

Discovery of Genes Involved in *F. tularensis* Survival in Freshwater

Aisling Macaraeg

Project Start Date: December 1st, 2022

Project End Date: May 2nd, 2023

Project Description

Background:

Francisella tularensis is a Gram-negative pathogenic bacterium that is the causative agent of tularemia. It is highly infectious to humans and animals and is considered a potential biological weapon by the US Centers for Disease Control and Prevention (Hennebique et al., 2019). *F. tularensis* can live outside of host cells in aquatic reservoirs, which can lead to infection in humans and rabbits via ingestion. Currently, there is research trying to understand both the environmental conditions and genetically-encoded factors required for survival of *F. tularensis* in water (Golovliov, 2021). Specifically, the connection between the aquatic cycle and terrestrial cycle of hares and mosquitoes, which are two common vectors of tularemia, is clear but poorly understood and would benefit from further study. This research is important due to recent outbreaks in Norway, Kosovo, Bulgaria, Spain, and Turkey can be traced to aquatic reservoirs (Maurin & Gyuranecz, 2016). I have started to address these questions regarding survival in freshwater by determining optimal temperature conditions in the laboratory. I have found that 4°C is the optimal temperature for long term survival with an average of 37.3 days in sterile freshwater. This preliminary data lays the groundwork for the project that I am proposing.

Goal:

My goal is to identify the specific gene(s) that are involved in the survival of *F. tularensis* Live Vaccine Strain (LVS) at 4°C in freshwater using a technique called transposon insertion sequencing (Tn-Seq). Using this technique, we can compare survival of thousands of different mutant cells between two conditions (here, in freshwater versus on a petri dish) using genetic manipulation followed by modern DNA sequencing techniques.

Project Objectives:

At the conclusion of this project, I would expect to have the following completed: (1) have performed the Tn-Seq protocol (Goodman et al., 2009); (2) have analyzed data that compares which mutants can grow on agar plates compared to those that can survive in freshwater; (3) created a list of genes that impact survival in freshwater, which will lead to further analysis; (4) validated the importance of a candidate gene necessary for survival in freshwater.

Project Plan:

The primary method to answer the question is transposon insertion sequencing (Tn-Seq). This is a common method used to identify genes necessary for survival in a particular condition. First, a mutant library is made using a vector that contains a transposon, a piece of DNA that encodes resistance to a selectable marker and is large enough to disrupt a gene, and a transposase, which is an enzyme that specifically cuts out the transposon and inserts it into the chromosome. This transfer of DNA from the vector to the chromosome only occurs a single time for each cell. After the transposon is inserted into the chromosome, the bacteria are now considered a mutant, as the original DNA has been modified, and if the transposon inserted into a gene, that gene is now inactivated. We hope to generate a large number of mutants (6,000 in this case), in the hopes of assessing every gene in the genome (there are approximately 2,000 genes). Then we will pool together all the bacteria to generate a library of these mutants and expose these mutant libraries to two conditions. One condition is “normal” (i.e. grown on agar plates) and is used as a control, and the other condition is a stressor of interest (i.e. grown in freshwater at 4°C). Following incubation in the two conditions, DNA is purified from the bacteria. Using molecular biology techniques, we can amplify and add adapter sequences to the location where the transposon inserted. This will allow us to identify all the transposon insertion sites using next-generation technology. We can then determine which mutants could live on the agar plate (normal) and which mutants were unable to survive in freshwater (the stressed condition). This allows us to identify which genes are considered essential for the condition of interest.

Starting in December, I will complete a pilot Tn-Seq experiment in which I complete all the steps outlined above, except only with the initial mutant library under no stress condition other than ability to survive on a nutrient rich agar plate. This will give an opportunity to optimize the method and evaluate any issues that may appear. After optimization is complete, I will proceed with the full-scale experiment in January. This experiment will consist of putting the mutant library in sterile freshwater at 4°C for 14 days and collecting samples of the bacteria to analyze at day 0, 7, and 14. The timepoints were chosen based on my previous findings. These data will be compared to each other, and day 0 will act as a control. I will purify DNA from the surviving cells at each of these time points. In collaboration with Dr. Ramsey, we will add adapters and

barcodes to create a library from the purified DNA, to be sequenced by the RI-INBRE Molecular Informatics Core. I plan to have sequencing data returned by February, which will then need to be analyzed. As I have not completed any bioinformatics work previously, this will be a great learning opportunity, but will also take a longer time for me to complete with no experience. Analysis of the data will include filtering out background noise, and mapping the sequence reads to the genome. The final result will be a list of genes that are essential for survival in freshwater. Dependent on how the entire process goes, if there is remaining time in March and April, I would like to select a gene candidate from the data analysis to create a knockout mutant, in which I will edit the DNA of *F. tularensis* to remove the candidate gene completely, and test for survival in freshwater. The purpose of creating a knockout mutant is to validate the findings. The expectation would be the cells do not survive in freshwater.

Your Contribution to the Project Concept:

This project concept was initially constructed during a conversation between Dr. Ramsey and I, when I was looking for an independent honors project. I was interested in survival of *F. tularensis* in freshwater and wanted to complete a viability assay, which is growing *F. tularensis* in freshwater and assessing its survival. I became interested in this topic from completing a project on *F. tularensis* from an epidemiological standpoint and read about how it is endemic in different aquatic reservoirs in Scandinavian countries. Dr. Ramsey suggested that I challenge myself a bit more and try to determine the optimal laboratory environment for freshwater survival as well as the genes involved. As an expert in Tn-Seq, Dr. Ramsey was the main contributor of experimental design. I will complete the experimental work of this project and will help troubleshoot as problems (inevitably) arise.

Plans for Results:

I will benefit the most from my work, and the scientific community will benefit as well, as we expect to publish our findings which may eventually contribute to disease mitigation. For the past year and a half, I have had the opportunity to continue to work on this project, and I have gained and will continue to gain many professional and scientific skills, such as DNA isolation, creation of a mutant library, and Tn-Seq. Additionally, my professor and I hope to publish a paper on this work. This project will improve current knowledge of how *F. tularensis* behaves in the aquatic reservoir as this is a current gap in existing research. The gene(s) found will be able

to contribute as novel data to how *Francisella tularensis* is able to survive for long periods of time in freshwater.

Value of the Project to You:

In the future, I am planning on applying to graduate school for molecular biology or microbiology. I have a strong interest in pathogenic microbiology and having the opportunity to continue to work with *F. tularensis* will create many learning experiences. I will be able to continue my first independent research project and improve my current skill set of critical thinking, analyzing, and solving problems. Additionally, I will learn more fundamental microbiology techniques, such as plating to identify viable bacteria as well as Tn-Seq protocols and analysis. Working on this project will give me valuable skills and opportunities to grow as a student researcher that will be directly applicable to my future endeavors in graduate school.

References

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November 14, 2022

Undergraduate Research Initiative Committee
University of Rhode Island
Kingston, RI 02881

Dear Committee Members,

I am extremely pleased to offer my support to Aisling Macaraeg for her undergraduate research project, "Discovery of Genes Involved in *F. tularensis* Survival in Freshwater." My laboratory studies the molecular mechanisms that lead to pathogenicity of the human pathogen *Francisella tularensis*. While this organism is highly pathogenic to humans, my laboratory takes advantage of the model organism *F. tularensis* subsp. *holarctica* LVS (Live Vaccine Strain), which does not infect or cause disease in humans and we do not work with any of the highly pathogenic strains.

Aisling is an academically outstanding upper-level student, with GPA of 3.91, and has been an active researcher in my laboratory since the fall of 2021. On her first research project, she worked collaboratively with another undergraduate researcher, a project ultimately funded by a (URI)² undergraduate research grant. That work focused on understanding the regulation of a ribosomal protein gene. The results that Aisling and her collaborator obtained revealed unexpected complexity that initiated a new line of investigation in my laboratory currently pursued by a Master's student.

In the spring of 2022, Aisling worked both on the ribosomal gene regulation project as well as her independent honors project. The goal of her independent honors project was to identify what laboratory conditions might mimic the long-term persistence of *F. tularensis* in freshwater in the environment. We know very little about the survival of *F. tularensis* in the environment, so this project provided critical initial data to create a lab-based model for *F. tularensis* environmental persistence. Aisling successfully completed multiple months-long experiments to obtain these data during the spring semester and over the summer, during her RI-INBRE Summer Undergraduate Research Fellowship (SURF).

Aisling is interested in further exploring which specific genes allow *F. tularensis* to persist over long periods of time (21 – 56 days) in freshwater. In our discussions about this project, Aisling has demonstrated that she is aware of prior research identifying a specific gene necessary for *F. tularensis* to survive the transition from high to low osmolarity, as occurs when *F. tularensis* enters the freshwater environment. She hypothesized that additional genes may be necessary, a hypothesis we can test using a state-of-the-art approach referred to as "Tn-Seq." Aisling's work over the summer has already prepared her to use this approach, as she modified a genetic tool to make downstream steps more cost-efficient. I am an expert in the use of the Tn-Seq method, so am well-qualified to train Aisling. I enthusiastically support the continued development and undertaking of her independent research project.

Together with my graduate students, I will supervise all the proposed work in this project to ensure safety, rigor, and reproducibility. The success of this project will be based on Aisling's ability to (i) learn and implement research protocols, (ii) critically analyze the resulting data, (iii)

make connections between the resulting data and the current scientific literature, and (iv) effectively communicate research findings, both within and outside our research group.

Aisling is an outstanding student with great enthusiasm for laboratory research. Her undergraduate research experience in my laboratory, including the exciting work proposed here, will prepare her to achieve her future goal of entering graduate school in biomedical research. She is extremely well-prepared to carry out the proposed experiments and I support her application for an undergraduate grant for original student research without reservation and with enthusiasm.

Sincerely,

A handwritten signature in dark ink, appearing to be 'KR' followed by a horizontal flourish.

Kathryn M. Ramsey, PhD
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