Assessing promoter sequences important for regulation of the *rpsU2* gene in a pathogenic bacterium

Aisling Macaraeg and Dan Floyd

Project Start Date: December 1st, 2021

Project End Date: May 2nd, 2022

Project Narrative

Statement of Preparation:

Aisling:

I am a third-year student currently studying Cell and Molecular Biology on the Microbiology track who has a strong interest in pathogenic bacteria. I am prepared to complete the proposed research due to the courses that I have taken, as they have provided valuable background knowledge and skills. Courses I have taken successfully include organic chemistry (CHM 227 and CHM 228), integrative microbiology (CMB 211), honors introductory biochemistry (CMB 311H), and pathogenic bacteriology (CMB 432). I am currently taking principles of cell biology (BIO 314), immunology and serology (CMB 333), introductory diagnostic microbiology (CMB 483), and the organic chemistry lab (CHM 226). Having taken these courses, I feel confident that I have gained the foundational knowledge about the functions, mechanisms of actions, and interactions of bacteria as well as eukaryotes. Along with traditional academics, I have gained research experience outside of the classroom laboratory setting that prepared me to successfully perform this project.

I was a biomedical research intern during the entirety of the semester of Spring 2021 at Berkeley Pharma Tech. This company is a relatively new startup based in California. There I completed a grant proposal for research into a new probiotic that may boost the immune system in the elderly with a group of peers. Additionally, I worked in Dr. Jenkins's lab this past summer at URI. There, I helped with the Sea Grant research, which included filtering water from the GSO Time Series, performing chlorophyll measurements, and assisting with PCR/other sequencing procedures. My current work in Dr. Kathryn Ramsey's lab positions me well to complete the proposed studies. With all these different experiences, I can complete the proposed project that interests me and furthers me in an area that I hope to continue my education.

Dan:

I am a fourth-year student studying Cell and Molecular Biology on the Biochemistry track with a strong interest in genetics. In the past, I have successfully completed relevant coursework including: organic chemistry lectures (CHM 227 and 228) and laboratory (CHM 226), introductory biochemistry lecture (CMB 311) and laboratory (CMB 312), integrative microbiology (CMB 211), general genetics lecture (352), immunology and serology (CMB 333), proteins and enzymes (CMB 482), and physical biochemistry (CMB 421). Additionally, I am currently enrolled in advanced microbiology lecture and laboratory (CMB 413 and 415, respectively), genetics laboratory (CMB 353) and a CMB seminar (CMB 495). The knowledge and experience I have gained through the completion of these courses provides me confidence in the genetic and biological sciences necessary to complete our proposed project.

Along with this coursework, I am also working in Dr. Kathryn Ramsey's research lab which provides me even more experience in relevant lab techniques and methods outside the classroom. Furthermore, I have become proficient at analyzing and interpreting scientific literature which will make gathering additional data for this project more efficient.

Project Proposal:

Background:

Francisella tularensis is a Gram-negative pathogenic bacterium responsible for causing the disease tularemia, which can be potentially fatal in both humans and other animals (Peterson, 2009). This microorganism has been the subject of numerous studies and countless hours of research due to its ability to survive in a wide variety of environments and its high virulence (Sjostedt, 2007). Today, there is a live vaccine strain of *F. tularensis* that has been attenuated, significantly reducing its virulence and can act as a model organism for genetic research, including the work proposed in our project. This study focuses on the regulation of a gene important for virulence of *F. tularensis*, *rpsU2*, which encodes the small ribosomal protein, bS21-2. Although this study will be conducted solely on *F. tularensis* LVS, the data collected and conclusions reached may be useful for the studies of many other microorganisms, especially those similar to *F. tularensis.*

To survive in an environment, all cells must carry out various biochemical processes to produce the constituents necessary for cellular functions, with most of these processes being achieved through the use of proteins. A frequently regulated, first step in creating proteins is the transcription of a gene into mRNA. Transcription is the "reading and copying" of the information coded within DNA, performed by the enzyme RNA polymerase. The presence of sequences known as promoters guide the RNA polymerase into the correct position on DNA to start transcription. Specifically, RNA polymerase recognizes and binds the DNA sequences referred to as -10 and -35 elements (Kanhere, A. & Bansal, M., 2005). Along with RNA polymerase, other proteins can regulate transcription by binding to this area of the DNA and either increasing or decreasing the rate of transcription.

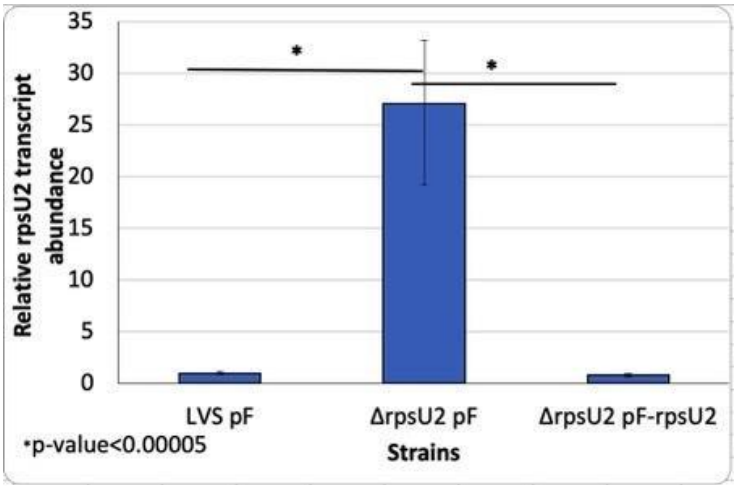

Figure 1: The figure above illustrates the relationship between *rpsU2* in the genome and the relative abundance of *rpsU2* transcripts when the gene is present or not. When *rpsU2* is present in a strain, the amount of *rpsU2* transcripts, and therefore the amount of bS21-2, is relatively low. When *rpsU2* is not present in the cell, the amount of *rpsU2* transcripts is much higher, leading to increased bS21-2 translation.

Ribosomes are responsible for the production of protein that is encoded by the mRNA transcript. Ribosomes are complex structures present in all organisms that contain some of the same molecules such as ribosomal proteins (r-proteins) and ribosomal RNAs (rRNAs), but can differ in the content of these molecules (Xue, S., & Barna, M. 2012). In *F. tularensis*, there are three homologs of the ribosomal protein bS21 (bS21-1, bS21-2, and bS21-3). The Ramsey lab has found that the bS21-2 protein, encoded by *rpsU2*, is important for virulence of *F. tularensis* and self-regulates *rpsU2* through unknown mechanisms. Thus, understanding how production of bS21-2 is regulated can lead to further understanding about virulence of *F. tularen*sis.

The Ramsey lab has also found that abundance of the *rpsU2* mRNA transcript is affected by presence of the protein it encodes, bS21-2. The figure to the left shows the relative amount of *rpsU2* transcript in three different strains: wild-type cells containing *rpsU2* (LVS pF), cells without bS21-2 (Δ*rpsU2* pF), and cells lacking bS21-2 with bS21-2 restored (on a plasmid; Δ*rpsU2* pF-*rpsU2*). In cells without bS21-2, there is a significant increase in *rpsU2* transcript. This suggests that bS21-2 may negatively regulate its own transcription.

Aims:

We hypothesize that the presence of bS21-2 inhibits transcription of *rpsU2*. Promoter sequences are known to be important to regulate transcription but which part of the *rpsU* promoter is responsible for regulation by bS21-2 is unknown and is the subject of our study. We can determine this through mutating the DNA sequences before, between, and after the -10 and -35 elements of the promoter so bS21-2 may not recognize and affect regulation of the gene. We will fuse this promoter to the *lacZ* gene to measure gene expression via a beta galactosidase assay; if the promoter works, this assay will show the activity of the gene sequence, allowing us to see whether transcription is occurring or not. This proposed method will be done on two *F. tularensis* strains: a wild-type control and our experimental mutated strain, without the *rpsU2* gene which codes for the bS21-2 ribosomal protein.

Results:

Since the beginning of the semester, we have generated plasmids to help assess the regulation of *rpsU2* expression. To do so, we have to amplify segments of DNA (using polymerase chain reactions, PCR). Specifically, we amplified the *rpsU2* promoter, the *tul4* promoter (a promoter unaffected by bS21-2), the 5´untranslated region (UTR) of *rpsU2*, and the 5´ UTR of *tul4*. We then completed a digestion, which uses enzymes to create "sticky" ends on each insert and a plasmid backbone that includes the *lacZ* reporter gene. We then performed a ligation to attach the insert and the backbone together. Our goal is to have cloned three different plasmids to help validate our hypothesis about which part of the DNA is responsible for regulating expression of the *rpsU2* gene by the end of the semester. Currently, we have successfully created two plasmids (pKR121 and pKR123) and are using a new PCR method to create the desired DNA segment for what would have been pKR122. These plasmids and the DNA segment will be inserted into *F. tularensis* LVS to create new strains of the bacteria. After these strains are created a β-galactosidase assay can be completed to determine if the promoter of *rpsU2* contains sequence elements important for the self-regulation. Once this is determined, we will be able to mutate the specific parts of the promoter DNA to determine which sequences are important for regulation by bS21-2, which is the goal of this project.

Procedures:

Dan and Aisling have the following plan for this project. As they have a different schedule and the steps need to be followed in a specific order, they will complete each step either individually or together depending on who is available in the lab. For example, if Aisling completes the first step on Monday, then on Tuesday Dan will complete the second step. They will communicate with each other about what they accomplish in the lab each day, so they are aware of what step they are currently working on. Specifically, this will be our workflow:

1. PCR to add and amplify mutations in the *rpsU2* promoter
2. Digest the amplified region from the PCR and a separate backbone component, which contains lacZ gene, to give each a "sticky" end so they can come together in the following step
3. Ligate the sticky ends of the mutation insert and the backbone to create the desired plasmid
4. Transform the ligations into *E. coli* to amplify the ligated plasmid
5. Purify plasmid DNA from *E. coli* using a "mini prep" protocol so the DNA is pure and concentrated to be sequenced
6. Sequence through INBRE Core to ensure that the correct insert is present in the backbone and there are no mutations
7. Once confirmed, electroporate into *F. tularensis* LVS wild type and Δ*rpsU2* cells so that the DNA is integrated onto the chromosome using the Tn7 system (Lovullo, 2009)
8. Complete a β-galactosidase assay to compare results found in semester Fall 2021. Analyze.

Original ideas:

We have learned a lot in the past few weeks throughout working in the lab and in our classes. In the lab we have learned many experiential skills that will be used throughout this project. For example, we will be completing PCRs, ligations, and culturing, as well as lab safety and following protocols. What we have learned in our classes has coincided well with what we are learning in the lab, which gives us the confidence and additional knowledge to create and accomplish our goal of mutating the promoter of the *rpsU2* gene. We have proposed to Dr. Ramsey mutating the regions in the promoter around the -10 and -35 elements. We may also shorten the promoter region to see if regions upstream from the -10 and -35 elements contribute to regulation. Another idea would be to delete a nucleotide in the sequence, or to validate that we have identified the correct promoter region by changing the -10 region and the -35 region.

Value of Project:

We are both planning on attending graduate school in the near future, and this project will provide key scientific training. This project will help hone our hands-on-skills by giving us opportunities to practice PCR, digestion, ligation, transformation, and beta-galactosidase assays. These are transferable skills that are frequently used in all molecular biology laboratories and that we will need in graduate school and potentially in future industry-based positions. Additionally, we will gain analytical and critical thinking skills throughout this project through troubleshooting and analyzing our results.

This project will also impact current knowledge on gene regulation mechanisms within *F. tularensis* and potentially other organisms that encode bS21-2. While the Ramsey lab has found that bS21-2 is important for virulence in *F. tularensis*, it is currently unknown how the bS21-2 protein impacts transcription and translation within the cell. Our project will provide evidence for how bS21-2 impacts transcription of its own gene, *rpsU2*. This project will contribute to our knowledge about a protein that is key for bacterial virulence in *F. tularensis* and is found in many other bacteria.

Reference List:

Lovullo, E. D., Molins-Schneekloth, C., Schweizer, H. P., & Pavelka, M. S. (2009). Single-copy chromosomal integration systems for Francisella tularensis. Microbiology, 155(4), 1152. doi:10.1099/mic.0.022491-0

Petersen JM, Mead PS, Schriefer ME. Francisella tularensis: an arthropod-borne pathogen. Vet Res.2009;40(2):07. PMCID: PMC2695023

Sjostedt, A. (2007). Tularemia: History, Epidemiology, Pathogen Physiology, and Clinical Manifestations. Annals of the New York Academy of Sciences, 1105(1), 1–29.

Xue, S., & Barna, M. (2012). Specialized ribosomes: A new frontier in gene regulation and organismal biology. Nature Reviews Molecular Cell Biology, 13(6), 355–369.

Duin, J., & Wijnands, R. (1981). The Function of Ribosomal Protein S21 in Protein Synthesis. European Journal of Biochemistry, 118(3), 615–619.

Kanhere, A., & Bansal, M. (2005, June 6). *Structural properties of promoters: Similarities and differences between prokaryotes and eukaryotes*. Nucleic acids research. Retrieved October 14, 2021, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1143579/.