Title of Proposed Project: Regulation of OmpA and Its Effect on Shigella Virulence

Describe the proposed research or creative project:
(Please note: Avoid jargon or terms unique to the discipline. If you must use such terms, please define them so all committee members can understand your proposal.)

Introduction
Shigellosis is an infectious diarrheal disease caused by bacteria of the genus Shigella (S. dysenteriae, S. flexneri, S. boydii, and S. sonnei). This potentially fatal infection is characterized by fever, abdominal cramps, and bloody diarrhea. According to conservative estimates, there are 165 million annual cases of shigellosis worldwide, resulting in 1.1 million fatalities each year [1].

While shigellosis occurs mostly in the developing world, cases in the US are not uncommon. The CDC estimates 300,000 cases of shigellosis in the US annually [2]. Additionally, Franklin County, Ohio, has been experiencing a recent outbreak of shigellosis with a record 771 cases in 2012 [3].

Because of growing antibiotic resistance and no vaccine, the development of new methods to treat shigellosis is vital [4-6]. Outer membrane protein A, or OmpA, is required for Shigella virulence and may be a good target for inhibitory drugs. Before OmpA can be a viable target for new anti-shigellosis agents, we need to understand the regulation of OmpA production and its role in virulence.

Shigella Invasion and Spread
Shigella can cause disease in a healthy person with only 10 to 100 organisms.

![Shigella Life cycle](image)

**Fig. 1: Shigella Life cycle.** While traveling through the lumen of the large intestine, some bacteria enter microfold cells (M cells), where Shigella are recognized as foreign invaders and are delivered to macrophages (pink asymmetrical shapes). These cells engulf Shigella, allowing their movement into the sub mucosa, where the bacteria lyse the macrophage before Shigella degradation can occur. Freed bacteria invade the epithelium, replicate, and spread.
Comparatively, the infectious dose for *Vibrio cholerae* ranges from $10^3$ to $10^8$ organisms [7].

*Shigella* bacteria are transmitted via an oral-fecal route, which is a common mode of transmission for other bacteria, such as *Escherichia coli*. In oral-fecal transmission, bacteria shed in the feces of an infected individual gain entry into the digestive tract of a new host, usually through the consumption of contaminated food or drink. For example, an adult may neglect to wash his or her hands after handling an infected baby’s diaper. If this adult were to prepare a meal with contaminated hands, it is possible for the bacteria to be transmitted to the food and into the adult’s digestive system. Given the small infectious dose required to initiate infection and the ease of transmission, shigellosis can quickly spread in places such as daycare centers, military units, and refugee camps. Once ingested, *Shigella* travel to the lumen of the large intestine where they invade the epithelium, replicate within the human cell, and spread from one cell to neighboring cells (Fig. 1) [1].

**Protein Production**

While there are a variety of factors that facilitate *Shigella* infection, proteins are especially common components. Protein production involves two processes: transcription and translation. During transcription, information from DNA, the genetic blueprint, is transferred to messenger RNA, or mRNA. Then, a ribosome translates the mRNA into a sequence of amino acids during the process of translation. Finally, the amino acid chain is freed from the ribosome and folds into a protein. The steps of transcription and translation occur for protein-coding gene expression in all cells, and each step can be subject to specific regulation. This process is depicted in Fig. 2.

**Regulatory Elements Affect Translation**

A system of RNA sequences called regulatory elements controls the progression of *Shigella* infection [8]. These regulatory elements can increase or decrease the expression of certain genes, such as outer membrane protein A, in order for the initiation and progression of an infection within a host. Because these elements regulate *Shigella* virulence, their identification and characterization may lead to potential targets in treating
shigellosis. Understanding these mechanisms of control may help with the development of new antimicrobial therapies designed to disrupt these precise mechanisms of gene regulation.

In bacteria, translation usually occurs simultaneously with transcription. Consequently, the amount of a particular bacterial protein would be expected to be proportional to the amount of transcribed mRNA. Regulatory elements can affect this balance by altering the translation efficiency.

Regulatory elements function by different mechanisms to control target gene expression. For instance, a protein or molecule may bind to a regulatory element and physically prevent the translation of nearby genes by blocking ribosomal binding. Additionally, a regulatory element may change its shape in response to environmental conditions, such as temperature. This structural adaptation may allow or prevent the expression of genes by permitting or blocking ribosomal binding to a given mRNA molecule.

This project will focus on a specific type of regulatory element called an RNA thermometer, which functions to regulate translation in response to temperature.

**Temperature-Dependent Regulatory Elements**

Like many other bacteria that are transferred via the oral-fecal route, *Shigella* must adapt to different environmental conditions in the outside and host environments. Important environmental factors, such as acidity, iron levels, and temperature, can help regulate gene expression and virulence [8]. For instance, the temperature inside of a human host measures to be approximately 37°C while room temperature is usually about 25°C. As *Shigella* travel into the higher temperature of the human host, the temperature change acts as an environmental clue that the bacteria have entered the human body. In response to this increased temperature, *Shigella* increase the expression of genes which facilitate infection. One mechanism by which *Shigella* regulate gene expression in response to temperature is by RNA thermometer.

**RNA Thermometer**

RNA thermometers are temperature-dependent, cis-acting regulatory elements. These regulatory elements are located in the 5' untranslated region, or 5' UTR, of the target mRNA. The 5' UTR is a section of RNA at the front of the mRNA that contains the ribosomal binding sequence, or RBS. The RBS is also known as the Shine-Delgarno sequence, or SD. In order for translation to occur, a ribosome must attach to the RBS (Fig. 2).

RNA thermometers are able to control the binding of a ribosome to the RBS by altering mRNA structure. When the environmental temperature is cooler (25°C), the thermometer's structure is such that the RBS is not in a single-strand, available conformation. Instead, the RBS is bound to nearby ribonucleic acids, creating a lollipop shape with a single-strand loop on top of a double-strand stem (Fig. 3A). If the environment warms (37°C), the thermometer melts, and the RBS is freed (Fig. 3B). Thus, a ribosome preferentially binds to the mRNA molecule at higher temperatures, and translation can occur more efficiently.
This project will focus on a specific type of RNA thermometer, a FourU RNA thermometer. A FourU RNA thermometer is characterized by four Uracil bases that bind to the RBS at low temperatures [9]. FourU RNA thermometers have been found in other bacteria, such as *Salmonella enterica* [10].

Andrew Kouse of Dr. Murphy’s lab at Ohio University has identified a FourU RNA thermometer in *S. dysenteriae* that increases translation efficiency at 37°C as compared to 25°C [11]. Additionally, Kouse identified 20 other potential *Shigella* FourU RNA thermometers with a computer program called Mfold. The *ompA* RNA thermometer was chosen for further study because OmpA is known to influence *Shigella* virulence [12, 13].

**OmpA Protein**

Outer membrane protein A, or OmpA, is crucial for *Shigella* virulence, specifically for cell-to-cell spread within the epithelium [12, 13]. Without OmpA, *Shigella* successfully invades human cells but cannot spread from one cell to a neighboring cell. Without cell-to-cell transfer, *Shigella* cannot cause disease. This data suggests that inhibition of OmpA production may be a way to treat shigellosis. Before the development of drugs to inhibit OmpA production is possible, it is essential that we understand the mechanisms controlling this process.

**Temperature-Dependent Regulation of *E. coli* OmpA**

The production of *E. coli* OmpA is highly regulated in response to temperature and other environmental conditions. Compared to *E. coli* grown at 25°C, *E. coli* grown at 37°C have less *ompA* mRNA but the same amount of OmpA protein. The same amount of protein with less mRNA (the molecular template for making the protein) indicates that translation (the process of building the protein from the mRNA template) is more efficient at 37°C [14]. While the mechanism of this temperature-dependent regulation of OmpA translation is unknown, the Murphy lab’s *in silico*
analysis predicts the presence of an RNA thermometer within the \textit{ompA} mRNA molecule (Fig. 4). An RNA thermometer alters translation efficiency in response to temperature by forming an inhibitory structure at low temperatures that prevents binding by the ribosome (the protein complex required to synthesize protein from the mRNA template). At higher temperatures, this inhibitory structure “melts out,” and translation proceeds uninhibited. 

\textit{Shigella} is highly related to \textit{E. coli}, and the Murphy lab’s analysis shows that the putative RNA thermometer is present in both species. Given the significance of OmpA to \textit{Shigella} virulence, I will focus my investigations on this pathogenic species.

**Describe the end goal, specific aim or hypothesis of the proposed research or creative project:**

**Goal:** This project aims to determine the mechanism controlling the temperature-dependent regulation of OmpA translation and the effect of this regulation on \textit{Shigella} virulence.

**Objective 1:** The first objective is to determine the impact of the putative FourU RNA thermometer in the temperature dependent regulation of OmpA within the context of the chromosomally encoded \textit{ompA} gene.

**Hypothesis 1:** An RNA thermometer located within \textit{ompA} mRNA molecule inhibits effective OmpA translation at 25°C.

**Approach 1:** In order to determine if the putative RNA thermometer is functional, the relative amounts of \textit{ompA} mRNA and OmpA protein will be compared between wild-type and mutant \textit{S. dysenteriae}. First, the relative amounts of \textit{ompA} mRNA will be measured with real time PCR, or real time Polymerase Chain Reaction, following the growth of wild-type \textit{S. dysenteriae} at 25°C and 37°C. Additionally, the relative amounts of OmpA protein will be measured by Western blot analysis. For details on the aforementioned assays, please refer to the “Methods” section.

Along with the wild-type \textit{S. dysenteriae}, two mutant strains will be analyzed. One mutant strain will have a stabilized FourU RNA thermometer; the other strain will have a destabilized FourU RNA thermometer. The relative levels of \textit{ompA} mRNA and OmpA protein will be measured for each strain after growth at 25°C and 37°C. Real time PCR and Western blot analysis will be used.

**Expected Outcomes 1:** It is expected that the wild-type will produce less mRNA at 37°C as compared to 25°C, but the same amount of protein will be made at each temperature.

The stabilized mutant is predicted to make less protein at 37°C as compared to the wild-type at 37°C. Additionally, it is thought that the stabilized mutant at 37°C will have the same amount of mRNA as wild-type at 37°C.

The destabilized mutant is expected to make more protein at 25°C as compared to wild-type at 25°C. Additionally, it is expected that the destabilized mutant at 37°C will have the same amount of mRNA as wild-type at 37°C.
Objective 2: The second objective is to characterize the contribution of the RNA thermometer to *Shigella* virulence.

Hypothesis 2: *Shigella* virulence is influenced by the activity of the *ompA* RNA thermometer.

Approach 2: The ability of wild-type *S. dysenteria* to invade, replicate, and spread will be measured using the virulence assay as described in the “Methods” section. These measurements will be compared to those of the stabilized and destabilized mutants.

Expected Outcomes 2: It is predicted that the wild-type will be able to invade, replicate, and spread. In other words, the wild-type will form plaques.

The stabilized mutant is expected to form no plaques or small plaques, looking like an *ompA* mutant.

The destabilized mutant at 37°C is predicted to form plaques like those of the wild-type at 37°C.

**Describe the method you will use to accomplish the end goal or specific aim of the proposed research or creative project:**

The aforementioned objectives will be accomplished through the following 4 steps: chromosome mutation, Western blot analysis, quantitative real time PCR analysis, and virulence assays. All experimental procedures will take place in the mentor’s lab, which is a certified Biological Safety 2 Lab. Training will be conducted by Dr. Erin Murphy and the Department of Environmental Health and Safety.

Chromosomal Mutations: Mutations will be introduced into the chromosomally located *ompA* RNA thermometer using site-directed mutagenesis as previously performed in Dr. Murphy’s lab and detailed in Broach *et al.*[23]. One chromosomal mutation will stabilize the inhibitory structure within the RNA thermometer, potentially preventing ribosomal binding at normally permissive temperatures. The second chromosomal mutation will destabilize the RNA thermometer structure, mimicking the “melting out” observed at

**Fig. 5: Chromosomal Mutations.** The RBS is labeled. The mutations are marked with black arrows.
high temperatures, and thus allow ribosomal binding at normally inhibitory temperature. These mutants will be used in subsequent experiments. Fig. 5 shows the exact mutations that will be made.

**Western Blot Analysis:** Wild-type *Shigella* and each constructed mutant will be cultured to early logarithmic phase at 25°C and 37°C, and OmpA protein levels will be measured using Western blot analysis as previously performed in Dr. Murphy's lab. Western blot analysis consists of separating proteins using gel electrophoresis and then transferring these proteins to a nylon membrane. Finally, antibodies are used to label and visualize a specific protein, which is OmpA in this project. If the temperature dependent regulation of *ompA* translation is mediated by the identified RNA thermometer, then it is expected that the stabilized mutant will have lower OmpA levels as compared to those in wild-type following growth of each strain at the permissive temperature of 37°C.

**Quantitative Real Time PCR:** Wild-type *Shigella* and each constructed mutant will be cultured to early logarithmic phase at 25°C and 37°C, and *ompA* mRNA levels will be measured using quantitative real time PCR (qPCR) analysis as previously implemented in Dr. Murphy's lab [23-25]. qPCR is a method of quantifying the relative amount of a specific mRNA molecule. If the temperature-dependent regulation of *ompA* translation is mediated by the identified RNA thermometer, then it is expected that mutations in the RNA thermometer will have no impact on *ompA* mRNA levels. RNA thermometers regulate after transcription and, therefore, do not influence the level of mRNA. Instead, RNA thermometers alter the efficiency of translation from the mRNA molecule.

**Virulence Assay:** *In vitro* plaque assay, as performed previously by the Murphy lab, will be used to investigate the effect of the *ompA* RNA thermometer on *Shigella* virulence. The wild-type, stabilized, and destabilized *Shigella* will be transferred to plates of HeLa cell monolayers. Plaque formation, indicating the bacteria's ability to spread and kill surrounding cells, will demonstrate levels of bacterial virulence. If virulence is affected by the RNA thermometer, it is expected that the mutants will impact (positively or negatively) the ability of *Shigella* to form plaques.

**Describe the significance of your research or creative project. Why is it important to the discipline?**

This project attempts to determine the regulation of OmpA and the role of this protein, which is thought to be critical to the virulence of not only *Shigella* but also various other human pathogens, such as *Yersinia pestis, Escherichia coli,* and *Vibrio cholerae* [15-19; 20-22]. Because of this protein's universality, knowledge about OmpA's regulation and its effect on *Shigella* virulence has the potential to aid in the study of other pathogenic bacteria. Additionally, regulation by RNA thermometers, such as that studied in this project, is suggested to be more widespread than previously thought. Consequently, a better understanding of this particular RNA thermometer could potentially provide useful information in the study of another organism's regulation.

*Shigellosis* is causing 1.1 million fatalities worldwide and is greatly affecting the wellbeing of developing countries [1]. This project aims to discover new information
about *Shigella* and the factors that control its virulence. Without a shigellosis vaccine, it is imperative that *Shigella*’s mechanisms and processes, including the regulation and role of OmpA, are researched. New treatments and vaccines can only be developed once this virulent bacterium is understood.

If this putative RNA thermometer is functional and influences virulence, it may be used to develop antibiotic treatments for shigellosis. A molecular structure may be developed that attaches to the double-strand stem of the RNA thermometer. This structure would stabilize the thermometer, and prevent ribosomal binding to the RBS (Fig. 3C). Since ribosomes would be unable to bind, *ompA* would not be translated into protein. Without OmpA protein, *Shigella* would not be able to spread in the human host.

Furthermore, the research of this project will be shared at two microbiological conferences (in Ohio and Colorado), the Student Research & Creative Activity Expo, and a thesis presentation. The audiences will range from experienced microbiologists to curious members of the Ohio University and Athens communities.

**(Answer ONLY if your project is part of, or fits within the research being conducted by a faculty member or your faculty advisor.) Describe specifically how our project fits within or is distinguishable from your faculty member’s research.**

The goal of all research in Dr. Murphy’s lab is to understand RNA mediated regulation of *Shigella* gene expression and the role of this regulation in the bacteria’s response to environmental conditions in the human host. The lab studies cis- and trans-acting regulatory RNA molecules; my project focuses on characterizing a specific cis-acting RNA thermometer. Mine is discrete project that fits into the overarching focus of the Murphy Lab.

Murphy’s lab was the first to identify and characterize a functional RNA thermometer in any Shigella species. The information, techniques, and experience from this initial analysis of a *shuA* RNA thermometer in *Shigella* will allow the successful and efficient completion of my project.

While I will be using the same techniques that have been applied numerous times in Murphy’s lab, I will be focused on the regulation of a different gene (*ompA*).
Bibliography


Requested Funding

Please note:
- If you request funds to purchase expensive equipment, such as computers, hardware, printers, cameras, etc., you must justify that the equipment does not exist elsewhere on campus for your use. If such equipment is funded, it will not become your personal property; it must remain in the academic department.
- If you request funds to pay for tuition at another institution, you must justify why your project cannot be completed unless you attend another school.
- If you request funds for a hotel room while at a conference or for research-related work, the committee will award funding for only two nights in a hotel.
- If you request funds for travel to present your research results or creative activity, indicate the name and date of the conference and provide specific travel plans.

Itemize each amount needed for each purpose. Be specific!

<table>
<thead>
<tr>
<th>Amount:</th>
<th>Item:</th>
<th>Justification for this expenditure:</th>
</tr>
</thead>
<tbody>
<tr>
<td>$800</td>
<td>General lab supplies</td>
<td>These are supplies required for basic bacteriology research. They include bacterial growth media, gloves, petri dishes, pipette tips, tubes, and DNA modification enzymes. Although these supplies can be found in the mentor’s lab, this project will deplete the available resources. Funds for general research supplies are requested to cover the replacement costs for these materials.</td>
</tr>
<tr>
<td>$200</td>
<td>Real time PCR reagents</td>
<td>These reagents include enzymes, cDNA synthesis kit, optical grade plates, and oligonucleotides. The techniques that require these supplies are critical to the project. Comparing the levels of protein and mRNA will allow the temperature-dependent regulation of OmpA translation to be observed.</td>
</tr>
<tr>
<td>$500</td>
<td>Travel</td>
<td>Funds are requested for travel to the Wind River Conference on Prokaryotic Biology. This is an annual international meeting that</td>
</tr>
</tbody>
</table>

offers a venue for students to gain new knowledge and to build connections with accomplished microbiologists. In 2014, the conference will be held in Colorado. The mentor and the lab have previously attended this conference. The results of this project will be presented at the Wind River Conference. Please see additional notes on the conference.

Travel to the Wind River Conference on Prokaryotic Biology:
This annual conference will be held in Estes Park Colorado from June 4th to June 8th of 2013. Additional information on this conference can be found on the office web-page: http://sciences.unlv.edu/wriver. Money from this award will be used to purchase the registration for this conference and a portion of the airfare. In this way the money will be spend before the termination of the award even though the meeting will not have taken place yet.

NOTE: If you are requesting travel funding only, please explain how you funded the other parts of your research project.

Have you requested funds for this project from your department or college? __yes X no

Are you receiving funding for this project from your college, school, department or any other source? __X yes _no If yes, amount you will receive: $6,000

I have received $6,000 for this project from a Student Enhancement Award. I have talked with Roxanne Male'-Brune and in the event that I am fortunate enough to receive this PURF award she has agreed to work with me to ensure that all guidelines for all awards are followed.

Provide an expected timetable for research (e.g., when it will start, how long it will take, when it will be completed?)

Fall Semester:
September: I started preliminary tests and research.
September, October, and November: I will be making mutants.
November and December: I will be analyzing the mutants.

Spring Semester:
January, February, and March: I will be characterizing virulence.
March, April, and May: I will be writing my thesis.
May: I will turn in my thesis.