THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

IDENTIFICATION AND CHARACTERIZATION OF *TRANS*-TRANSLATION INHIBITORS

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A thesis
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ABSTRACT

Individuals around the world are acquiring deadly infections from previously "common" and "non-lethal" sicknesses because common antibiotics are proving to be ineffective. The urgency for development of novel antibiotics is tremendous. In the early 20th century, antibiotics were viewed as the saving remedy to many ailments that would otherwise lead to death such as diarrhea and pneumonia. Now, the world is witnessing an increasing emergence of resistant bacterial pathogens that will cause more global deaths than any other condition by 2050.[1]

This work aimed at utilizing *trans*-translation as a target pathway for novel antibiotic development. This is an essential pathway in many bacterial pathogens that rescues ribosomes that stall during translation due to the formation of nonstop complexes. These complexes form when no stop codon is present at the 3' end of an mRNA message being translated.[3] This disables the termination of the message which is needed to produce a functional protein. If *trans*-translation is activated, its main machinery consisting of tmRNA and SmpB protein identifies the stalled ribosome and, through several steps, tags the protein for degradation.[3]

The first project featured in this work highlighted the process of identifying inhibitors of this pathway in resistant bacterial pathogens. Characterization of such inhibitors was explored to determine potential candidates for novel antibiotic development. The second project aimed to reveal molecular mechanisms occurring during *trans*-translation, as knowledge of this entire process has not been exhausted. This was initiated by completing a cloning project to begin ribosome profiling in a *C. crescentus* model. The profiling will provide information about mRNA sequences present in ribosomes while nonstop complexes are present during *trans*-translation and what conditions these sequences are dependent on. Therefore, continuation of both projects will aid in the movement to combat antibiotic resistance through research efforts.

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Chapter 1

Information

Antibiotic Resistance: A Global Crisis

The global phenomenon of antibiotic resistance has been estimated to lead to more deaths than cancer, 10 million annually, by 2050.[1] Visually, this value is striking in **Figure 1** compared to other conditions leading to global deaths. The figure exemplifies the recent and tremendous failure of many antibiotics which are chemical compounds utilized to kill or prevent the growth of certain bacteria. It also highlights the powerful reality resulting from deadly bacterial infections not responding to treatment.

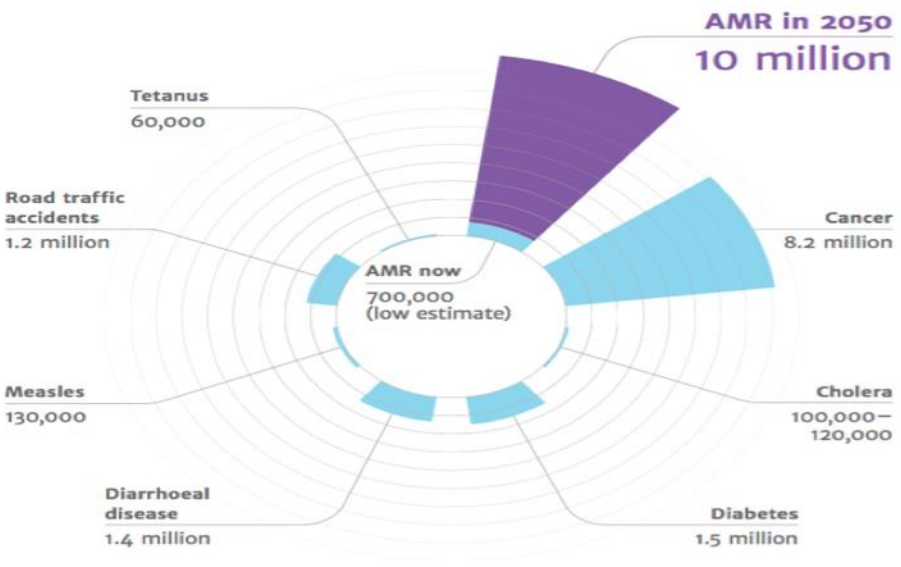

Figure 1. Projected Global Deaths from Antibiotic Resistance (AMR) in 2050. This figure has been adapted from O'Neil, J. (2016). Review on Antimicrobial Resistance: Tackling drug-resistant infections globally. *UK Department of Health.*

The overuse of antibiotics has contributed to the phenomenon of bacteria developing ways to overcome the killing mechanisms of antibiotics along with other contributing factors: patients not finishing prescription treatments, overuse of antibiotics in livestock, poor infection control in health facilities, lack of hygiene, and lack of new antibiotics being developed.[2] Throughout this process, bacterial pathogens become capable of surviving, proliferating, and spreading their resistance mechanisms.[2] Furthermore, a remarkable example of how such processes influence humanity is found in the statistic that over 1.8 billion people are infected with *M. tuberculosis* (MTB) worldwide which results in 1.5 million deaths annually.[3] New studies and experiments need to be conducted because pathogens like MTB are becoming resistant to all available treatments.[1] Therefore, a focus of developing new potential antibiotics targeting new molecular components is a priority in research against all other priority pathogens listed by the Center for Disease Control and Prevention.[2]

trans-Translation: A Pathway for Novel Antibiotic Development

The Keiler lab has been part of this movement to combat antibiotic resistance with recent studies focusing on identifying and characterizing drug candidates that act as inhibitors to a specific, essential process in many bacterial pathogens: *trans*-translation. This pathway, not found in human cells, provides bacteria with a method of rescuing ribosomes from nonstop translation complexes that occur when no stop codon is present at the 3' end of an mRNA.[3] The main steps of *trans*-translation are depicted in **Figure 2**.

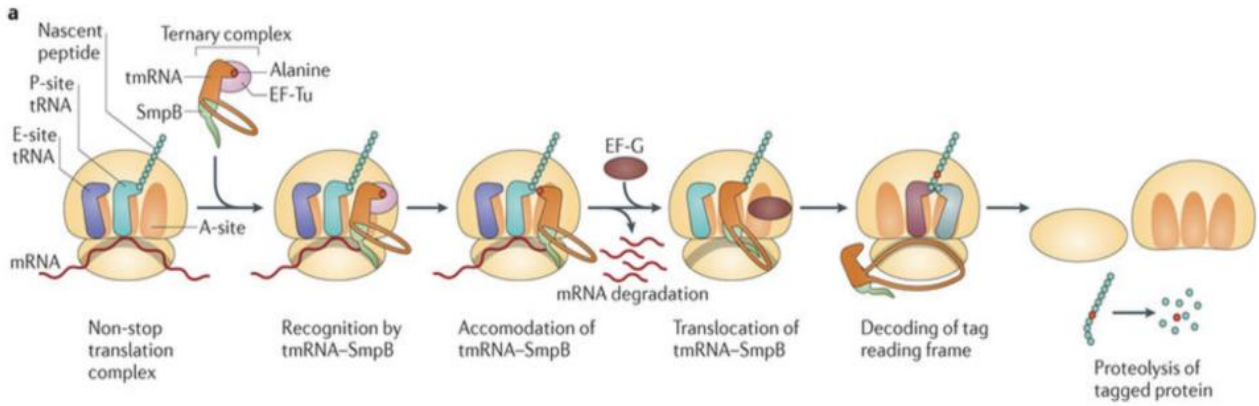

Figure 2. Schematic of Key Steps during *trans*-Translation. This figure has been adapted from Keiler, Kenneth. (2015). Mechanisms of ribosome rescue in bacteria. *Nature Reviews Microbiology*. 13, 285-297.

The process involves a ribonucleoprotein complex composed of transfer-messenger RNA (tmRNA) and a SmpB protein. These two components are part of a ternary complex with elongation factor Tu-GTP (EF-Tu). The formation of a nonstop complex stabilizes the subunits of the ribosome (in bacteria 30S and 50S) preventing their dissociation.[4] Once a nonstop translation complex is identified, the carboxy-terminal tail of SmpB binds to the empty mRNA channel, and the ribonucleoprotein complex enters the ribosome by imitating aminoacylated tRNA in the ribosomal A site.[3] The nascent polypeptide is then transported to the tmRNA followed by the peptidyl-tmRNA-SmpB translocating to the P site which is initiated by elongation factor G (EF-G).[4] This movement also enables the insertion of a terminating sequence into the mRNA channel with a stop codon.[3] The presence of the stop codon in the tag reading frame prevents translation from continuing.[4] The ribosome is forced to release the non-functional protein which now contains a tag for degradation recognized by proteases in a bacterial cell such as ClpXP.[3]

trans-Translation provides a novel pathway for targeting with novel antibiotics. This is due to the fact that human cells do not contain this ribosome rescue process. Therefore, the risk of off-target effects with administration of such an inhibitor of *trans*-translation is substantially

lowered. The potential for broad-spectrum use against various bacterial pathogens is significant because the machinery for *trans*-translation, specifically the *ssrA* and *smpB* genes encoding tmRNA and SmpB protein, have been identified in >99% of sequenced bacterial genomes.[4] The projects that will be explained thoroughly in this work aim to exploit the components surrounding *trans*-translation to help identify potential inhibitors of this process and to help advance our knowledge of each step regarding this process overall.

trans-Translation Project #1: Inhibitor Identification

This work will focus on two different projects that focus on *trans*-translation but have different aims. The first project that will be summarized in Chapters 2 and 3 and will be focused on identifying potential inhibitors of *trans*-translation through a high-throughput screen of ~13,000 chemical compounds. This screen utilized a luciferase-based assay that will be described in great detail in Chapter 2. "Hit" compounds were identified through this screen, and these were compounds considered to be potential inhibitors of *trans*-translation. In order to confirm inhibition and determine which "hits" would be studied further, mCherry fluorescence assays were utilized for this purpose.

Once these initial experiments were completed, the next step was to test the top "hit" compounds. KKL-4669, KKL-11311, and KKL-11986 were tested with different bacterial pathogens in minimum inhibitory concentration (MIC) experiments. In order to grasp the potential broad-spectrum use of these compounds, the growth inhibition trials were conducted in both Gram-negative and Gram-positive pathogens. The Gram-negative bacteria tested were *Escherichia coli, Pseudomonas aeruginosa, Salmonella enterica,* and *Neisseria gonorrhoeae*. The Gram-positive

bacteria tested consisted of *Bacillus anthracis* and *Staphylococcus aureus*. As a result of these experiments, it was concluded that KKL-4669 was the most active compound against various pathogens since it led to low MIC and IC_{50} measurements. Therefore, characterization of only this "hit" compound was pursued.

The next major concern for KKL-4669 was to identify what component of *trans*-translation it was targeting and during what step this targeting took place. In vitro transcription/translation reactions were initiated in order to confirm that KKL-4669 was targeting *trans*-translation and not simply translation. Control in vitro studies were performed before trials with inhibitor were present. This entailed purifying components of the *trans*-translation machinery, tmRNA and SmpB protein. These were then tested for functionality and tagging efficiency of a radiolabeled protein, dihydrofolate reductase (DHFR), where *trans*-translation was active. The trials only led to this initial control trial where it was confirmed that the purified components were active. Future in vitro studies with KKL-4669 will be needed to continue the target identification process.

trans*-Translation Project #2: Ribosome Profiling in *Caulobacter crescentus

As the Keiler lab continues attempting to target *trans*-translation for development of novel antibiotics, they would also like to advance their understanding of the process of *trans*-translation in its entirety. Even though much research has discovered these key components of *trans*-translation, all aspects underlying these mechanisms have yet to be uncovered. And these facts are the driving force for this second project.

For example, investigation about features such as the mechanism of initiating *trans*-translation when there is mRNA extending past the A site or at various lengths is not fully

understood.[5] Additionally, the number of nucleotides present in the mRNA channel during *trans*-translation has not been confirmed. Currently, preliminary data for ribosome profiling in *E. coli* suggests that ~19 nucleotides may be present when a nonstop complex forms. It is necessary to confirm this observation and to discover whether or not the formation of nonstop complexes is dependent on specific sequences being conserved across a variety of genes. Contrastingly, the question of whether or not the formation of nonstop complexes will only occur if certain genes are present in the mRNA channel should also be explored.

To complement and support the studies being conducted in *E. coli*, the use of *C. crescentus* as a model was pursued in this second project. It is a Gram-negative bacteria and "powerful model" for high-yield of gene and protein expression.[6] This project aimed at utilizing MS2-tagging, ribosome pull-down, and ribosome profiling methodology to observe mRNA sequences occupying the ribosome in *C. crescentus* during *trans*-translation and inhibition of *trans*-translation conditions. Before it could reach the actual ribosome profiling experiment, several deoxynucleic acid (DNA) cloning stages needed to be completed to attain ideal constructs in *Caulobacter* where the *ssrA* gene was attached to a MS2 tag, which originated from bacteriophage. This entailed producing clones in *E. coli* first prior to producing clones in *C. crescentus*. The methodology for this endeavor is explained extensively in Chapters 5 and 6.

Overview

This work contains the methodology of two projects both focusing on learning more about *trans*-translation as a molecular process and about how it can be manipulated and targeted by novel antibiotics. Both efforts hold the same overarching urgency of combating the global crisis of

antibiotic resistance by developing new ways of fighting various resistant pathogens which is affecting ~ 2 million individuals annually in the United States alone.[2]

Chapter 2

Materials and Methods for Identification of *trans*-Translation Inhibitors

The bacterial strains utilized in the process of identifying and characterizing potential *trans*-translation inhibitors are listed in **Table 1** below.

Table 1. Bacterial Strains

Bacterial Strain	Description
E. coli	MG1655 Wild-type (WT); no plasmid KCK #125 strain
E. coli ΔssrA	MG1655; no plasmid KCK #186 strain Chloramphenicol cassette (ssrA::chlor) Deletion in *ssrA* gene, encodes for tmRNA
E. coli smpB	BL21(DE3) Contains pET28b-smpB-tmRNA Contains kanamycin resistance marker
E. coli luc	MG1655 (*need to find genotype info)
E. coli luc-trpAT	MG1655 KCK #488 strain Contains pRrluctag LuctrpAt Contains ampicillin resistance marker
E. coli mCherry	DH5 α KCK #322 strain Contains pRSETB-mCherry Contains ampicillin resistance marker
E. coli mCherry-trpAT	Sb75 KCK #373 strain Contains pTrcmChAA; contains reporter tag with nonstop codon Contains ampicillin resistance marker
E. coli ΔtolC	MG1655 KCK #482 strain Contains ampicillin resistance marker Deletion in *tolC* gene, encodes efflux pumps
E. coli DHFR	DH5 α KCK #374 strain Contains pM2DHFR Contains ampicillin resistance marker
B. anthracis	WT; Sterne KCK #355 strain
S. aureus	WT
P. aeruginosa	WT; PAO-1 KCK #375 strain
S. enterica	ATCC strain
N. gonorrhoeae	WT

Growth Conditions

Overnight cultures (typically 4-5 ml for all bacteria) of *E. coli luc, E. coli luc-trpAT*, and *E. coli* Δ*tolC* were grown at 37ºC in Lysogeny Broth (LB) for 15-17 hours in the presence of ampicillin (100 μg/ml). *E. coli mCherry* and *E. coli mCherry-trpAT* were also grown at 37ºC in EZ Rich Media in the presence of ampicillin (100 μg/ml). *E. coli* WT, *E. coli* Δ*ssrA*, and *B. anthracis* were grown in LB at 37ºC for about 15-17 hours. *E. coli smpB* was grown in the presence of kanamycin (30 μg/ml) for 15-17 hours. *S. aureus*, *P. aeruginosa*, and *S. enterica* were grown in Mueller-Hinton (MH) broth at 37ºC for 15-17 hours. *Neisseria gonorrhoeae* required a special growth protocol as described below. *E. coli ssrA* (WT) 5 ml overnight LB cultures were utilized for amplification and purification of the *ssrA* gene. 5 ml overnights of *E. coli DHFR* and *E. coli DHFR-ns* in LB were grown at 37 ºC for 15-17 hours.

Luciferase Assay: High-Throughput Screen of ReFrame Library

Overnight cultures of *E. coli luc* and *E. coli luc-trpAT* were diluted to an OD_{600}= 0.40. At this final concentration, induction of protein expression with isopropyl ß-D-1 thiogalactopyranoside (IPTG) at 1 mM was initiated. Induced *E. coli luc-trpAT* was thoroughly vortexed, and 50 μl of culture was aliquoted into each well of a 96-well plate from the ReFrame Library and mixed thoroughly. This library was provided by Calibr and the Gates Foundation and contained approximately 13,000 small molecules spotted in 96-well plates at 20 μM. On the days of screening, plates needed to be thawed out (~30 min) and spun down prior to beginning the luciferase assay. Each plate had empty wells for a negative control, dimethyl sulfoxide (DMSO), and the positive control, KKL-35. Once controls were added, the plate was covered with adhesive

seal, vortexed thoroughly for 30 sec, and spun down for one minute. The samples were incubated at room temperature for 1.5 hours on benchtop. During this incubation, Bright-Glo luciferase reagent, stored at -80 ºC, was thawed out for each new day of screening. After the incubation, 50 µl reagent was aliquoted to each well. The plate was sealed, vortexed for 30 sec, spun down for one minute, and incubated at room temperature for 10 min. Luminescence was measured at 560 nm utilizing the Spectramax i3 (Molecular Devices). Luciferase assays with "hit" compounds, whose luminescence measurements were three standard deviations above the mean, were repeated in a dose-response format.

Confirmation Screen: mCherry Fluorescence Assay

E. coli mCherry and *E. coli mCherry-trpAT* overnight cultures were then diluted to an OD_{600}= 0.02 in 10 ml for Δ*ssrA* and 20 ml for WT. Both diluted *E. coli* mCherry strains, were grown in a 37ºC shaker until reaching an OD_{600}= 0.20. Once at this final concentration, the bacteria were induced for protein expression with IPTG at 1 mM. The 20 ml culture of WT *E. coli* was divided into two 10 ml samples. Drug was added to one sample at 10 µM. KKL-35 was utilized as a positive control for a *trans*-translational inhibitor. In the other WT sample, DMSO was added as negative control. No additional compounds were added to the Δ*ssrA* strain. Fluorescence and absorbance (at 600nm) recordings were taken at T=0 hours and then each hour for 4 hours utilizing the Spectramax i3 (Molecular Devices). These were measured utilizing 100 µl of each strain in 96-well plates.

Minimum Inhibitory Concentration (MIC)

MIC assays were repeated to acquire a total of three trials with each compound-pathogen pairing. The most active compounds identified in the luciferase HTS, KKL-4669, KKL-11986, and KKL-11311, were all utilized in the experiment. The overnight cultures of each pathogen were back diluted to an OD_{600}= 0.002. In a 96-well sterile polystyrene u-bottom plate, 2 µl of each drug were placed in Row A in separate wells leading to a starting concentration of 200 µM in the first row. DMSO and KKL-35 were utilized as negative and positive controls, respectively. 100 µl of the bacterial culture was dispensed into the Row A, and 50 µl was dispensed into each well for every other row. Two-fold dilutions were performed down the plate. Only one pathogen was used per 96-well plate. After the dilutions were completed, each plate was covered with adhesive seal and incubated at 37ºC for 20 hours. MICs were then observed and recorded. For *S. aureus*, *P. aeruginosa*, and *S. enterica*, biohazard safety was enforced, and experiments were conducted in the biosafety cabinet.

MIC Protocol for *N. gonorrhoeae*

Biohazard safety was enforced, and experiments with *N. gonorrhoeae* were conducted in the biosafety cabinet. For the strain of *N. gonorrhoeae* utilized for these MIC assays with KKL-4669, KKL-11986, and KKL-11311, Thayer-Martin plates were streaked with sample from its frozen stock and grown at 37ºC in the presence of carbon-dioxide for approximately 20 hours. After this incubation, sample was inoculated into 3 ml of phosphate buffered saline(PBS). This starter culture was needed for optimal growth throughout the experiment. This culture was used to acquire 10 ml of a fastidious broth (FB) solution at OD_{600}= 0.4-0.5. Note that when using FB

medium, additional 0.1 M nicotinamide adenine dinucleotide (NAD) solution and 0.1% wt/vol pyrodoxine must be added. After 3-4 hours incubation in the 37ºC shaker, the culture was back diluted to an OD_{600}= 0.2 in FB medium. The rest of the experiment was conducted as described above with the other pathogens with the exception of the overnight incubation of the 96-well plate being in the presence of CO_2 at 37ºC.

SmpB Protein Purification

50 ml of phosphate salt solution was dispensed into 600 ml of Terrific Broth and was mixed thoroughly. 25 ml of this salt/broth mixture was separated, and 500 µl of the *E. coli* WT overnight culture was added along with kanamycin (30 µg/ml). This sample was then incubated in a 37ºC shaker until it reached an OD_{600} between 0.60-1.00. This dense culture was then induced with IPTG at 1 mM and left to grow in the 37ºC shaker for 3 hours. 2 ml of culture was separated into smaller aliquots that were spun down, and the pellets were stored at -20ºC. The rest of the culture was harvested by centrifugation for 10 min, and the large pellet was frozen down in -80ºC.

Before proceeding with the purification process with the large pellet, sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) was performed with the smaller pellets that were thawed and resuspended in 200 µl of LB followed by vortexing. 50 µl of these resuspensions were spun down for 1 min at max speed. Supernatant was discarded, and 50 µl of 1X SDS Buffer was added to each pellet. The samples were placed in a 95ºC water bath for 3 min. These warmed samples were then separated on a gel: 10 ml 12% resolving and 4 ml stacking. The gel was stained with Coomassie Blue and destained with gel equilibration buffer. It was visualized via ultraviolet light exposure.

All the steps for the rest of the Smp protein purification were under denaturing conditions. The large *E. coli* pellet was unthawed and resuspended in 40 ml wash buffer (20 mM imidazole). The cells were lysed by sonication and then were centrifuged two times at 7,000 x *g* for 10 min each run. Simultaneously, a nickel-nitrilotriacetic acid (Ni-NTA) agarose resin was prepared. 200 µl of resin/ethanol solution was centrifuged for 1 min. The liquid was removed, and four additional 1 ml-washes with binding buffer (no imidazole) and additional spins were performed. The resin was added to the lysed cells, and this sample was placed on a rocker at room temperature for 20 min-1 hr. Then this mixture was run through a column, and the flow through was stored at 4ºC. 20 ml of wash buffer (20 mM imidazole) was then run through the column, and this flow-through was also stored at 4ºC. 20 ml of elution buffer (250 mM imidazole) was applied to the column, and the eluate was collected in small 1.5 ml increments which were stored at 4ºC. The samples were analyzed by SDS-PAGE to ensure that a pure protein was present that appeared to be around 18 kDa in mass in all of the samples. The same conditions as previously described above for SDS-PAGE were utilized.

All of the pure protein fractions at this mass were combined and dialyzed in the presence of 50 mM 4-(2-hydroxyethyl-1-piperazineethanesulfonic acid) (HEPES), 100 mM potassium chloride (KCl), 10 mM magnesium chloride ($MgCl_2$), 10 M potassium hydroxide KOH, and 7 mM beta-mercaptoethanol (BME) as a denaturant. Four rounds of dialysis were performed to ensure removal of salts from the protein. SDS-PAGE was performed again to ensure a purified protein was present after dialysis. The protein samples were concentrated using 3 kDa filter columns, spinning samples at max speed, and collecting filtrate every 10 min for 30 min. The samples were analyzed by SDS-PAGE to ensure that the pure protein was present in the concentrated material.

In Vitro Transcription of tmRNA (RNAse-free conditions)

The *ssrA* gene was amplified from *E. coli* WT using polymerase chain reaction (PCR) with Phusion DNA polymerase. This PCR product was purified with the Qiagen PCR Purification kit under RNAse free conditions. The purified template was then utilized in the 500µl in vitro transcription reaction outlined below in **Table 2**.

Table 2. In Vitro Transcription Reaction Ingredients

500µl In Vitro Transcription Reaction
5X Fidelity Buffer- 1M HEPES, 1M Dithiothreitol (DTT), 1M $MgCl_2$, 0.5M Spermidine, 100µg/µl Bovine Serum Albumin (BSA), RNAsfree H_2O
1M DTT
1M $MgCl_2$
Purified *ssrA* DNA template
Inorganic Pyrophosphate (T1PP)
10X NTP Mix
RNAse free H_2O
T7 Ribonucleotide (RNA) Polymerase

This reaction occurred in a 37ºC waterbath for 2 hours followed by DNase I treatment. This treated transfer-messenger RNA (tmRNA) product was then run on a 40 ml 6% urea gel for ~6 hours. UV shadowing enabled identification of bands at a length of ~ 363 bp. These where cut and were incubated in diffusion buffer overnight at 37 ºC.

These samples were spun down at 16,000 x *g* for 10 min at 4ºC. 0.3 M sodium acetate was added to the samples, and the samples were vortexed. 200 proof ethanol was added to samples followed by vortexing. The samples were placed at -20ºC for 10 min. They were spun again for 10 min at 4ºC. Supernatant was removed, and the pellet was washed with 80% ethanol. The

pellets were allowed to dry for ~1 hr. 20 µl of sterile water was added to each pellet and left on ice for 15 min. The samples were vortexed, combined, and then left in ice. A 6% 10 ml urea gel was used to check that pure tmRNA was present after the extraction steps. Bands at ~363 bp were analyzed. The bands were cut from this gel and purified utilizing the NEB Monarch Gel Extraction Kit.

In Vitro Tagging Assay PCR Amplification of DHFR-nonstop (DHFR-ns)

The full-length *DHFR* gene was amplified from *E. coli DHFR* utilizing Phusion DNA Polymerase. A nonstop reverse primer and the T7 universal forward primer used in this PCR reaction led to the formation of a DHFR-ns template. This provided a DNA template for a control in vitro tagging reaction. The PCR product was extracted from a 1% agarose gel and purified utilizing the NEB Monarch Gel Extraction Kit. Bands ~500 bp were isolated.

The PURExpress In Vitro Protein Synthesis Kit E6800S was utilized to perform the tagging assay. Three 10 µl reactions were conducted with the following components: Solution A, Solution B, DNA template with *DHFR-ns*, purified tmRNA, purified SmpB, and [^{35}S]-Met. One of the three reactions was a negative control reaction and contained anti-ssrA oligonucleotide (5 µM). Another reaction contained purified SmpB protein, and the last reaction contained concentrated SmpB protein. The tmRNA and SmpB were mixed together before combining other ingredients of the reaction. The radiolabeled methionine was added last in a radioactive safety cabinet. The reaction occurred for 2.5 hours at 37ºC. After this incubation, the reactions were placed on ice for 10 min after adding cold acetone to each.

The samples were then spun at 16,000 x *g* for 10 min. All of the liquid was removed from each reaction, and the pellets were left to dry for 15 min. 12μl 1X SDS buffer was added to each reaction, and they were incubated on a 95ºC heat block for 5 min. These warmed samples were then separated on a gel: 5.00 ml 15% resolving and 3 ml stacking. The gel ran for 1.5 hours at 100 V, and was then dried for 2 hours at 80ºC. The gel was visualized via exposure to a phosphor screen overnight and was subsequently analyzed by using the Typhoon Gel scanner.

Chapter 3

Results and Conclusions for Identification of *trans*-Translation Inhibitors

Luciferase Assay: High-Throughput Screen of ReFrame Library

The high-throughput screen tested approximately 13,000 compounds in the luciferase assay. A schematic of the luciferase assay is shown in **Figure 1**. The luciferase-based assay utilized the mechanisms regarding expression of the *luc* gene in *E. coli* which encoded for firefly luciferase. The luciferase-based reporter utilized for this project contained a copy of the *trpAT* gene encoding the transcriptional terminator. It was inserted before the stop codon of *luc* on a multicopy plasmid.[3] This luc-trpAT reporter was important because it led to the production of a *luc* mRNA without a stop codon which initiated an active *trans*-translation system tagging and degrading the luciferase protein.[3] This highlight of the luciferase-based assay provided a way to determine if *trans*-translation was specifically being inhibited: higher luminescence signaled greater inhibition of *trans*-translation as shown in **Figure 3**. If *trans*-translation was not inhibited, the luciferase protein made during translation would have been successfully tagged for degradation.

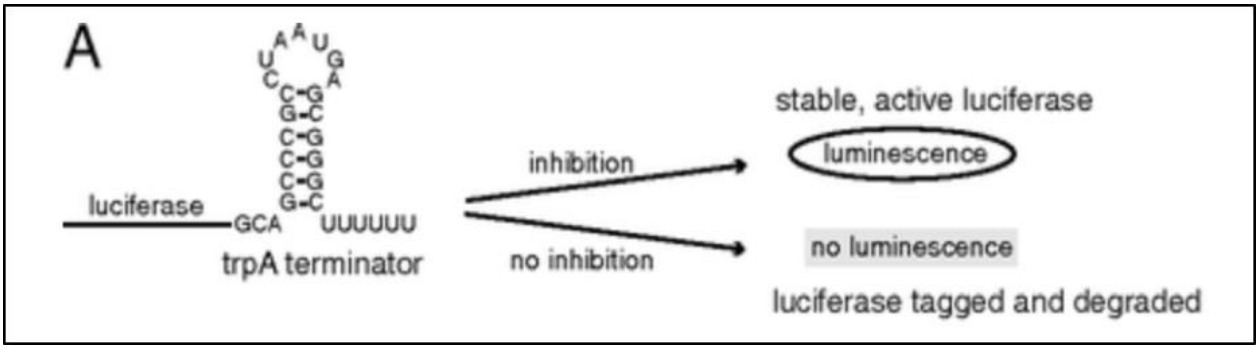

Figure 3. Luciferase Assay in HTS. A) Active *trans*-translation led to the luciferase protein being tagged and degraded from the *trans*-translation machinery leading to low luminescence levels. Inhibition of *trans*-translation prevented this degradation process allowing luciferase protein to interact with luciferin and leading to higher luminescence measurements. This figure has been adapted from Ramadoss, N. S., et al. (2013). Small molecule inhibitors of *trans*-translation have broad-spectrum antibiotic activity. *PNAS. 110*(25), 10282–10287.

Furthermore, control luciferase-based assay screenings with *E. coli luc-trpAT* and *E. coli luc*, which produced *luc* mRNA with a stop codon, had also been performed to determine the luminescence threshold that signaled inhibition. Additionally, the Bright-Glow reagent utilized in these experiments contained the substrate for luciferase, luciferin. Therefore, this substrate-enzyme interaction led to the production of the luminescence when functional luciferase was produced due to inhibition. Luminescence for each well in the 96-well plates was measured and plotted as shown in the example in **Figure 4**.

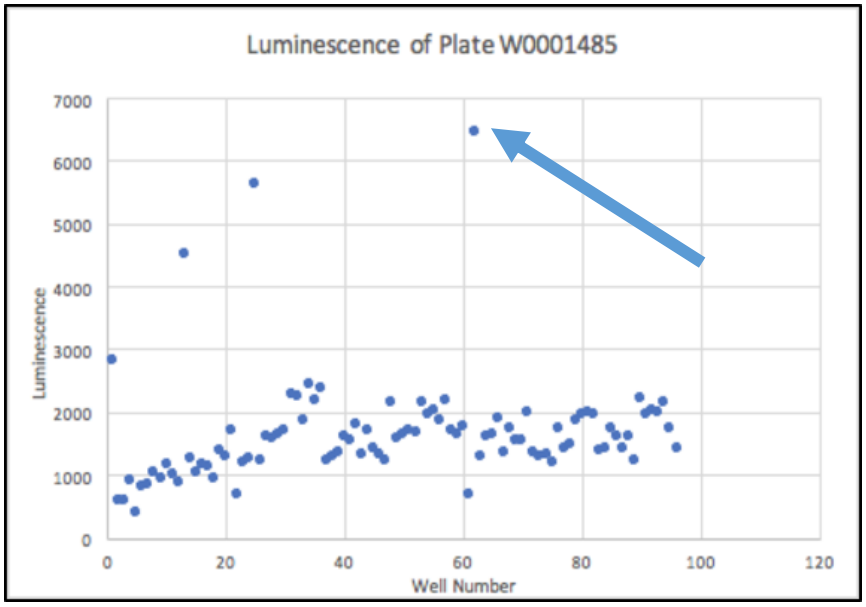

Figure 4. Representation of Results for Data obtained from the HTS. Luminescence was measured for each well in the Spectramax i3 (Molecular Devices), and the arrow indicates a "hit" compound that was visibly three standard deviations above the mean of luminescence for all compounds in the plate.

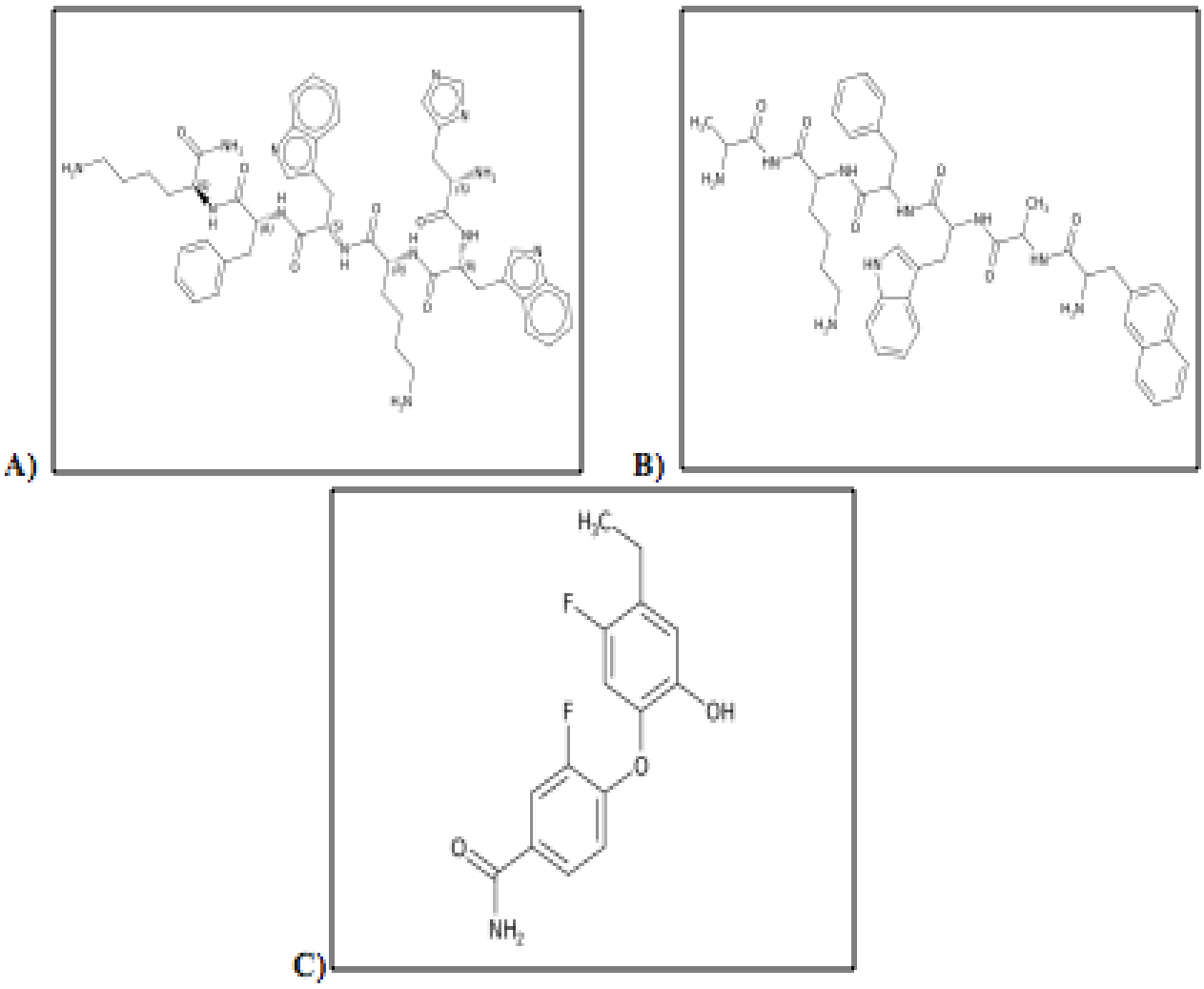

Figure 5. Identified Inhibitors of *trans*-Translation from the HTS. Chemical structures of the top three hit compounds in the HTS with the highest inhibition activity. A) KKL-11311. B) KKL-11986. C) KKL-4669. The chemical compounds were previously characterized and structures were previously known.

70 "hit" compounds were observed to inhibit *trans*-translation. 40 out of these 70 displayed the most promising results. Compounds were considered "hits" if luminescence levels were three standard deviations above the mean of the rest of the compounds in the screen. An example of data from one 96-well plate that contained a hit compound is depicted in **Figure 4.** Three hit compounds were determined to have the highest percentage of inhibition of *trans*-translation:

KKL-4669, KKL-11986, KKL-11311. These compounds are shown in **Figure 5**. These compounds were tested at various concentrations in subsequent dose assays, and the half-maximal inhibitory concentrations, IC_{50}, values were determined. The dose response curves are depicted in **Figure 6**.

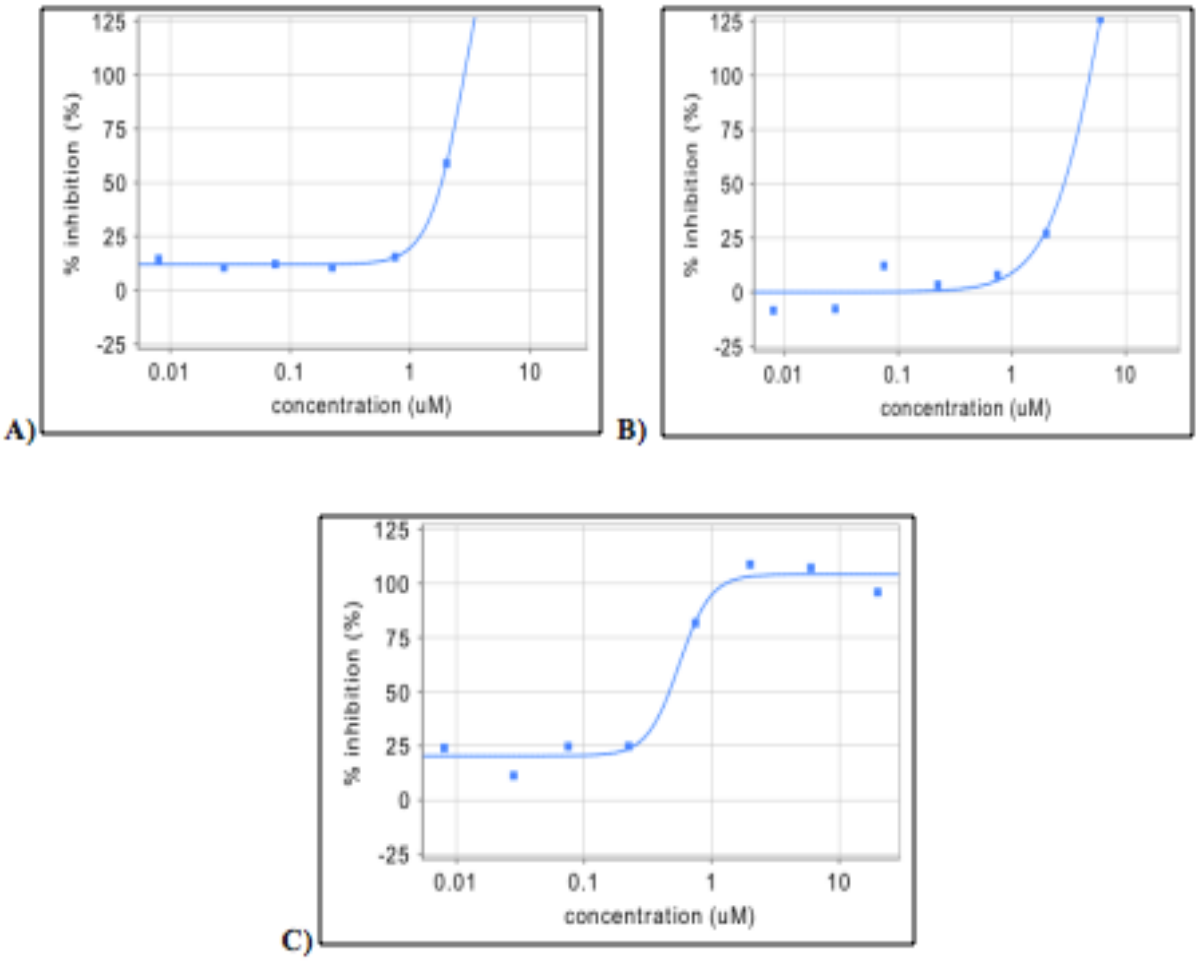

Figure 6. Dose Response Curves of Top Three "Hit" Compounds Identified in the HTS. Each dose response curve related % Inhibition versus Increasing Concentration (μM) for each compound based on luminescence measured in luciferase assay utilizing the SpectraMax i3 (Molecular Devices). A) KKL-11311. B) KKL-11986. C) KKL-4669.

IC_{50} assays were utilized to observe the potency of the chemical compound's effect on inhibiting the growth of *E. coli*. Data from these assays in **Figure 6** show that KKL-4669 had the lowest, and therefore most promising, IC_{50} of ~0.60 μM. The upper baseline on the plots for KKL-11311 and KKL-11986 were not attainable preventing an IC_{50} value for either to be determined. Low IC_{50} measurements indicated that lower concentrations of a drug were needed to inhibit growth. Additionally, KKL-4669 exhibited a sigmoidal dose response as the concentration of drug increased. Therefore, this is an optimal and effective scenario for a lead compound. These three compounds were utilized in several other experiments with goals of confirming their mode of action of inhibiting *trans*-translation and characterizing them.

Confirmation Screen: mCherry Fluorescence Assay

Fluorescence reporter assays were utilized to assess the compounds' ability to inhibit *trans*-translation. The mCherry-trpAT reporter was important because it encoded for an mCherry mRNA without a stop codon which would lead to an active *trans*-translation system tagging and degrading the mCherry protein. This highlight of the assay provided a way to determine if *trans*-translation was specifically being inhibited: higher fluorescence signaled greater inhibition of *trans*-translation. If *trans*-translation was not inhibited, the mCherry protein made during translation would have been successfully tagged and processed through proteolysis.

Observations of bacterial growth and average fluorescence were noted for the experimental trials of the three compounds with *E. coli mCherry-trpAT*. These results were compared to the positive control strain *E. coli mCherry* Δ*ssrA* which was considered to be 100% fluorescence since no *trans*-translation should have been occurring. After normalizing the fluorescence to absorbance

(RFU/OD_{600}), these values were utilized to quantify inhibition of *trans*-translation. All of these analyses are shown in **Figure 7**. Differences between all of the trials were not apparent until 5 hours. It appeared to be evident that the positive control emitted the largest fluorescence values compared to WT or no drug conditions in *E. coli-trpAT*. KKL-11311 and KKL-11986 trials led to larger inhibition compared to WT but lower than the Δ*ssrA* positive trial. KKL-4669 did not show substantial inhibition compared to WT in this assay despite leading to highest inhibition in the luciferase screen above.

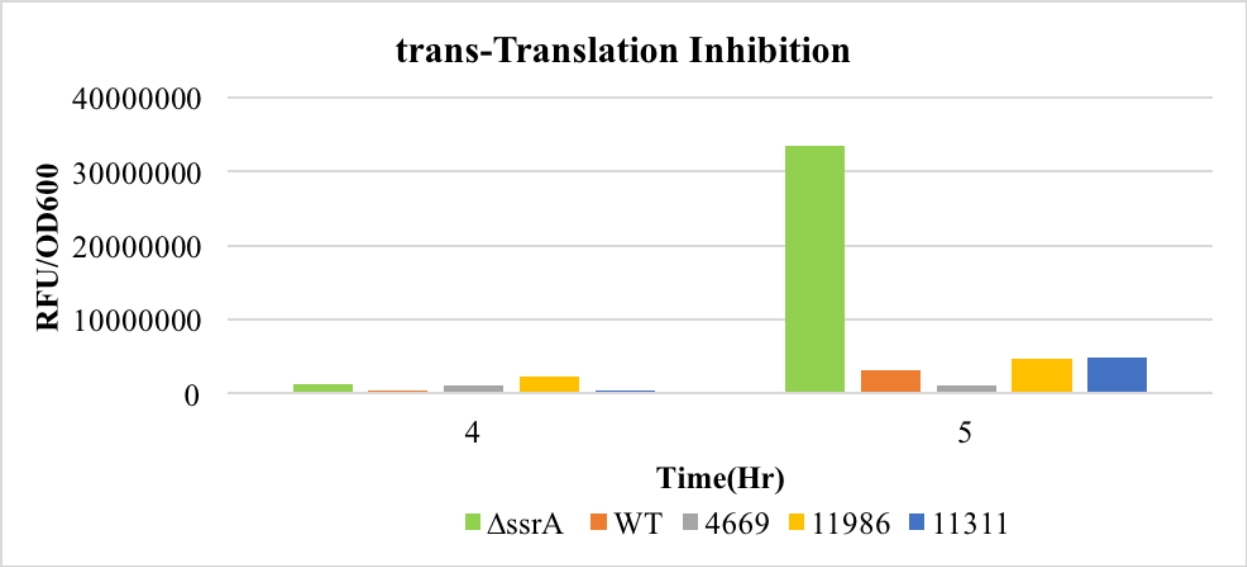

Figure 7. Characterization *trans*-Translation of Hit Compounds. All measurements were acquired from the SpectraMax i3. Bacterial growth was monitored via OD_{600} and average fluorescence was determined for each trial by normalizing fluorescence to 100% *E. coli* Δ*ssrA* fluorescence. Inhibition of *trans*-translation was determined through normalization of RFU/OD_{600} which was plotted against the 4 hours and 5 hours time points. Positive control, *E. coli mCherry* Δ*ssrA*; Negative control, *E. coli mCherry*; KKL-4669, 11986, and 11311, Experimental hit compounds.

Minimum Inhibitory Concentration (MIC) Experiments

Characterization of these compounds continued by way of conducting MIC experiments against various pathogens of interest for antibiotic development. In **Table 3**, it is evident that KKL-4669 had the most significant inhibitory activity against *E. coli WT*, *E. coli* Δ*tolC, S. aureus*, *S. enterica*, and *N. gonorrhoeae*, due to relatively low MICs respectively: 1.56-3.13 µM, 0.53 µM, 1.56-3.13 µM, 100-200 µM, and 0.63-1.25 µM. These lower MIC values suggest that KKL-4669 was actively inhibiting growth at low concentrations which is a quality paramount of antibiotic activity. Several compounds resulting from the high-throughput screen were characterized as having inhibitory activity against *trans*-translation, and KKL-4669 seemed most promising to pursue in subsequent characterization due to its dose-response behavior and inhibition of various pathogens.

Table 3. MIC Results of Hit Compounds Across Pathogens

Pathogen	KKL-35	KKL-4669	KKL-11311	KKL-11986
E. coli WT	1.56 µM	1.56-3.13 µM	>200 µM	>200 µM
E. coli Δtolc	0.30 µM	0.53 µM	125 µM	125 µM
S. aureus	≤1.56 µM	1.56-3.13 µM	>200 µM	>200 µM
P. aeruginosa	>200 µM	>200 µM	>200 µM	>200 µM
S. enterica	100-200 µM	100-200 µM	>200 µM	>200 µM
N. gonorrhoeae	0.08 µM	0.63-1.25 µM	100-200 µM	>200 µM
B. anthracis	3.00 µM	>200 µM	>200 µM	>200 µM

In Vitro Tagging Assay with DHFR-ns, tmRNA, and SmpB Protein

In vitro transcription reactions for production and purification of tmRNA along with purification of the SmpB protein were necessary for performing in vitro tagging assays that would provide additional insight into whether or not KKL-4669 could be confirmed as inhibiting *trans*-translation specifically and not simply translation. **Figure 8** depicts the purified tmRNA and purified SmpB protein (18 kDa) isolated for the tagging assay. The in vitro tagging control assay included a gene encoding dihydrofolate reductase (DHFR) with the stop codon removed to create a DHFR-ns template. This was used in a coupled in vitro transcription/ translation reaction with *E.* coli. It had been shown previously that in vitro expression of DHFR-ns led to full-length DHFR protein and that when tmRNA-SmpB was incorporated in the reaction, larger amounts of tagged DHFR were produced.[3] Therefore, if large amounts of untagged DHFR were produced as a result of the tagging reaction, it would have suggested that the compound did not inhibit translation but inhibited *trans*-translation.

Using the purified products in **Figure 8** and the reaction protocol in the methods section, a control in vitro tagging assay was performed without KKL-4669. The results of the SDS-PAGE conducted with the DHFR-ns template in various trials consisting of different purified tmRNA and SmpB protein are shown in **Figure 9**. When tagging is active, most of the DHFR protein produced has the tmRNA-encoded peptide as a result of *trans*-translation being activated. This leads to the tagged DHFR protein migrating more slowly on an SDS-PAGE gel. Ultimately, the goal of such an assay was to have a control before conducting the same reaction where inhibitors blocking tagging were added.

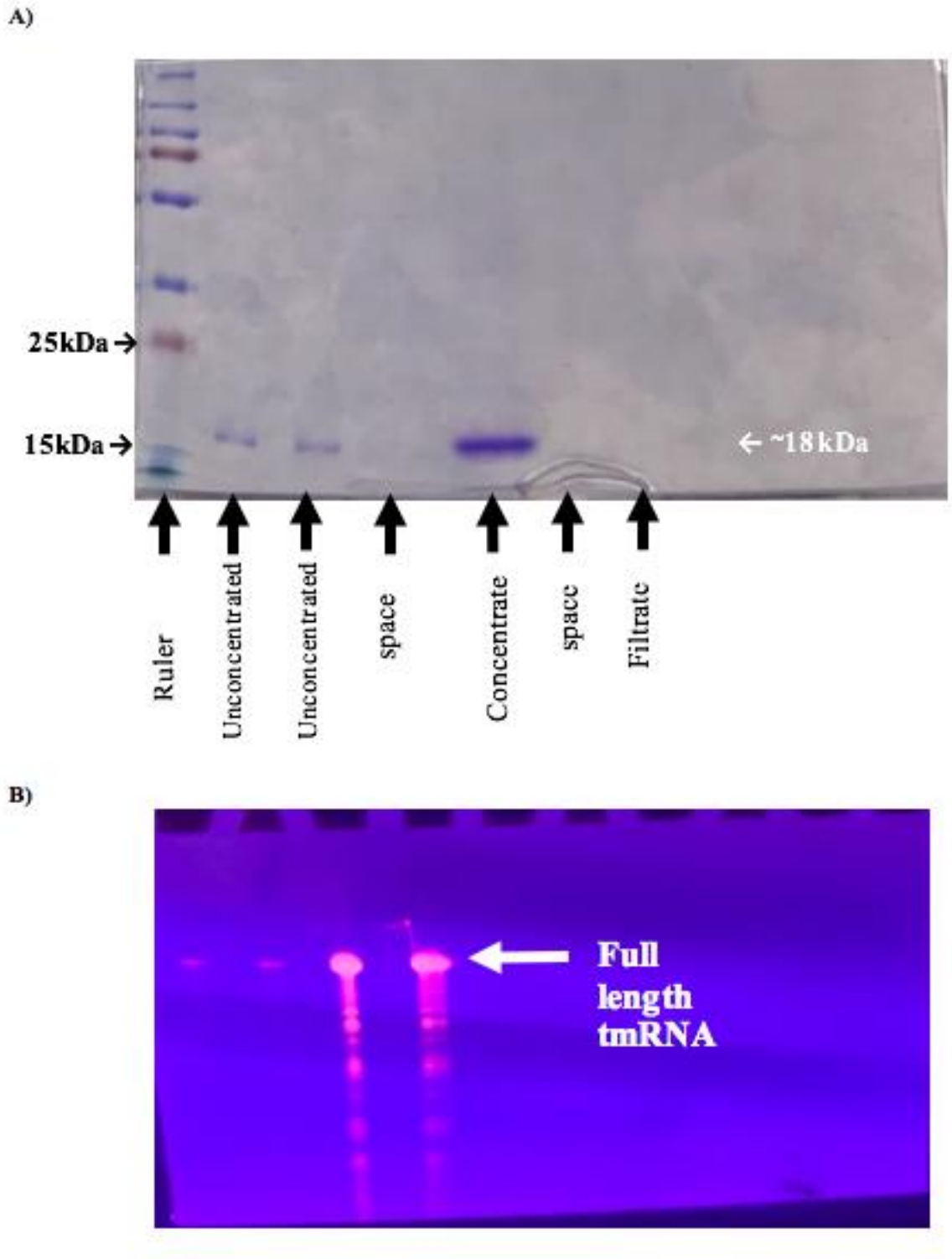

Figure 8. Purification of SmpB Protein and tmRNA for In Vitro Tagging Assay. A) Purified SmpB protein product from His-tag/Ni-NTA Purification; The concentrated ~18 kDa product is above. B) Full length tmRNA product from in vitro transcription.

Inhibition of *trans*-translation tagging would decrease the amount of tagged protein and increase the amount of untagged DHFR protein marking inhibition of *trans*-translation. The SDS-PAGE gel exposed to the phosphor screen and scanned by the Typhoon Gel Scanner is shown in **Figure 9**. The anti-*ssrA* oligonucleotide present in the reaction in lane 1 inhibited background *trans*-translation activity from the tmRNA-SmpB in the kit.[3] The negative control lane 2 shows results when tmRNA and SmpB were not present. Lane 3-5 show the trials where *trans*-translation appeared to be active since the thick bands on the gel correlate to the tagged DHFR protein.

The greatest tagging efficiency occurred with Lane 4 at >90% which consisted of AD tmRNA (purified during this project) and JA-1 SmpB protein (purified previous to this project). The reaction with both AD components was not far from this activity and led to a slightly lower efficiency below 90%. These percentages are significantly higher (nine-fold) than the ~10% efficiency of background of the negative control. The presence of such high efficiency suggests that these components were optimally functional at conducting *trans*-translation and tagging the DHFR protein. Comparison of DHFR template (with stop codon) to DHFR-ns template or with inhibitors was not conducted, but the functionality of the purified components was determined.

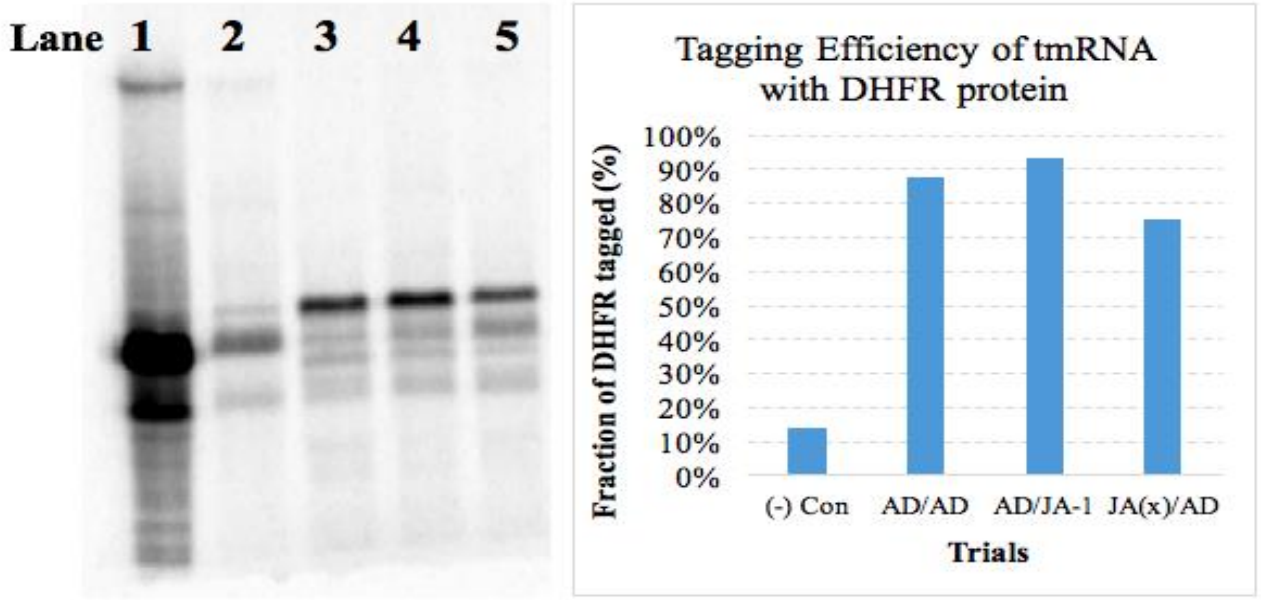

Figure 9. In Vitro Tagging *trans*-Translation Reactions Testing if Purified Components were Functional and Active. Left: SDS-PAGE gel after exposure to phosphor and scanned via Typhoon Gel scanner. "AD" denotes the components used in this project and "JA" denotes components purified previously. Lane 1: Anti-ssrA. Lane 2: No tmRNA or SmpB protein in reaction. Lane 3: AD tmRNA/AD SmpB. Lane 4: AD tmRNA/ JA-1 SmpB. Lane 5: JA(x) tmRNA/ AD tmRNA. **Right**: Quantification of tagging efficiency is shown which was calculated as the percentage of total DHFR protein in the tagged DHFR band.

Overview

This chapter focused on the results from drug discovery efforts beginning with high-throughput screening methods particularly targeting *trans*-translation inhibition. The luciferase assay utilized in the HTS along with the mCherry assay confirmation assays enabled three compounds to be considered for subsequent experimentation: KKL-4669, KKL-11311, and KKL-11986. Characterization of their effects on the growth of various bacterial pathogens led to the determination that KKL-4669 was the most promising candidate for antibiotic development. Preparation for in vitro transcription/ translation assays was conducted through purification of

trans-translation machinery, tmRNA and SmpB protein. Control in vitro reactions established the functionality of this machinery. Furthermore, all of these steps accelerated this project closer to target identification for KKL-4669.

Chapter 4

Materials and Methods for Ribosome Profiling in *C. crescentus*

Bacterial Strains

The bacterial strains utilized to conduct ribosome profiling in *C. crescentus* are listed in **Table 4** below.

Table 4. Bacterial Strains, Plasmids, Primers for Ribosome Profiling in *C. crescentus*

Bacterial Strains	**Description**
Escherichia coli	• Contains plasmid pKK838 (pJS14 + ssrA) • KCK #123 strain • Chloramphenicol resistance marker • Contains WT *C. crescentus* promoter
Caulobacter crescentus WT	• CB15N(BE1000) • KCK #1 strain • DPH8 parental strain containing pDH2
Caulobacter crescentus ΔssrA	• CB15NDssrA • KCK #116 strain • Spectinomycin resistance marker replaced ssrA gene
Plasmids	
pJET1.2/blunt	• Cloning vector containing two BglII recognition sequences flanking the insertion site and a T7 promoter • Positive selection for recombinant plasmids • Ampicillin resistance marker
pKK838	• Contains WT *C. crescentus* promoter • Chloramphenicol resistance marker
Primers for *ssrA-MS2*	
Forward	5'AACTAGTGGATCCTGGGCCTGGTGGAGCTGT-3'.
Reverse with MS2 tag	5'TGGTAAGCTTACATGGGTGATCCTCATGTAGGTGG AGGGATTCTGTTGGCAGGCTCCCT-3'

Growth Conditions

E. coli DH5α (KCK strain #15) cells were grown overnight in 5 ml LB cultures at 37ºC incubating for ~15-17 hours. *E. coli* pKK838 was grown in 5 ml LB cultures at 37ºC in the presence of chloramphenicol (20 μg/ml) for ~15-17 hours. *Caulobacter ssrA* (WT and KCK strain #1) and into *Caulobacter crescentus* Δ*ssrA* (KCK strain #116) cells were grown in 5 ml overnight cultures containing Peptone Extract Yeast (PYE) media in the presence of chloramphenicol (1 μg/ml) at 30ºC. These strains took longer to grow than *E. coli* and were therefore incubated ~24 hours.

1.Cloning of *ssrA-MS2* into pJET1.2 with *E. coli*

Initially, the MS2 tag, 24 RNA stem loop residues, was added to *ssrA* through PCR amplification with Taq DNA polymerase. A reverse primer listed in **Table 4** added the tag and a forward primer also aided with the amplification *ssrA*. This PCR product, ~504 bp, was isolated and extracted from a 1.5% 50 ml agarose gel. The PCR product running at ~500 bp was extracted and purified with the NEB Monarch Gel Extraction kit. This insert and purified pJET1.2/blunt vector DNA were contained in a T4 ligation reaction that incubated overnight at 16ºC. The 20 μl ligation product was transformed into 50 μl of DH5α calcium competent cells. The cells were incubated on ice for 30 min followed by heat shock at 42ºC for 50 sec. They were then incubated on ice for 2 min, and 1 ml of LB was added to the sample. After incubation at 37ºC for one hour occurred, 100 μl of sample was plated onto LB agar plates with ampicillin (50 μg/ml) for overnight incubation.

Colony growth signaled the presence of the pJET-ssrA-MS2 construct since the pJET plasmid contained an ampicillin resistance marker. Colony PCR was performed with several "positive" colonies with Taq polymerase and both primers listed above. The products of this PCR were visualized on a 1.5% 50 ml agarose gel. The "positive" colonies that led to a 504 bp insert band were also used to set up 5 ml LB overnight cultures with ampicillin (50 μg/ml) which were inoculated with these positive colonies. pJET-ssrA-MS2 DNA was purified from *E. coli* via the NEB Miniprep Kit. This DNA was sequenced, and this purified plasmid was digested with HindIII and BamHI restriction enzymes. The digestion reaction ran for 20 min and was then placed in the 80ºC heatblock for 10 min. The sample was then visualized as an insert band ~500 bp on a 1.5% 50 ml agarose gel. This insert was isolated and purified using the NEB Monarch Gel Extraction Kit.

2.Cloning of *ssrA-MS2* into pKK838 with *E. coli*

Plasmid was isolated from *E. coli* pKK838 and purified via the NEB Miniprep Kit. The isolated prep, which was a vector for this cloning, was digested with HindIII and BamHI restriction enzymes as previously described for the insert DNA in the previous section. This vector DNA was then visualized as a band ~3.5 kB on a 1.5% 50 ml agarose gel. This vector was isolated and purified using the NEB Monarch Gel Extraction Kit. This pKK838 vector and *ssrA-MS2* insert were ligated together in a T4 ligation reaction with the same conditions as was previously described. The 20 μl ligation product was transformed into 50 μl of calcium competent *E. coli* pKK838 cells following the same procedure as shown above. The only difference with this transformation was that LB agar plates containing chloramphenicol (20 μg/ml) were used since

the pKK838 plasmid contained a chloramphenicol resistance marker. Colony PCR was also performed with several "positive" colonies with Taq polymerase and both primers listed above. The "positive" colonies that led to the visualization of a 504 bp insert band were also used to set up 5 ml LB overnight cultures with chloramphenicol (20 μg/ml) which were inoculated with these positive colonies. pKK838-ssrA-MS2 DNA was purified from *E. coli* via the NEB Miniprep Kit, and this DNA was sequenced.

3.Cloning of pKK838-ssrA-MS2 into WT and Δ*ssrA C. crescentus*

The major obstacle with working with *C. crescentus* had been its different growth patterns compared to *E. coli*. The growth of overnight cultures required longer periods of time. It also seemed to depend heavily on whether the overnight cultures were inoculated with sampled from an agar plate or from a frozen stock. Inoculating from an agar plate led to the most efficient results. 5 ml PYE overnight cultures for WT *C. crescentus* took approximately 17-20 hours. Δ*ssrA C. crescentus* cultures took longer around 20-23 hours. All incubations occurred at 30 ºC. These incubations represent inoculations from agar plates. In order to acquire competent cells, *Caulobacter* cells had to be electroporated shortly before transformation occurred.

Acquiring competent cells required monitoring growth so that overnight cultures were used at an OD_{600}= 0.3-0.4. If the cultures were left to grow past this range, they could be back diluted and allowed to recover. However, this only was appropriate if the OD_{600} did not reach 0.8 because at this point, recovery was very difficult. Therefore, once the correct OD_{600} was acquired the culture was divided into 1.50 ml aliquots and centrifuged for 5 min at max speed and at 4 ºC. It was crucial from this point on that all steps were performed under this cold temperature. The

supernatant was removed, and the pellets were resuspended in 1 ml of ice-cold sterile water. The samples were then spun down again for 5 min. This wash step along with the subsequent spin was repeated two more times for a total of 3 washes. After the last spin, supernatant was removed, and the pellets were resuspended in 30 µl of ice-cold sterile water.

1 µl of plasmid was then added to these samples. A positive control consisted of the pKK838 vector only being introduced. A negative control was sample without plasmid where 1 µl of water was a substitute. The experimental plasmid was the purified pKK838-ssrA-MS2 construct isolated from *E. coli* in the previous cloning experiments. Each 30 µl sample was transferred to a sterile cuvette for electroporation at *E. coli* 2 setting. Immediately after shocking the cells, 1 ml of PYE media was added to the cuvettes containing the sample. Each sample was then incubated for 1.5 hours at 30ºC. After this incubation, 250 µl of each sample was plated on PYE agar plates containing chloramphenicol (1 µg/ml) since a successful transformation would allow only cells containing the pKK838 plasmid with a chloramphenicol resistant marker to grow. "Positive" colonies from the vector plates and experimental clone plates for both *Caulobacter* strains were isolated and plasmids from each were purified via NEB Miniprep Kit. PCR reactions were conducted utilizing the same primers used with *E. coli* cloning protocols. Products were run on 1.5% 50 ml agarose gels, and ~504bp bands were visualized via UV exposure and purified through utilizing the NEB Monarch Gel Extraction Kit.

Chapter 5

Results and Conclusions for *Caulobacter* Ribosome Profiling

Cloning of the *ssrA* gene with a MS2 tag into *Caulobacter crescentus* was completed through a sequence of steps. The first cloning project of amplifying the *ssrA* gene from *E. coli* pKK838 while adding the MS2 tag is schematically shown in **Figure 10**. Subsequent purification and restriction digestion steps led to the *ssrA-MS2* product to have been ligated with the pJET1.2/blunt vector, also shown in **Figure 10**. The final construct was transformed into *E. coli* DH5α, isolated, purified, and digested as shown in **Figure 11** which depicts the beginning of the second cloning project. The pKK838 vector in **Figure 11** was isolated from *E. coli* and digested. This linear vector and the *ssrA-MS2* insert from the pJET1.2/blunt cloning were ligated, and the new pKK838-ssrA-MS2 construct was transformed back into *E. coli* pKK838 cells as depicted in **Figure 11**. The plasmid isolated from a positive colony after this transformation was used in PCR amplification of the target insert. The insert amplified from this colony was visualized as shown in **Figure 12**. The target construct (pKK838-ssrA-MS2) was then transformed into *C. crescentus WT* and *C. crescentus ΔssrA* strains in the third stage to this cloning project which is shown in **Figure 13**. The genetic material of the clones in *Caulobacter* was isolated and visualized as shown in **Figure 14** to ensure that *ssrA-MS2 (*~500bp) was present in the target clones.

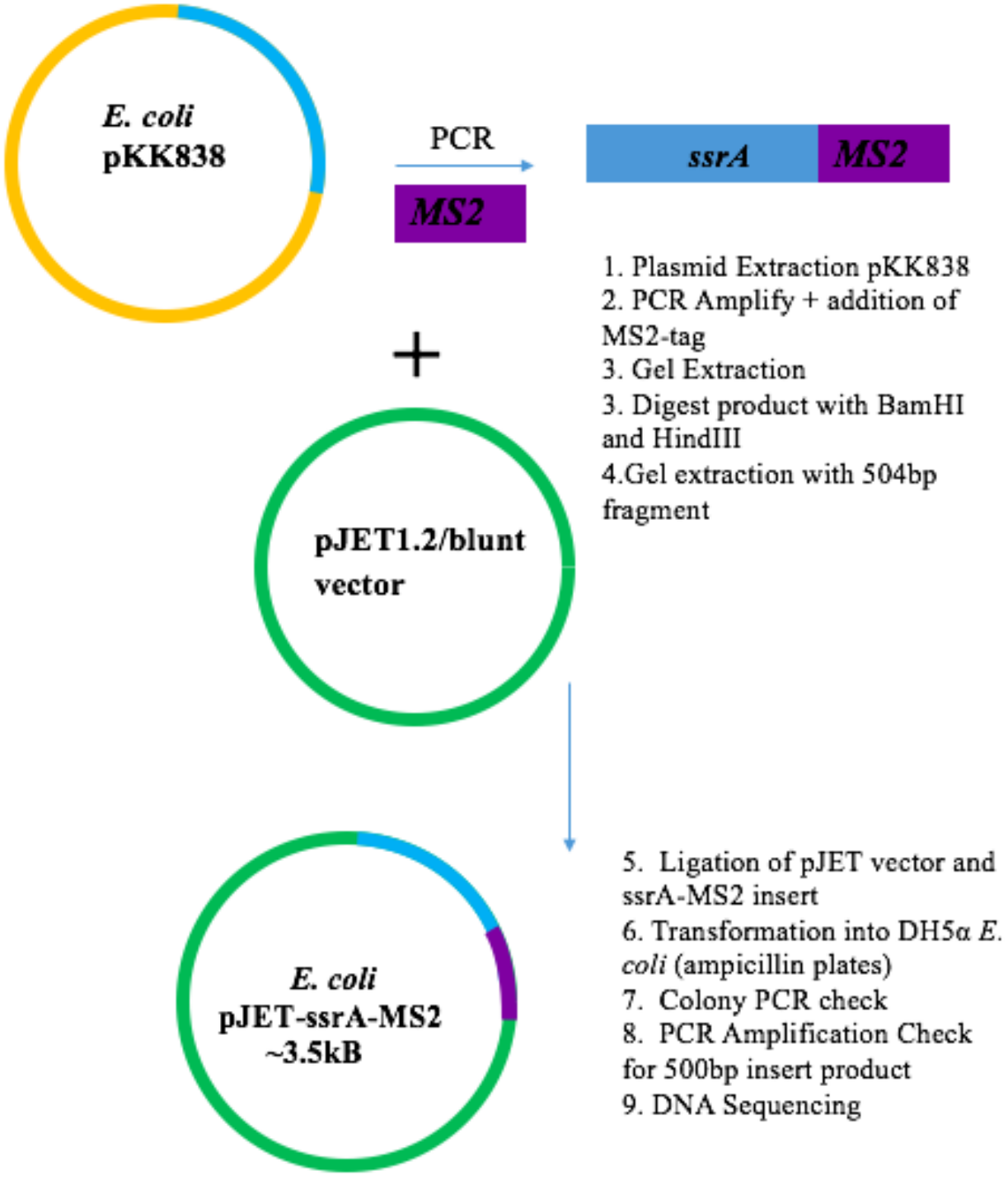

Figure 10. Cloning Project Stage #1. The steps leading to the growth of pJET-ssrA-MS2 *E. coli* clones are outlined here.

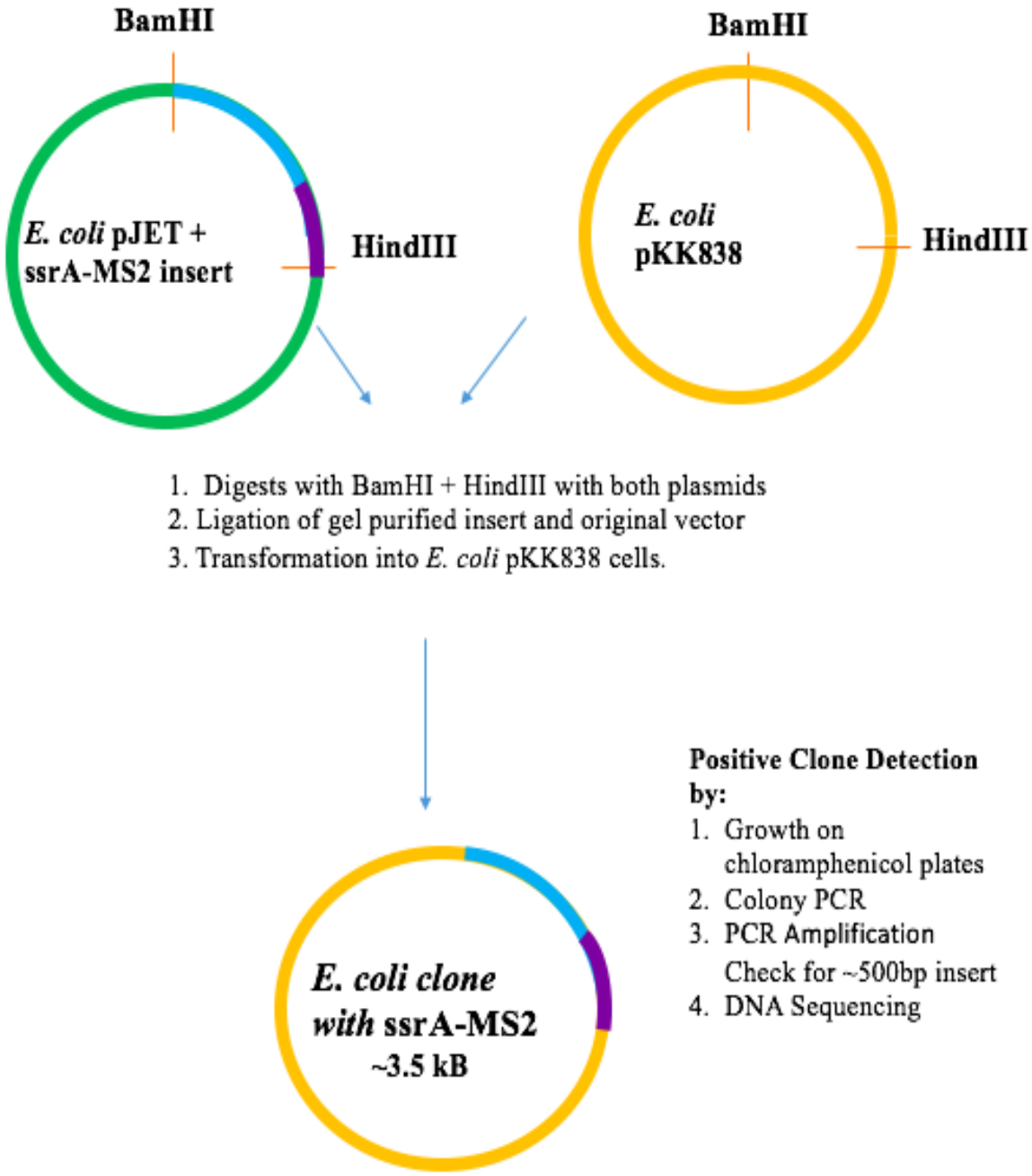

Figure 11. Cloning Project Stage #2. The steps leading to the growth of pKK838-ssrA-MS2 *E. coli* clones are outlined here.

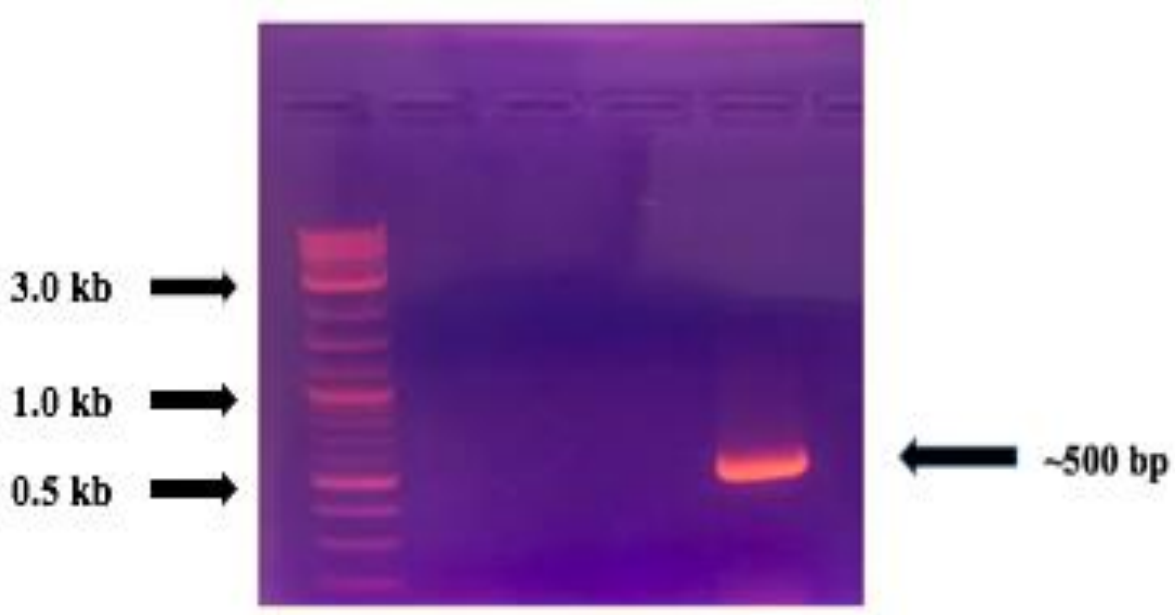

Figure 12. PCR Amplification of *ssrA-MS2* insert. Plasmid was isolated from a positive *E. coli* pkk838-ssrA-MS2 colony on chloramphenicol plate after transformation. The *ssrA-MS2* insert PCR products shown in lane 5 ~504bp.

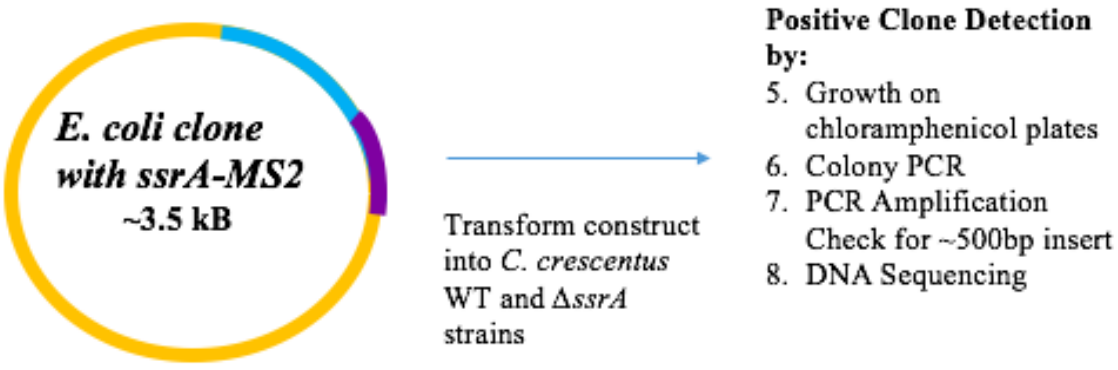

Figure 13. Cloning Project Stage #3. The steps leading to the growth of pKK838-ssrA-MS2 *C. crescentus* clones are outlined here.

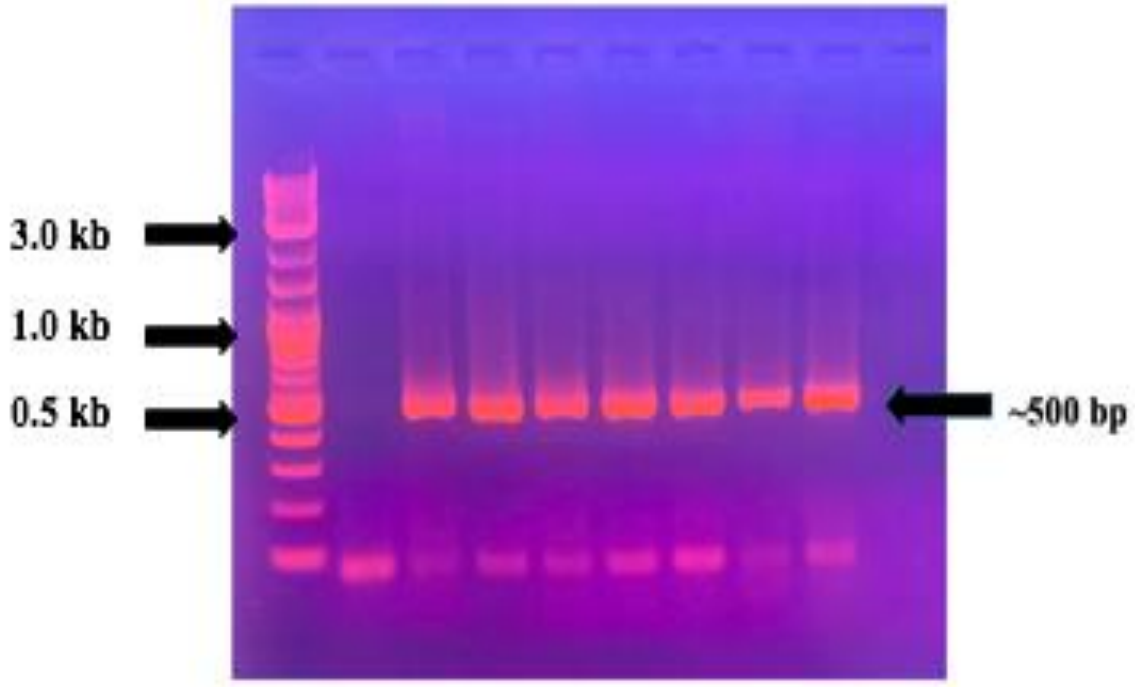

Figure 14. PCR Amplification of *ssrA-MS2* insert. Plasmid was isolated from positive *C. crescentus* pkk838-ssrA-MS2 colonies on chloramphenicol plates after transformation for both the WT and Δ*ssrA* strains. DNA from positive controls such as *Caulobacter* and *E. coli* colonies with the empty pKK838 vector along with the *E. coli* clones from Stage #2 was also isolated and compared. The *ssrA-MS2* insert PCR products shown in lane 5 ~504bp.

Attaining these *Caulobacter* clones will now enable the next steps in ribosome profiling to be conducted involving MS2-tagging, ribosome pull-down, and ribosome profiling methodology to observe mRNA sequences occupying the ribosome in *C. crescentus* during *trans*-translation and inhibition of *trans*-translation conditions.

Chapter 6

Discussion and Future Directions

It is evident that the year 2050 is not far away and that new antibiotic development is urgently needed more than ever. It was not necessarily foreseen that such a pharmacological revolution as antibiotic development would experience such a global phenomenon as resistance worldwide. The projects described in this work aimed at contributing to the effort of identifying potential new antibiotics and at expanding knowledge about *trans*-translation overall. These goals have not ultimately been completed; however, significant steps towards these aims have been achieved.

The high-throughput screen was successful in identifying several compounds that appeared to inhibit *trans*-translation which led to subsequent studies particularly with compound KKL-4669. This enabled the main "hit" compound in the screen to be characterized against various bacteria where low MIC values in many Gram-negative (*E. coli*, *S. enterica*, and *N. gonorrhoeae*) pathogens was significant. In the process towards confirming KKL-4669 as an inhibitor of *trans*-translation and identifying its molecular target, control in vitro assays were performed that required much methodology in purification of *trans*-translation machinery, tmRNA and SmpB protein. The functionality of these components to promote *trans*-translation activity was evident in the high tagging efficiencies observed in the in vitro tagging assay. The future direction of this project involves confirmation of inhibition of *trans*-translation via in vitro tagging assays with KKL-4669. Once this is achieved, the molecular target within the *trans*-translation process will be identified through mutagenesis and click chemistry methodologies.

The ribosome profiling project in *Caulobacter* has had a successful beginning at achieving the *ssrA-MS2* clones needed in *E. coli* and, most importantly, in *Caulobacter*. This

was confirmed through UV visualization of agarose gels, positive colony appearance through antibiotic selection after transformation, and sequencing. Future directions for this project include ribosome pull-down assays, mRNA footprinting, and ribosome profiling methodology to analyze mRNA sequences.

It is known across the literature and within the field that novel drug and antibiotic development can take anywhere from 10-30 years until individuals can actually benefit in the clinic from such drugs. Therefore, it is understandable that a new antibiotic has not been developed in the three-year duration of these projects. Nonetheless, much valuable information has been attained that has expanded the overall field of knowledge for bacterial pathogens' responses to certain compounds such as KKL-4669, for example. The continuation of both projects described in this work could have substantial and beneficial consequences. If KKL-4669 is characterized further, it may serve as a candidate for further antibiotic development. If the ribosome profiling project continues to utilize the MS2-tagging, ribosome pull-down, and ribosome profiling methodologies, the observation of mRNA sequences occupying the ribosome in *C. crescentus* during *trans*-translation and inhibition of *trans*-translation conditions can be attained. This could provide invaluable insight into *trans*-translation and aid in completing the entire picture of this process which could ultimately lead to new targeting focuses for future novel antibiotic development.

Efforts must continue in this area of research in order to get closer to combatting the tragic reality of antibiotic development. Antibiotic resistance is leading to ~50,000 deaths each year in Europe and the United States alone.[1] The bacteria may be mutating and becoming resistant faster each year, but research can also keep improving simultaneously. Furthermore, the findings and potential of these two projects targeting *trans*-translation show how such work can

be part of this development that could one day be looked back on as the beginning of work that led to saving and improving individuals' lives across the globe.

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National Society of Collegiate Scholars Member Spring 2017-May 2020

Penn State University Student Engagement Network Grant Fall 2018, Spring 2020

NASA Pennsylvania Space Grant Undergraduate Scholarship Fall 2018, Spring 2019

RESEARCH EXPERIENCE

The Pennsylvania State University University Park, PA
Independent Research in Antibiotic Resistance 2017-May 2020
Dr. Keiler Lab, Department of Biochemistry and Molecular Biology

- Conducted high-throughput screen of a 13,000-compound library using a luciferase reporter assay and characterized inhibitors of *trans*-translation through in vitro assays with various bacterial pathogens
- Ribosome profiling in *C. crescentus*

Penn State College of Medicine Hershey, PA
MD/PHD Schreyer Honors College Exposure Internships Summer 2016/Summer 2017
Dr. Parent Lab, Department of Medicine

- Conducted experiments involving Rous sarcoma virus (RSV) and its nuclear interactions between with host factors
- Utilized RNA fluorescent *in situ* hybridization (FISH) and DNA FISH methods, cloning, cell synchronization, and confocal microscopy imaging

The Commonwealth Medical College Scranton, PA
Research Fellowship 2014

- Observed efforts with *Drosophila*, with breast cancer cells, and in the animal vivarium

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- Confocal/ Fluorescence Microscopy with RSV
- DNA FISH and RNA FISH with RSV
- Microscopy of *Drosophila melanogaster* and neuronal analysis
- In vitro assays and radiolabeling in bacterial pathogens
- Fluorescent Reporter Assays; spectrophotometric analysis
- Cloning (PCR, gel electrophoresis, restriction digests)
- Bacterial growth inhibition assays
- DNA isolation and purification
- Transformation and Transfection methodology
- Bacterial and Mammalian Cell Culture/ MIC assays (bacterial resistant pathogens)

PRESENTATIONS

Davison, A., Maldonado, R., Rice, B., Musier-Forsyth, K., Parent, L. Nuclear Interactions between Retroviruses and a Host Factor Required for Reverse Transcription. Poster presentation delivered at the Summer Undergraduate Research Symposium, Penn State College of Medicine, Hershey, PA, August, 2017.

Davison, A., Maldonado, R., Rice, B., Musier-Forsyth, K., Parent, L. Nuclear Interactions between Retroviruses and a Host Factor Required for Reverse Transcription. Poster presentation delivered at the Eberly College of Science Poster Exhibition at the Science Benefactor's Reception, Penn State University, University Park, PA, October, 2017.

Brogan, A., **Davison, A**. Identification of *trans*-Translation Inhibitor Compounds.
Poster Presentation delivered at the Spring Undergraduate Exhibition, Penn State University, University Park, PA, April, 2018.

Davison, A. Investigating RSV Proviral Integration at the Nuclear Periphery. Oral Presentation delivered at the 2018 Summer Undergraduate Research Symposium, Penn State College of Medicine, Hershey, PA, August 2018.

Davison, A. KKL-4669: Inhibitor of *trans*-Translation. Poster Presentation delivered at the Spring Undergraduate Exhibition, Penn State University, University Park, PA, April, 2019.

Davison, A. Ribosome Profiling during *trans*-Translation to Combat Antibiotic Resistance. Recorded Poster Presentation submitted to virtual Spring Undergraduate Exhibition, Penn State University, University Park, PA, April, 2020.

ABSTRACTS

Davison, A., Maldonado, R., Rice, B., Parent, L. Nuclear Interactions between Retroviruses and a Host Factor Required for Reverse Transcription. Abstract of the Summer Undergraduate Research Symposium, Penn State College of Medicine, Hershey, PA, August, 2017.

Davison, A., Maldonado, R., Rice, B., Parent, L. Subcellular Interactions between Retroviruses and Host Factors Required for Successful Integration and Infection**.** Abstract of the Summer Undergraduate Research Symposium, Penn State College of Medicine, Hershey, PA, August, 2018.

Rai, P., Chen, E., **Davison, A.,** Maldonado, R., Parent, L. Visualizing co-transcriptional splicing events Rous sarcoma virus. Abstracts of the Graduate Student Research Forum, Penn State College of Medicine, Hershey, PA, March, 2019.

PROFESSIONAL EXPERIENCES

Penn State Cancer Institute Hershey, PA
Clinical Shadowing Summer 2017
Dr. Rebecca Phaeton, Department of Obstetrics and Gynecology

- Observed treatment of primarily patients with ovarian cancer; Scrubbed in the OR for a laparoscopic hysterectomy

Penn State Neuroscience Institute Hershey, PA
Clinical Shadowing Summer 2018
Dr. Mark Stahl, Department of Neurology: Movement Disorders

- Observed diagnosis, treatment, and therapeutic options for patients with Essential Tremor and Parkinson's Disease

LEADERSHIP EXPERIENCES

The Luzerne Foundation Luzerne, PA
Director of Youth Advisory Committee 2013-2016

- Oversaw grant proposals, distributed grant monies to non-profit organizations, and speaker for Annual Report meetings

Pontifical Mission Society Walls, MS
Mission Trip to Walls, MS 2015

- Renovated damaged homes in impoverished Mississippi, served at local soup kitchen, and worked at women's shelter in Memphis, TN

Schreyer Honors College Student Council University Park, PA
Member 2016-2018

- Participated in PSU community service projects and fundraising efforts

Eberly College of Science Student Council University Park, PA
Member 2016-2018

- Contributed in organization of events and fundraisers

Project Cahir University Park, PA
Member of PSU Scholarship Initiative Spring2019- May 2020

- Organizing efforts, events, and an awareness campaign with a goal of educating others about student hunger, homelessness, and poverty and helping our own students in need

Discovery Space State College, PA
Volunteer Fall 2018/Spring 2019

- Hands-on science center with interactive exhibits and programs for children ages 14 and under