide concentrations, HsPth1 and HsPth2 will be screened for inhibition against all 16 nucleotides with a major focus on ATP and GDP inhibition as no other nucleotide species showed inhibition against Pth1. HsPth2 was not expected to be susceptible to nucleotide inhibition based on this current hypothesis. In order to accomplish this, HsPth1 and HsPth2 will be cloned into pKQV4 and pET28b vectors, respectively, in order to have hexahistidine tagged HsPth1 and HsPth2 (His-HsPth1 and His-HsPth2).
APPENDICES
APPENDIX A:
LIST OF NPPE USED IN NPPE INHIBITION SCREEN

Table A.1: List of NPPE used in NPPE inhibition screen (Top 25 shown in Figure 3.7)

<table>
<thead>
<tr>
<th>Natural Product Name</th>
<th>Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Ardisia revoluta bark</td>
<td>Acetone</td>
</tr>
<tr>
<td>2. Ardidendron vaillanti bark</td>
<td>EtOH</td>
</tr>
<tr>
<td>3. Mandevilla veraguasenii</td>
<td>Acetone</td>
</tr>
<tr>
<td>4. Inga sierra bark</td>
<td>MeOH</td>
</tr>
<tr>
<td>5. Ardisia compressa</td>
<td>CHCl$_3$</td>
</tr>
<tr>
<td>6. Exothea paniulata bark</td>
<td>EtOH</td>
</tr>
<tr>
<td>7. Salacia liana bark</td>
<td>CHCl$_3$</td>
</tr>
<tr>
<td>8. Lonchocarpus orotinus</td>
<td>EtOH</td>
</tr>
<tr>
<td>9. AlDrymonia conchocalyx vine</td>
<td>EtOH</td>
</tr>
<tr>
<td>10. Cestrum racemosum bark</td>
<td>Acetone</td>
</tr>
<tr>
<td>11. Myorica sp “fuzzy leaf” leaf</td>
<td>Acetone</td>
</tr>
<tr>
<td>12. Drypetes lasiogyna ver australasia bark</td>
<td>CHCl$_3$</td>
</tr>
<tr>
<td>13. Mallotus paniculate bark</td>
<td>EtOH</td>
</tr>
<tr>
<td>14. Albizia adenocephalia</td>
<td>DMSO</td>
</tr>
<tr>
<td>15. Ocistea floribunda Bark</td>
<td>Acetone</td>
</tr>
<tr>
<td>16. Acacia aulacocarpa</td>
<td>DMSO</td>
</tr>
<tr>
<td>17. Conestegba xalapensis bark</td>
<td>DMSO</td>
</tr>
<tr>
<td>18. Urera carcasasma</td>
<td>EtOH</td>
</tr>
<tr>
<td>19. Psychotria parvislora</td>
<td>Acetone</td>
</tr>
<tr>
<td>20. Sityrax argenteus bark</td>
<td>CH$_2$Cl$_2$</td>
</tr>
<tr>
<td>21. Cinnamomum tonduzii bark</td>
<td>EtOH</td>
</tr>
<tr>
<td>22. Syzygium johnsonii bark</td>
<td>EtOH</td>
</tr>
<tr>
<td>23. Ocotea “los llamas” bark</td>
<td>Acetone</td>
</tr>
<tr>
<td>24. Polysoma alangiacea bark</td>
<td>CHCl$_3$/EtOH</td>
</tr>
<tr>
<td>25. Grevilla lilliana bark</td>
<td>CHCl$_3$/EtOH</td>
</tr>
<tr>
<td>26. Neolitsea dealbata bark</td>
<td>EtOH</td>
</tr>
<tr>
<td>27. Endiandra wolfei bark</td>
<td>CHCl$_3$/EtOH</td>
</tr>
<tr>
<td></td>
<td>Plant Name</td>
</tr>
<tr>
<td>---</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>28.</td>
<td><em>Amphitecna haberii</em> leaf</td>
</tr>
<tr>
<td>29.</td>
<td><em>Albizia julibrissin</em></td>
</tr>
<tr>
<td>30.</td>
<td><em>Turpinia occidentalis</em> leaf</td>
</tr>
<tr>
<td>31.</td>
<td><em>Zanthoxylum</em> bark</td>
</tr>
<tr>
<td>32.</td>
<td><em>Conradina canescens</em></td>
</tr>
<tr>
<td>33.</td>
<td><em>Agathis atropurpurea</em></td>
</tr>
<tr>
<td>34.</td>
<td><em>Sweitenia mohogoni</em> Bark</td>
</tr>
<tr>
<td>35.</td>
<td><em>Byronima crassifolia</em> bark</td>
</tr>
<tr>
<td>36.</td>
<td><em>Ruyschia phyllandra</em> bark</td>
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<td>37.</td>
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<td>38.</td>
<td><em>Machaerium biovalatum</em> bark</td>
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<td>39.</td>
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</tr>
<tr>
<td>40.</td>
<td><em>Cryptocarya corrugata</em> bark</td>
</tr>
<tr>
<td>41.</td>
<td><em>Diospyres digyna</em> bark</td>
</tr>
<tr>
<td>42.</td>
<td><em>Galanthymine 0.1 mg/ml</em></td>
</tr>
<tr>
<td>43.</td>
<td><em>Quercies insignis</em> bark</td>
</tr>
<tr>
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<td><em>Cedrela tonduzi</em> bark</td>
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<td>45.</td>
<td><em>Lonchocarpus oliganthus</em> bark</td>
</tr>
<tr>
<td>46.</td>
<td><em>Corothon monteverdensis</em> bark</td>
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<td>47.</td>
<td><em>Tapirira Mexicana</em> bark</td>
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<td>48.</td>
<td><em>Verberina tarbacensis</em> bark</td>
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<td>49.</td>
<td><em>Balapus australiana</em> bark</td>
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<td>50.</td>
<td><em>Styphnolobium munteveridis</em> bark</td>
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<td>51.</td>
<td><em>Myrcianthus</em> “black fruit” bark</td>
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<td>52.</td>
<td><em>Drypetes lasiogyna</em> ver australasica* bark</td>
</tr>
<tr>
<td>53.</td>
<td><em>Myorica</em> sp “fuzzy leaf” leaf</td>
</tr>
<tr>
<td>54.</td>
<td><em>Beilschidia</em> “concho blanco” bark</td>
</tr>
<tr>
<td>55.</td>
<td><em>Bursera</em> bark</td>
</tr>
<tr>
<td>56.</td>
<td><em>Bocconia frutescens</em> bark</td>
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</table>
Figure B.1: Vo determination of MtuPth1: Initial $V_o$ for MtuPth1 was determined at each bulk peptidyl-tRNA concentration. The slope of the tangent line at time zero was equal to $V_o$ for that substrate concentration. Red, black and blue represents 28 μM peptidyl-tRNA, 56 μM, and 112 μM, respectively.
Table B.1: Determination of initial *M. tuberculosis* Pth1 kinetic parameters

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<tr>
<th>$S_o$</th>
<th>$V_{max,obs}$</th>
<th>$x = V^2$</th>
<th>$y = V^2/S$</th>
<th>$\alpha_1$</th>
<th>$\beta_1$</th>
<th>$\gamma_1$</th>
<th>$\delta_1$</th>
<th>$\epsilon_1$</th>
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</tr>
<tr>
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<td>0.18</td>
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<td>0.15</td>
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<td>0.94</td>
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<td>Sums</td>
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<td>1266.60</td>
<td>6.06</td>
<td>20.49</td>
<td>88.56</td>
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</tbody>
</table>

$\Delta_1 = \alpha_1 \epsilon_1 - \gamma_1 \delta_1 = (303.61 \times 0.70) - (6.06 - 20.49) = 88.56$

$V_{max}^0 = (\beta_1 \epsilon_1 - \delta_1 \gamma_1) / \Delta_1 = ((1266.60^* 0.70 - 20.49) / 88.56) = 5.28 \mu\text{mol/min}$

$K_m^0 = (\beta_1 \gamma_1 - \alpha_1 \delta_1) / \Delta_1 = ((1266.60^* - 6.06) - (303.61^* 20.49)) / 88.56 = 16.45 \mu\text{M}$

Table B.2: Determination of *M. tuberculosis* Pth1 Kinetic Parameters

Fine Adjustment of Provisional *M. tuberculosis* Pth1 Parameters: Least Squares Nonlinear Regression Analysis

<table>
<thead>
<tr>
<th>$S_o$</th>
<th>$V_{max,obs}$</th>
<th>$S + K_m^0$</th>
<th>$f = V_{max}^0 S / (S + K_m^0)$</th>
<th>$f' = V_{max}^0 S / (S + K_m^0)^2$</th>
<th>$\alpha_2$</th>
<th>$\beta_2$</th>
<th>$\gamma_2$</th>
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<tr>
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<td>19.95</td>
<td>0.92</td>
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<td>0.86</td>
<td>2.0 $\times 10^{-3}$</td>
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<td>1.80</td>
<td>23.45</td>
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<td>5.0 $\times 10^{-3}$</td>
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<td>2.84</td>
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<td>30.45</td>
<td>2.42</td>
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<td>5.90</td>
<td>6.0 $\times 10^{-3}$</td>
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<td>5.83</td>
<td>-0.19</td>
</tr>
<tr>
<td>28</td>
<td>3.10</td>
<td>44.45</td>
<td>3.32</td>
<td>-0.07</td>
<td>11.10</td>
<td>6.0 $\times 10^{-3}$</td>
<td>-0.24</td>
<td>10.31</td>
<td>-0.23</td>
</tr>
<tr>
<td>56</td>
<td>3.80</td>
<td>72.45</td>
<td>4.08</td>
<td>-0.06</td>
<td>16.70</td>
<td>3.0 $\times 10^{-3}$</td>
<td>-0.23</td>
<td>15.51</td>
<td>-0.21</td>
</tr>
<tr>
<td>84</td>
<td>4.53</td>
<td>100.45</td>
<td>4.41</td>
<td>-0.04</td>
<td>19.51</td>
<td>2.0 $\times 10^{-3}$</td>
<td>-0.19</td>
<td>20.01</td>
<td>-0.20</td>
</tr>
<tr>
<td>112</td>
<td>4.73</td>
<td>128.45</td>
<td>4.60</td>
<td>-0.04</td>
<td>21.21</td>
<td>1.0 $\times 10^{-3}$</td>
<td>-0.17</td>
<td>21.78</td>
<td>-0.17</td>
</tr>
<tr>
<td>Sums</td>
<td>77.70</td>
<td>250.00 $\times 10^{-3}$</td>
<td>-1.18</td>
<td>77.00</td>
<td>-1.16</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$\Delta_2 = \alpha_1 \beta_1 \gamma_1 \delta_1 = 0.55; \ B_1 = (\beta_1 \gamma_2 \delta_2 \epsilon_2) / \Delta_2 = 1.01; \ B_2 = (\alpha_1 \beta_1 \gamma_1 \delta_1) / \Delta_2 = 0.90$

$V_{max} = B_1 V_{max}^0 = 5.30 \mu\text{mol/min} = 5.30 \mu\text{mol/min}^*$

$K_m = K_m^0 = B_2 / B_1 = 17.35 \mu\text{M} = 17.00 \mu\text{M}^*$

Standard Error ($V_{max}$) = $V_{max}^0 \sigma (\beta_1 / \Delta_2) = 0.21$

Standard Error ($K_m$) = $\sigma / B_1 V (\alpha_1 / \Delta_2) = 2.24$

$*$refined further with Excel solver function

$\sigma^2 = \Sigma (V_{obs}^2 - B_1 \delta_2 - B_2 \epsilon_2) / (n-2) = 0.035$

112
Figure B.2: \( V_0 \) determination of SaPth1: Initial \( V_0 \) for SaPth1 was determined at each bulk peptidyl-tRNA concentration. The slope of the tangent line at time zero was equal to \( V_0 \) for that substrate concentration. Red, black and blue represents 28 μM peptidyl-tRNA, 56 μM, and 112 μM, respectively.

Table B.3: Determination of initial \( S. aureus \) Pth1 kinetic parameters

<table>
<thead>
<tr>
<th>( S_o )</th>
<th>( V_{max_{obs}} )</th>
<th>( x = V^2 )</th>
<th>( y = V^2/S )</th>
<th>( \alpha )</th>
<th>( \beta )</th>
<th>( V_0 )</th>
<th>( \delta )</th>
<th>( \epsilon )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3.5</td>
<td>1.63</td>
<td>2.66</td>
<td>0.76</td>
<td>4.33</td>
<td>7.10</td>
<td>1.24</td>
<td>2.02</td>
<td>0.58</td>
</tr>
<tr>
<td>7</td>
<td>2.50</td>
<td>6.25</td>
<td>0.90</td>
<td>15.63</td>
<td>39.05</td>
<td>2.23</td>
<td>5.58</td>
<td>0.80</td>
</tr>
<tr>
<td>14</td>
<td>3.60</td>
<td>12.96</td>
<td>0.93</td>
<td>46.65</td>
<td>167.96</td>
<td>3.33</td>
<td>12.00</td>
<td>0.86</td>
</tr>
<tr>
<td>28</td>
<td>4.37</td>
<td>19.10</td>
<td>0.68</td>
<td>83.45</td>
<td>364.69</td>
<td>2.98</td>
<td>13.02</td>
<td>0.46</td>
</tr>
<tr>
<td>56</td>
<td>5.20</td>
<td>27.04</td>
<td>0.48</td>
<td>140.61</td>
<td>731.16</td>
<td>2.51</td>
<td>13.05</td>
<td>0.23</td>
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<tr>
<td>84</td>
<td>5.45</td>
<td>29.70</td>
<td>0.35</td>
<td>161.88</td>
<td>882.23</td>
<td>1.93</td>
<td>10.50</td>
<td>0.13</td>
</tr>
<tr>
<td>112</td>
<td>5.60</td>
<td>31.36</td>
<td>0.28</td>
<td>175.62</td>
<td>983.44</td>
<td>1.57</td>
<td>8.79</td>
<td>0.07</td>
</tr>
<tr>
<td>Sums</td>
<td>628.17</td>
<td>3175.63</td>
<td>15.79</td>
<td>64.96</td>
<td>3.13</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\[
\Delta_1 = \alpha \cdot \epsilon_1 - \gamma_1 \cdot (\epsilon_1) = (628.17 \cdot 3.13) - (15.79 \cdot 64.96) = 941.90
\]

\[
V_{max}^o = (\beta_1 \cdot \epsilon_1 - \delta_1) \Delta_1 = (3175.63 \cdot 3.13) - (64.96^2) / 941.90 = 6.08 \text{ μmol/min}
\]

\[
K_m^o = (\beta_1 \cdot \gamma_1 - \alpha \cdot \delta_1) / \Delta_1 = ((3175.63 \cdot 15.79) - (628.17 \cdot 64.96)) / 941.90 = 9.91 \text{ μM}
\]
Table B.2: Determination of *S. aureus* Pth1 Kinetic Parameters

<table>
<thead>
<tr>
<th>S₀</th>
<th>Vm₀</th>
<th>S + Km₀</th>
<th>f = Vm₀*S/S + Km₀</th>
<th>( f' = -Vm₀*S/(S + Km₀)^2 )</th>
<th>( \alpha₂ \Sigma(f²) )</th>
<th>( \beta₂ \Sigma(f²'') )</th>
<th>( \gamma₂ \Sigma(f''') )</th>
<th>( \delta₂ \Sigma(vⁿf) )</th>
<th>( \epsilon₂ \Sigma(vⁿf'') )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.5</td>
<td>1.63</td>
<td>13.41</td>
<td>1.59</td>
<td>-0.12</td>
<td>2.52</td>
<td>1.40 x 10⁻²</td>
<td>-0.20</td>
<td>2.59</td>
<td>-0.19</td>
</tr>
<tr>
<td>7.5</td>
<td>2.50</td>
<td>16.91</td>
<td>2.52</td>
<td>-0.15</td>
<td>6.34</td>
<td>2.0 x 10⁻²</td>
<td>-0.37</td>
<td>6.29</td>
<td>-0.37</td>
</tr>
<tr>
<td>14</td>
<td>3.60</td>
<td>23.91</td>
<td>3.56</td>
<td>-0.15</td>
<td>12.68</td>
<td>2.0 x 10⁻²</td>
<td>-0.53</td>
<td>12.82</td>
<td>-0.54</td>
</tr>
<tr>
<td>28</td>
<td>4.37</td>
<td>37.91</td>
<td>4.49</td>
<td>-0.12</td>
<td>20.17</td>
<td>1.40 x 10⁻²</td>
<td>-0.53</td>
<td>19.62</td>
<td>-0.52</td>
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<tr>
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<td>5.20</td>
<td>65.91</td>
<td>5.16</td>
<td>-0.08</td>
<td>26.69</td>
<td>0.61 x 10⁻²</td>
<td>-0.40</td>
<td>25.83</td>
<td>-0.40</td>
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<tr>
<td>84</td>
<td>5.43</td>
<td>93.91</td>
<td>5.44</td>
<td>-0.06</td>
<td>29.58</td>
<td>0.34 x 10⁻²</td>
<td>-0.31</td>
<td>29.53</td>
<td>-0.31</td>
</tr>
<tr>
<td>112</td>
<td>5.60</td>
<td>121.91</td>
<td>5.59</td>
<td>-0.05</td>
<td>31.20</td>
<td>0.21 x 10⁻²</td>
<td>-0.26</td>
<td>31.28</td>
<td>-0.26</td>
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<tr>
<td>Sums</td>
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<td></td>
<td></td>
<td></td>
<td>129.16</td>
<td>8.00 x 10⁻²</td>
<td>2.60</td>
<td>127.96</td>
<td>2.59</td>
</tr>
</tbody>
</table>

\[ \Delta₂ = \alpha₂\beta₂, \gamma₂ = 4.08; \quad B₁ = (\beta₂\delta₂, \gamma₂, \epsilon₂)/\Delta₂ = 0.99; \quad B₂ = (\alpha₂\epsilon₂, \gamma₂, \delta₂)/\Delta₂ = -0.17 \]

\[ \text{Vmax} = B₁^*\text{Vmax}^\text{obs} = 6.01 \mu\text{mol/min} = 6.00 \mu\text{mol/min}^* \]

\[ \text{Standard Error (Vmax)} = \text{Vmax}^\text{obs}^*\sigma(\beta₂/\Delta₂) = 0.10 \]

\[ K_m = K_m^\text{obs} + B₂/B₁ = 9.73 \mu\text{M} = 10.00 \mu\text{M}^* \]

\[ \text{Standard Error (K_m)} = \sigma/B₁^*\sigma(\alpha₂/\Delta₂) = 0.40 \]

*refined further with Excel solver function*

\[ \sigma^2 = \Sigma(\text{Vm}_\text{obs}^2 - B₁\delta₂ - B₂\epsilon₂)/(n-2) = 0.005 \]
WORKS CITED


Singh, Nongmaithem Sadananda, and Umesh Varshney. “A physiological connection between tmRNA and peptidyl-tRNA hydrolase functions in Escherichia


Yaginuma, Hideyuki, Shinnosuke Kawai, Kazuhito V. Tabata, Keisuke Tomiyama, Akira Kakizuka, Tamiki Komatsuzaki, Hiroyuki Noji, and Hiromi Imamura. 2014. “Diversity in ATP Concentrations in a Single Bacterial cell population revealed by quantitative single-cell imaging.” *Scientific Reports* 4:6522. DOI: 10.1038/srep06522