In the bacteria *Pseudomonas aeruginosa*, the *mexAB-oprM* operon encodes for an efflux pump that is responsible for resistance to multiple antimicrobials (1). The efflux pump consists of the inner membrane protein MexB, the periplasmic adaptor protein MexA, and the outer membrane protein OprM (2). In addition to being found in *P. aeruginosa*, homologs of proton-dependent efflux pumps exist in distantly related bacteria such as *Escherichia coli*, as well as in different strains of *Pseudomonas* (3,4). Multiple MexAB-OprM homologs are found in a single genome and are expressed in a context-dependent manner. The exception in *P. aeruginosa* is MexAB-OprM itself, which is constitutively expressed during its growth phase and represents one of the first lines of defense against antimicrobials. Mutations in the upstream proximal regulator MexR and other regulators can lead to high levels of MexAB-OprM expression, resulting in a concomitant increase in resistance to antimicrobials. Additionally, the proximal regulator can also be activated or repressed by certain antimicrobials and other compounds, further modifying expression. Bioinformatic analysis of closely related *Pseudomonas* species have shown that the MexAB-OprM homolog with the highest identity and similarity to MexAB-OprM can have proximal regulators that are of a different family but still be constitutively expressed. Recently, we identified a MexAB-OprM homolog in *P. stutzeri* with the highest identity to MexAB-OprM that did not contain a proximal regulator. My project will be to characterize whether this efflux pump is constitutively expressed using reverse-transcription PCR (RT-PCR) and using gene deletion to test whether it effluxes known substrates of *P. aeruginosa* MexAB-OprM. This work will help to better understand the diversity of constitutive efflux pump operon structures and substrates within closely-related species and potentially lead to future studies identifying their regulator(s).

The first step in my project will be to isolate RNA from a strain of *P. stutzeri*, 57B2 during log-phase. I will then conduct complementary DNA (cDNA) synthesis followed by PCR to determine if the homolog MexAB-OprM is expressed at this time similar to *P. aeruginosa* (6). A *mexA* primer set will be used to confirm expression for MexAB-OprM in the cDNA samples. To control for log-growth expression, an *rpoD* primer set will also be used on the cDNA samples. To test for the presence of genomic DNA (gDNA) after RNA extraction, the two primer sets will also be used in an RNA only sample. To verify the PCR master mix was made correctly, a gDNA control for the two primer sets will also be used. I will perform this experiment three times to account for biological variation. If there is a band corresponding to the *mexA* gene length in the cDNA sample, then I will conclude that the homolog is constitutively expressed. However, if there is no band of this length, then the *mexA* gene is not expressed at this phase and I will test other candidate homologs.

Next, I will use SacB-mediated sucrose counter-selection to delete the gene segment between *mexA* and *mexB*. In order to do this, I will use Gibson assembly to insert a 100 bp section of DNA containing the first 50 bp of *mexA* and *mexB* genes into the pEX18 suicide plasmid (7). The ends of both the plasmid and the insert will be “chewed back” by T5 exonuclease which creates single-stranded DNA 3’ overhangs by removing 5’ nucleotides (7). This allows the ends of the DNA fragment to anneal to the complementary DNA segments on the ends of the plasmid, which causes the plasmid to re-circularize. The resulting pEX18-*mexAB* plasmid will be circular and contain a gentamicin resistance factor, and the *sacB* gene that, when expressed, kills the cell if exposed to sucrose (8). I will transform the plasmid into *E. coli* SM10.

Following Gibson assembly, I will perform sacB-mediated sucrose counter-selection. SM10-pEX18-*mexAB* plasmid will be conjugated with *P. stutzeri* using the protocol that I developed during the academic year. The plasmid is then integrated into the *P. stutzeri* chromosome via the first recombination event and the transformed colonies will be grown in the presence of gentamicin to select for cells that underwent recombination and have integrated the

plasmid successfully. Next, a second recombination event will occur. If this event occurs so that segments upstream of the deletion, the suicide plasmid (8) containing the *sacB* gene, the gentamicin resistance factor, as well as the deletion, will be looped out of the chromosome. If the second recombination event occurs downstream of the deletion, then the deletion remains in the chromosome while the *sacB* gene, gentamicin resistance gene, *mexA, mexB,* as well as the space between these two genes, will be looped out. These colonies will then be grown in the presence of sucrose to select for colonies that have looped out the suicide plasmid, as the colonies that did not undergo the second recombination event, and therefore still express the *sacB* gene, will be killed when grown with sucrose. This process is diagramed below in appendix 2. Finally, I will perform PCR on the colonies selected for via *sacB*-mediated sucrose counter-selection. If the resulting band is shorter than the wild type band, then I can conclude that the deletion was successful, and these colonies are expressing the deletion genotype.

The last step of this project is to test the minimal inhibitory concentration of the mutants expressing the desired deletion genotype using the antimicrobial triclosan (TCS) and the antibiotic tetracycline. This will be performed by growing 57B2Δ*mexA* colonies and wild-type colonies in increasingly dilute concentrations of TCS or tetracycline. If the mutant colonies have a lower MIC when compared to the wild type, then the deletion shows that the MexAB-OprM homolog contributes to resistance. This result would indicate further need to characterize atypical homologous operons and how they may be regulated to respond to antibiotics in the absence of a proximal regulator. However, if the mutant and wildtypes exhibit the same MIC for both TCS and tetracycline, it is likely that the efflux pump in *P. stutzeri* does not efflux TCS or tetracycline. This may be a result of the atypical operon structure and differences in sequence identity with *P. aeruginosa* may be explored to identify potential reasons for lack of substrate specificity to these two chemicals.

To prepare for this lab work, I have completed the biology 220 laboratory, general chemistry 121 laboratory, organic chemistry 230 laboratory sequences as well as courses in genetics, cell biology, physiology, animal physiology, biochemistry, organic chemistry, general chemistry and international public health. In addition to class work, I also have hands on laboratory experience being enrolled in civil and environmental engineering 399 with Dr. Hartmann for 3 quarters and conducting independent research in the lab. During my time researching at this lab, I have helped trouble shoot and develop a protocol for transposon mutagenesis in order to characterize the relationship between the *mexAB-oprM* operon and triclosan resistance in 56A10, an isolate of *P. fulva*. Finally, I am currently enrolled in advanced cell biology and microbiology and next quarter will be enrolled in immunobiology and molecular microbiology. Each of these courses has helped me learn the skills necessary to complete this research project. In the future, I would like to continue to study microbiology to better understand the mechanisms behind antibiotic resistance and the dangers of infectious pathogens. As both of these topics are an increasing threat to public health and are especially threatening to vulnerable populations such as hospitalized persons and those who are immunocompromised, I intend to pursue a degree in microbiology to further understand the pathogens posing these threats. I hope to use this information to discover new ways to combat antibiotic resistance and other virulent pathogens threatening our public and global health.

Appendix 1: References

1. Sánchez, Patricia, et al. "Transcriptional Regulation of MexR , the Repressor of Pseudomonas Aeruginosa MexAB-OprM Multidrug Efflux Pump." *OUP Academic*, Oxford University Press, 1 Jan. 2002, academic.oup.com/femsle/article/207/1/63/572231.
2. Chen, Hao & Yi, Chengqi & Zhang, Jin & Zhang, Wenru & Ge, Zhiyun & Yang, Cai-Guang & He, Chuan. (2010). Structural insight into the oxidation-sensing mechanism of the antibiotic resistance of regulator MexR. EMBO reports. 11. 10.1038/embor.2010.129.
3. Nikaido, Hiroshi. "Multidrug Efflux Pumps of Gram-Negative Bacteria." *Journal of Bacteriology*, Department of Molecular and Cell Biology, University of California, Berkeley, California , Oct. 1996, jb.asm.org/content/jb/178/20/5853.full.pdf.
4. Florence Jude, Corinne Arpin, Céline Brachet-Castang, Michèle Capdepuy, Pierre Caumette, Claudine Quentin; TbtABM, a multidrug efflux pump associated with tributyltin resistance in *Pseudomonas stutzeri*, *FEMS Microbiology Letters*, Volume 232, Issue 1, 1 March 2004, Pages 7–14,
5. Tian, Zhe-Xian et al. "CpxR Activates MexAB-OprM Efflux Pump Expression and Enhances Antibiotic Resistance in Both Laboratory and Clinical nalB-Type Isolates of Pseudomonas aeruginosa" *PLoS pathogens* vol. 12,10 e1005932. 13 Oct. 2016, doi:10.1371/journal.ppat.1005932
6. Biolabs, New England. "Reverse Transcription (CDNA Synthesis)." *New England Biolabs: Reagents for the Life Sciences Industry*, www.neb.com/applications/cloning-and-synthetic-biology/dna-preparation/reverse-transcription-cdna-synthesis.
7. "Gibson Assembly Cloning." *Addgene*, www.addgene.org/protocols/gibson-assembly/.
8. Huang, Weiliang and Angela Wilks. "A rapid seamless method for gene knockout in Pseudomonas aeruginosa" *BMC microbiology* vol. 17,1 199. 19 Sep. 2017, doi:10.1186/s12866-017-1112-5

Appendix 2: *sacB* mediated sucrose counter selection: