REGULATION OF *OMPA* AND ITS EFFECT ON *SHIGELLA* VIRULENCE

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Abstract

*Shigella* bacteria cause 165 million annual cases of Shigellosis, resulting in 1.1 million fatalities each year. With growing antibiotic resistance and no vaccine, new methods must be developed to treat Shigellosis. Outer membrane protein A, or OmpA, is required for *Shigella* virulence and may be a good target for inhibitory drugs. Before these new treatments can be developed, we need to understand the regulation of *ompA* and its role in virulence. In a previous study, it was found that *ompA* mRNA (the molecular template for making protein) levels in *E. coli* are decreased at higher temperatures while OmpA protein levels remain the same. This suggests that translation efficiency is affected by temperature dependent regulation. Protein is more efficiently made from the lesser amount of mRNA present at higher temperatures. This project determined that *S. dysenteriae ompA* has a similar temperature-dependent regulation. Future studies will be conducted to determine if this temperature dependent-regulation is mediated by a putative FourU RNA thermometer in the 5’ UTR of *ompA*. Additionally, future studies will examine the contribution of this putative RNA thermometer to *S. dysenteriae* virulence.
Regulation of OmpA and Its Effect on *Shigella* Virulence

Senior Thesis
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1. 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 approximately 165 million annual cases of shigellosis worldwide, resulting in 1.1 million fatalities each year [7].

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

![Figure 1: Artist’s rendition of *S. sonnei* [1].](image)
shigellosis with a record 771 cases in 2012 [3].

Because of growing antibiotic resistance among *Shigella* species and the lack of a vaccine, the development of new methods to treat shigellosis is vital [8-10]. Outer membrane protein A (OmpA) is required for *Shigella* virulence and may be a good target for inhibitory drugs. Specifically, if the production of OmpA can be inhibited, it would be expected that *Shigella* would not be able to cause disease. Before the production of OmpA can be a viable target for new anti-shigellosis agents, we need to understand how the production of this protein is regulated and how this regulation impacts the ability of the pathogen to cause disease.

![Figure 2: Image of S. sonnei growing on the surface of an agar plate containing Congo Red [2].](image)
1.1 *Shigella*

*Shigella* are Gram-negative, facultative anaerobes. They are bacillus, non-motile, and nonspore-forming [7]. *S. sonnei* is pictured in Figure 1. *Shigella* colonies, which grow red on Congo Red plates, are small and round with a shiny surface as shown in Figure 2.

1.2 *Shigella*-Mediated Diseases

*Shigella* infection in a healthy human host can result after ingestion of only 1 to 10 organisms. This is a very small infectious dose as compared to other diarrhea-causing pathogens. For example, *E. coli* infection can cause disease only if there are between $10^6$ and $10^8$ organisms [11].

1.2.1 Shigellosis

Shigellosis is a diarrheal disease that is characterized by fever and abdominal cramps. Each year, it causes 1.1 million fatalities worldwide [12]. While the disease is usually not lethal in the US, there are still 18,000 laboratory confirmed cases each year [13]. It is estimated that shigellosis cases often go unreported and the actual number of cases is more than 20 times that figure [12].
1.2.2 Underestimation of Shigellosis Cases

There are multiple reasons for shigellosis to be underreported. Firstly, people suffering from diarrhea often stay at home and self-medicate. Secondly, if an individual does seek a physician’s help, the doctor usually prescribes a broad-range antibiotic and instructs the patient to stay hydrated. In these cases, the causative bacterial species is not investigated. In severe cases, physicians may screen the bacteria’s genetics with polymerase chain reaction (PCR). However, *Shigella* is genetically similar to *E. coli*. In fact, enteroinvasive *E. coli* is more related to *Shigella* than non-invasive *E. coli*. Consequently, a *Shigella* infection may be misdiagnosed as an *E. coli* infection. A specific test can differentiate between *E. coli* and *Shigella*, but the extra test may not be implemented.

1.2.3 Shigellosis Outbreaks

Within the past two years, there have been shigellosis outbreaks in Cleveland, Ohio, and Columbus, Ohio. In both cities, the outbreak was centered on a number of daycare centers, which is not surprising [14, 15]. Shigellosis is often spread at daycare centers and children’s pools. Another course of infection is the ingestion of food contaminated during harvest or preparation. Food may be grown in a field with sewage, or it may be prepared with dirty water or unclean hands.
1.2.4 Prevention

*Shigella* infection can be prevented by taking proper sanitary precautions, including careful hand washing. Specifically, hands should be washed for 20 seconds with soap and warm water.

While an individual is traveling abroad, shigellosis can be avoided in the same manner that traveler’s diarrhea is prevented. For instance, only fruit with a heavy, removed peel should be consumed. These fruits include bananas and oranges. Additionally, water should be clean and boiled. Other beverages should be sealed in a clean bottle or can. Again, hands need to be frequently and properly washed.

1.2.5 Treatment

If an *S. dysenteriae* infection occurs, antibiotics are contraindicative. After 24 hours, the addition of antibiotic is sensed by the bacteria, which respond by up regulating production of the shiga toxin (Figure 3). This worsens the symptoms of shigellosis. If a patient seeks treatment within the first 24 hours, a doctor may prescribe fluoroquinolones, ceftriaxone, or azithromycin. Ampicillin and Trimethoprim-sulfamethoxazole are commonly avoided because of growing resistance in *Shigella* species and other bacteria. Antidiarrheal drugs such as loperamide (Imodium®) or diphenoxylate with atropine (Lomotil®) can make the illness worse. Usually, a patient is instructed to rest and drink water [16].
With severe cases of shigellosis, hospitalization and intravenous electrolyte treatments may be necessary. Severe cases of shigellosis are most likely to occur in children, elderly individuals, and immunocompromised people.

1.2.6 Post-Infection Sequelae

In a healthy human host with ample water and electrolytes, shigellosis usually resolves itself within 5 to 7 days [13]. However, *Shigella* infection can result in post-infection sequelae like hemolytic uremic syndrome and Reiter's syndrome, which cause damage to the kidneys and joints, respectively [17].
Shigella can bring about hemolytic uremic syndrome by causing the formation of thrombi, or blood clots. The clots form as a reaction of host antibody against shiga toxin. Additionally, shiga toxin damages the lining of capillaries. When the capillary lining is damaged, platelets flood the area to aid in the healing process. Hemolytic uremic syndrome, or HUS, is characterized by a low platelet count and kidney failure. Kidney failure results from clots in the organ. HUS symptoms include fever, nausea, and abdominal pain. There are no specific treatments for HUS. Transfusions and dialysis may be used to treat symptoms, but the host must heal itself [18].

Reiter’s syndrome is an autoimmune disease that is characterized by painful joints and pain during urination. Inflicted individuals may also suffer with pus-filled sores and conjunctivitis. In order for an individual to get Reiter’s syndrome, he or she must have a genetic predisposition. During an infection with Shigella, antibodies are made that attack the Shigella. In genetically predisposed individuals, these antibodies may also attack joint tissues. This arthritic disorder may be treated with non-steroidal, anti-inflammatory drugs, such as ibuprofen (Advil® and Motrin®) and naproxen (Aleve®). Also, a patient may be prescribed corticosteroids or drugs that suppress the immune system, such as sulfasalazine or methotrexate [19].
1.3 *Shigella* Pathogenesis

*Shigella* bacteria are transmitted via an oral-fecal route, which is a common mode of transmission for many bacteria that cause diarrheal disease, such as *Escherichia coli* (Figure 4). 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 properly 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 and invade the epithelium. [7]. *Shigella* enter the intestinal epithelium by triggering uptake into microfold cells (M cells), which continuously sample particles from the lumen. When *Shigella* are found, immune responses are induced. Consequently, macrophages engulf the bacteria and travel into the sub mucosa. In the sub mucosa, *Shigella* cause the apoptosis of the macrophages, freeing the bacteria from degradation. Then, *Shigella* enter the epithelium from the basolateral side. In the epithelium’s cytoplasm, the bacteria replicate and spread to neighboring cells via actin motility. During this process, *Shigella* cause severe tissue destruction of the epithelium, resulting in lesion formation and the production of the characteristic bloody diarrhea (Figure 5). [20]
1.4 Protein Production

While there are a variety of factors that facilitate Shigella infection, proteins are especially common components. Protein production involves two consecutive processes: transcription and translation. During transcription, information from DNA, the genetic blueprint, is transferred to messenger RNA, or mRNA. Next, 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 the production of every protein in all
cells, and each step can be subject to specific regulation. This essential process of
translation is depicted in Figure 6.

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 the corresponding mRNA molecule. Regulatory
elements can affect this balance by altering the efficiency of translation.

1.5 RNA Regulatory Elements Affect Translation

Ribo-regulators can control any step within the process of gene expression.
Ribo-regulators are RNA molecules or portions of RNA molecules that are not
translated into proteins. Instead, they function as an RNA entity to influence the expression of other genes [8]. These regulatory elements can increase or decrease the expression of certain genes, such as outer membrane protein A (OmpA). Because these elements often regulate *Shigella* virulence, their identification and characterization may lead to potential targets in treating shigellosis. Specifically, understanding these mechanisms by which ribo-regulators control target gene expression may help with the development of new antimicrobial therapies designed to disrupt these precise mechanisms.

Ribo-regulators can function by different mechanisms to control target gene translation. For instance, an RNA molecule may bind to an mRNA molecule and physically prevent the translation of the encoded gene by blocking ribosomal binding. Additionally, an RNA molecule regulatory element may change its shape in response to environmental conditions, such as temperature. This structural change may allow or prevent translation of the encoded gene by permitting or blocking ribosomal binding to a given mRNA molecule.

This project will focus on a specific type of ribo-regulator called an RNA thermometer, which functions to regulate translation efficiency in response to temperature.

### 1.6 Temperature-Dependent Regulatory Elements

Like many other bacteria that are transferred via the oral-fecal route, *Shigella* must survive environmental conditions that vary between the host environment
encountered during infection and the non-host environment encountered during transmission. Important environmental factors, such as acidity, iron availability, and temperature, vary between non-host and host environments. Differences in these conditions allow the environments to influence 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 indication that the bacteria have entered the human body. In response to this increased temperature, Shigella increase the expression of genes which facilitate the establishment and progression of an infection. One mechanism by which Shigella regulate gene expression in response to temperature is an RNA thermometer.

**1.7 RNA Thermometers**

![Image of RNA thermometer](image)

**Figure 7: Structural change of an RNA thermometer as temperature increases from a non-permissible temperature to a permissible temperature.** The structural change is gradual.
RNA thermometers are temperature-dependent, cis-acting regulatory elements. These regulatory elements are located in the 5’ untranslated region, or UTR, of the regulated mRNA itself. The 5’ UTR is a section of RNA at the front of the mRNA that contains the ribosomal binding sequence (RBS). In order for translation to occur, a ribosome must be able to bind directly to the RBS (Figure 6).

RNA thermometers are able to control the binding of a ribosome to the RBS by altering mRNA structure (Figure 7). When the environmental temperature is cooler (25°C), the thermometer’s structure is such that the RBS is occluded within a double-stranded inhibitory structure. This inhibitory structure takes the form of a single-strand loop on top of a double-strand stem (Figure 8A). If the environment warms (37°C), the thermometer melts, and the RBS is exposed (Figure 8B). Thus, a ribosome

![Figure 8: Schematic of a Basic RNA Thermometer. A. At non-permissive temperatures, an inhibitory structure is formed that prevents translation by blocking the ribosome (green oval) from binding to the RBS sequence (red line). B. At permissive temperatures, this inhibitory structure is destabilized, the ribosome binds, and translation proceeds. C. The orange barrel represents a theoretical compound that would inhibit gene expression by specifically binding to and stabilizing the inhibitory structure within the RNA thermometer. Figure is not drawn to scale.](image)

thermometer melts, and the RBS is exposed (Figure 8B). Thus, a ribosome
preferentially binds to the mRNA molecule at higher temperatures when the RBS is exposed, and translation occurs more efficiently. [21]

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 are contained within the inhibitory structure and bind to the RBS at low temperatures (Figure 9) [9]. FourU RNA thermometers have been found in other bacteria, such as Salmonella enterica [10]. This is just the second FourU RNA thermometer to be identified in any Shigella species.

Andrew Kouse of Dr. Murphy’s lab at Ohio University has identified a FourU RNA thermometer in S. dysenteriae that increases the translation efficiency of the regulated gene at 37°C as compared to that at 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].

![RNA Thermometer Diagram](image)

**Figure 9: Predicted RNA thermometer on the ompA mRNA molecule.** This structure is predicted to prevent binding of the ribosome to the mRNA molecule at low temperatures. The ribosomal binding site (RBS) is marked.
1.8 OmpA Protein

OmpA is a protein that is highly conserved among enterobacteria. This porin protein has an atomic mass of approximately 35 KDa. Its β-barrel structure is shown in Figure 10, and Figure 12 shows its placement in the bacteria’s bilipid membrane.

OmpA’s β-barrel structure and membrane placement allow the membrane to have low permeability; small, nutritional solutes can pass through the membrane. Additionally, OmpA helps membrane stability.

OmpA is crucial for *Shigella* virulence, specifically for cell-to-cell spread of the pathogen within the intestinal epithelium [22, 23]. Without OmpA, *Shigella* successfully invades human cells but cannot spread from one cell to a neighboring cell (Figure 5). Without cell-to-cell spread, *Shigella* cannot cause disease. This data

![Figure 10: Image of E. coli OmpA](image-url)
suggests that directed 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.

1.9 Shigella and E. coli

As previously mentioned, *Shigella* is highly related to *E. coli*. Both are enterobacteria with similar colonial morphology. They are rod-shaped, Gram-negative facultative anaerobes.

According to housekeeping gene sequences and a shared invasion plasmid, enteroinvasive *E. coli* is more related to *Shigella* than to non-invasive *E. coli*. Both cause dysentery using the same method of invasion (Figure 5). Figure 11 shows a phylogenetic tree containing *Shigella* and *E. coli*. 
Figure 11: Phylogenetic tree with *E.coli* and *S. dysenteriae* [6].
1.10 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 [24]. While the mechanism of this temperature-dependent regulation of OmpA translation is unknown, the Murphy lab’s *in silico* analysis predicted the presence of an RNA thermometer within the *ompA* mRNA molecule (Figure 13). An RNA thermometer alters translation efficiency in response to temperature by forming an inhibitory structure at low temperatures that prevents

Figure 12: Image of *E. coli* OmpA in a bilipid membrane [5].
binding by the ribosome (the protein complex required to synthesize protein from the mRNA template). At higher temperatures, this inhibitory structure is destabilized, or “melts out,” and translation proceeds uninhibited.

*Shigella* is highly related to *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 *Shigella* virulence, I focused my investigations on this pathogenic species.

### 2.0 Thesis Project

#### 2.1 Aims of Study

**Overall Hypothesis:** The expression of *S. dysenteriae ompA* is subjected to post-transcriptional temperature-dependent regulation mediated by a FourU RNA thermometer located within the 5’UTR of the transcript (Figure 13).

![Figure 13: Predicted location of the putative FourU RNA thermometer in the *ompA* gene of *S. dysenteriae*.](image)
Overall Goal: This project aimed to determine the mechanism controlling the temperature-dependent regulation of OmpA translation and the effect of this regulation on *Shigella* virulence. This goal will be reached by the completion of two aims.

**2.1.1 Aim 1: Determine if *Shigella* OmpA production is controlled by a temperature-dependent post-transcriptional mechanism.**

**Background and Significance:** *S. dysenteriae* is similar to *E. coli*, which is known to have a temperature-dependent regulation of *ompA*. Because OmpA is required for *Shigella* virulence, understanding how OmpA production is regulated may aid in the development of novel drugs.

**Approach:** In order to determine if *Shigella* OmpA production is controlled by a temperature-dependent post-transcriptional mechanism, relative amounts of *ompA* mRNA and OmpA protein from wild type *S. dysenteriae* grown at 25°C were compared to those measured following growth of the strain at 37°C. The relative amounts of *ompA* mRNA were measured with real time Polymerase Chain Reaction, or qPCR, following the growth at 25°C and 37°C. Additionally, the relative amounts of OmpA protein were measured by Western blot analysis.
Expected Outcomes: It was expected that wild type *S. dysenteriae* grown at 37°C would have less mRNA as compared to *S. dysenteriae* grown at 25°C. While it was expected that mRNA would be different, it was predicted that *S. dysenteriae* grown at 25°C and 37°C would produce equal amounts of protein.

Results:

*ompA* mRNA Levels

Figure 14 shows a qPCR that was performed on wild type *Shigella*. The graph was determined based on averages of a triplicate analysis. According to this data, *S. dysenteriae* grown at 25°C has more *ompA* mRNA than *S. dysenteriae* grown at 37°C.

![Figure 14](image-url)

*Figure 14: ompA* mRNA levels are higher following growth of *S. dysenteriae* at 25°C as compared to those following growth of the strain at 37°C. *S. dysenteriae* ompA mRNA levels were analyzed by Real-time PCR following growth of the organism at 25°C and 37°C. ompA levels were normalized to that of rrsA in each sample and are expressed relative to the amount measured in the 25°C. * denotes a significant difference from the amount of transcript measured at 25°C (p ≤0.01).
OmpA Protein Levels

Figure 15 shows a Western blot analysis that was performed on wild type *Shigella*. The samples shown are indicative of a triplicate analysis. According to this preliminary analysis, OmpA protein is produced at similar levels in *S. dysenteriae* grown at 25°C and 37°C.
Figure 15: A) OmpA protein is produced at equal levels in *S. dysenteriae* grown at 25°C and 37°C. *S. dysenteriae* were cultured to the mid-log phase of growth at 25°C and 37°C. Whole cell protein preparations were prepared from an equivalent number of bacterial cells grown under each experimental condition. Samples were run on a 7.5% acrylamide gel and subjected to Western blot analysis using a monoclonal anti-OmpA antibody. Lane 1: Protein markers; Lane 2: 25°C protein sample; Lane 3: 37°C protein sample. Data shown are representative of two independent triplicate analyses. B) Protein loading control. Lane 1: 25°C protein sample; Lane 2: 37°C protein sample.
2.1.2 Aim 2: Determine if the observed temperature-dependent regulation of OmpA production is mediated by a FourU RNA thermometer.

**Approach:** Two mutant strains of *S. dysenteriae* will be constructed and analyzed. One mutant strain will have a stabilized FourU RNA thermometer within the ompA gene; the other strain will have a destabilized FourU RNA thermometer within the ompA gene. The relative levels of *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.

**Background and Significance:** Two mutations will be introduced into the *S. dysenteriae* chromosome: one mutation will stabilize the ompA thermometer and one mutation will destabilize the thermometer. The exact base changes introduced for each of these mutants can be found in Figure 19. The stabilized mutant is expected to prevent the RNA thermometer from transitioning into the permissible structure. In contrast, the destabilized mutant is expected to facilitate the RNA thermometer in achieving the permissible structure.

If the putative RNA thermometer is involved, the mutations will have predictable consequences on expression. These consequences are detailed below in “Expected Outcomes.”
Expected Outcomes: The stabilized mutant *S. dysenteriae* grown at 37°C is predicted to have less protein than wild type *S. dysenteriae* grown at 37°C. The destabilized mutant *S. dysenteriae* grown at 25°C is predicted to have more protein than wild type *S. dysenteriae* grown at 25°C.

Results:

Figures 16-18 were show gels that were used during the mutant construction detailed in "Methods."

![Figure 16: Gel purification of upstream and downstream products used for SOEing.](image)

Lane 1: Markers; Lane 2: Destabilized upstream sample; Lane 3: Destabilized downstream sample; Lane 4: Stabilized upstream sample; Lane 5: Stabilized downstream sample. Data shown are representative of two independent triplicate analyses.
Figure 17: Gel purification of SOEing products. Lane 1: Markers; Lane 2: Destabilized sample; Lane 3: Stabilized sample.

Figure 18: Gel to screen *E. coli* DH5α mutants for inserted mutations. NotI digestion cuts the insert out of the pGEMT plasmid. Lane 1: Markers; Lane 2: Destabilized sample; Lane 3: Stabilized sample.
2.2 Significance of OmpA and RNA Thermometers

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* [25-29]. 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.

2.3 Future Drug Development

Shigellosis is causing 1.1 million fatalities worldwide and is greatly affecting the wellbeing of developing countries [7]. 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 (Figure 8C). 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.

2.4 Methods

These aims were accomplished through the following 3 steps: Western blot analysis, quantitative real time PCR analysis, and chromosome mutation. All experimental procedures took place in the mentor’s lab, which is a certified Biological Safety 2 Lab. Training was conducted by Dr. Erin Murphy and the Department of Environmental Health and Safety.

2.4.1 Chromosomal Mutations

Mutating the chromosomally located ompA RNA thermometer using site-directed mutagenesis was attempted as previously performed in Dr. Murphy’s lab and detailed in Broach et al.[30]. One chromosomal mutation was designed to stabilize the inhibitory structure within the RNA thermometer, potentially preventing ribosomal binding at normally permissive temperatures. The second chromosomal mutation was designed to destabilize the RNA thermometer structure, mimicking the “melting out” observed at high temperatures, allowing ribosomal binding at normally inhibitory temperature. Figure 19 shows the exact mutations that were planned. Each mutation was constructed in E. coli, but neither has been successfully inserted into the S. dysenteriae chromosome.
Both mutations were built with splicing by overlap extension, or SOEing. SOEing is a type of polymerase chain reaction, or PCR. PCR is a technique that amplifies a specific DNA sequence by thermocycling the original DNA with primers, DNA polymerase, and other chemicals. First, double stranded DNA is denatured at a high temperature. Next, the temperature is lowered so that specific primers can anneal to complimentary DNA. In SOEing, the 3’ end is complementary, but a mutation is located in the primer’s 5’ end. After the primers are attached, the temperature is raised, allowing polymerase to create a new double strand of DNA. This process is depicted in Figure 20.

This SOEing PCR required upstream and downstream templates. These templates were developed using Phusion® PCR, which utilizes a high fidelity polymerase. Two primers (ompA SOE 1 and ompA SOE 4), the outside primers, were the same for both mutations. The mutations were determined by only the inside primers. The stabilized mutation primers were ompA SOE 2s and ompA SOE 3s, and

![Figure 19: Chromosomal Mutations.](image)

The RBS is labeled. The mutations are marked with black arrows.
the destabilized mutation was made with ompA SOE 2 and ompA SOE 3. All primers were developed by Andy Kouse of the Murphy lab.

In Phusion® PCR, each reaction was a total of 50 µL. One µL of S. dysenteriae 04576S1-G was added to 10 µL 5X Phusion buffer, 4 µL 2.5 mM dNTPS, 1 µL of each of the two primers required for the desired mutation, 32 µL ddH₂O, and 1 µL of Phusion® polymerase. The polymerase was added to the reaction only after the mixture was heated to 98°C for 5 minutes. After the enzyme’s addition, the reaction is
heated to 98°C for 30 seconds. Next, the reaction was cycled through the following steps 30 times: 98°C for 10 seconds, 55°C for 15 seconds, 72°C for 1 minute and 45 seconds. Finally, the reaction was heated to 72°C for 5 minutes.

Resulting upstream and downstream templates were run out on a 0.8% agarose gel (Figure 16). Desired bands, measuring approximately 600 (downstream) and 700 (upstream) base pairs, were extracted using Omega’s E.Z.N.A® Gel Extraction Kit or Qiagen’s ®Gel Extraction Kit as instructed by the kit’s published procedure.

Then, constructed templates were used for SOEing PCR. Seventy-five ng of the desired mutation’s upstream and downstream templates were added to 10 µL 5x Phusion® buffer, 4 µL 2.5 mM dNTPs, 1 µL ompA SOE 1, 1 µL ompA SOE 4. The reaction was brought to 49 µL with the addition of ddH₂O. The reaction was heated to 98°C before 1 µL of Phusion® polymerase was added. After the enzyme’s addition, the reaction is heated to 98°C for 30 seconds. Next, the reaction was cycled through the following steps 30 times: 98°C for 10 seconds, 55°C for 15 seconds, 72°C for 1 minute and 45 seconds. Finally, the reaction was heated to 72°C for 5 minutes.

SOEing products were run out on a 0.8% agarose gel. Desired bands, measuring approximately 1200 base pairs, were extracted using Omega’s E.Z.N.A® Gel Extraction Kit or Qiagen’s® Gel Extraction Kit as instructed by the kit’s published procedure.

The extracted products were inserted into a pGEMt EZ plasmid by ligation. Seven µL of insert were mixed with 1 µL pGEMt EZ plasmid, 1 µL 10x ligase buffer,
and 1 µL ligase. The reaction was cooled to 16 °C for 18 hours and heated to 65°C for 20 minutes.

Ligated pGEMt EZ plasmid was transformed into *E. coli* DH5α. Frozen *E. coli* DH5α was thawed on ice. All ligated plasmid was mixed with the thawed *E. coli* DH5α. The mixture was heated to 42 °C for 1 minute and 15 seconds. Then, the mixture is put on ice for 15 minutes. The cells were transferred to a culture tube of 1 mL LB and incubated at 37 °C for 1 hour. After an hour, 100 µL of the culture were plated on LB with ampicillin (250 µg/mL) and X-gal (250 µg/mL). The plate was incubated overnight at 37°C.

Colonies were selected and transformation was confirmed through Not1 digest of isolated plasmids. Plasmids were isolated using Qiagen’s QIAprep® Spin Miniprep Kit. One mL of culture was pelleted and resuspended in 250 µL Buffer P1. Next, 250 µL Buffer P2 were added to the sample. The mixture was inverted 4 to 6 times and was left at room temperature for 5 minutes. Then, 350 µL Buffer N3 were added. Again, the sample was inverted 4 to 6 times. After, samples were centrifuged at 13,000 rpm for 10 minutes. Resulting supernatant was transferred to a QIAprep® spin column by pipetting. The column was centrifuged for 1 minute, and the flow-through was discarded. The column was washed with 750 µL Buffer PE and centrifuged for 1 minute. Again, flow-through was discarded. Samples were centrifuged again for 1 minute to remove residual buffers. Columns were placed in clean collection tubes. Forty µL ddH2O were added to each sample. Then, samples incubated at room temperature for 5 minutes. After, samples were centrifuged at 14,800 rpm for 1 minute.
Resulting flow-through was transferred back onto the column. Again, samples incubated at room temperature for 5 minutes. Finally, samples were centrifuged at 14,800 rpm for 1 minute, and flow-through, which now contained concentrated plasmid was collected. Resulting plasmid was used in Not1 digestion. Each reaction was 15 µL and consisted of 1.5 µL NEB 3 buffer, 1.5 µL 10X bovine serum albumin (BSA), 1 µL Not1 enzyme, and 11 µL of plasmid. Reactions were heated to 37°C for 3 hours and 65°C for 1 hour. Then, digested plasmids were run out on a 0.8% agarose gel at 60 V for 55 minutes. Gels were visualized with ultraviolet light, and bands measuring approximately 1200 bp, which is the estimated size of the inserts, were extracted. Bands were purified using QIAquick® Gel Extraction Kit. The band was placed in a 1.5 mL tube and 750 µL of Buffer QG were added. The mixture was incubated at 50°C until the gel completely dissolved, or approximately 5 to 10 minutes. After the gel dissolved, 100 µL isopropanol were added to the samples. A QIAquick spin column was placed in a 2 mL collection tube. Samples were applied to the column and centrifuged at 14,800 rpm for 1 minute to bind the DNA. Flow-through was discarded.

Next, 0.75 mL Buffer PE was added to the column. Then, the column incubated at room temperature for 5 minutes. After, samples were centrifuged for 1 minute, and flow-through was discarded. Then, the samples were centrifuged at 13,000 rpm for 1 minute in order to remove residual buffers. The column was placed into a clean collection tube, and 40 µL ddH₂O were added to the column. Next, the column incubated at room temperature for 5 minutes. After, the samples were
centrifuged at 14,800 rpm for 1 minute. Resulting flow-through was transferred back onto the column. Again, samples incubated at room temperature for 5 minutes. Finally, samples were centrifuged at 14,800 rpm for 1 minute, and flow-through, which now contained concentrated insert.

Incorporation into *E. coli* DH5α was the latest step in mutant development to be completed. Figure 21 shows both completed and future steps in mutant production.

**Figure 21: Completed and future steps in developing *S. dysenteriae* mutants.**
2.4.2 Bacterial Culture Growth

*S. dysenteriae* were grown to early logarithmic phase for the following experiments. Figure 22 depicts the process of growing these cultures.

**Figure 22: Sample preparation.** Frozen wild type *S. dysenteriae* is streaked for isolation on an agar plate with Congo Red. The plate is grown overnight at 37°C. Three colonies are selected and grown in 3 mL LB overnight at 30°C. Samples are taken from these cultures and grown to early logarithmic phase at 25°C or 37°C. RNA and total protein is isolated from each culture.
2.4.3 Protein Preparation

*S. dysenteriae* were cultured to the mid-log phase of growth at 25°C and 37°C. Whole cell protein preparations were prepared from an equivalent number of bacterial cells grown under each experimental condition. Bacteria were pelleted and resuspended in 200 µL of a buffer consisting of 100 µL ddH₂O, 95 µL Laemmli sample blue, and 5 µL of 2-Mercaptoethanol (BME). Mixtures were placed in boiling water for 10 minutes and then stored at -20 C.

2.4.4 Western Blot Analysis:

Wild-type *Shigella* was cultured to early logarithmic phase at 25°C and 37°C, and OmpA protein levels were measured using Western blot analysis. Samples were run on a 7.5% acrylamide gel. The bottom gel was made with 2.5 mL 30% Acrylamide:Bisacrylamide (29:1), 2.5 mL bottom buffer, 5 mL ddH₂O, 5uL Tetramethylethylenediamine (TMED), and 50uL 10% Ammonium persulfate (APS). The top gel consisted of 2.44 mL ddH₂O, 1 mL top buffer, 0.56 mL 30% Acrylamide:Bisacrylamide (29:1), 50 µL APS, and 5 µL TEMED. Running buffer was a 1:200 Sodium Dodecyl Sulfate (SDS) solution in ddH₂O. The samples were run at 100 V until the dye was within 1 to 2 mm of the bottom of the gel (approximately 7 cm), which was approximately 2 hours.

Next, the gel was subjected to Western blot analysis using a monoclonal anti-OmpA antibody. The membrane was run at 350 mA for 1 hour in transfer buffer,
which consisted of 39 mM glycine, 48 mM Trisbase, 0.037% SDS, and 20% methanol in ddH₂O.

After, the membrane was put into a 10% nonfat milk solution in Phosphate Buffered Saline with Tween (PBST) and left on a shaker overnight at 4°C. Next, the membrane was transferred into monoclonal anti-OmpA antibody and left on a shaker overnight at 4°C. The antibody was 1:1000 in a 5% nonfat milk solution in PBST. The next day, the membrane was washed in PBST 3 times for 5 minutes each time. After, the membrane was transferred into a 10% nonfat milk solution in PBST for 10 minutes at 4°C. Next, the membrane was transferred into secondary antibody, which was goat anti-mouse HRP. The secondary antibody was diluted to 1:10000 in a 5% nonfat milk solution in PBST. The membrane was in the secondary antibody on a shaker at 4°C for 1 hour. Next, the membrane was washed in PBST 3 times for 5 minutes each time.

Finally, the membrane was treated with a solution from Immunostar Western Kit® that was prepared according to its published procedure. The membrane was rocked in this solution for 5 minutes at room temperature.

ChemiDoc™ XRS+ imaging system with Image Lab™ image acquisition and analysis software was used to visualize the membrane.

2.4.5 RNA Preparation

*S. dysenteriae* were cultured to early logarithmic phase at 25°C and 37°C. Three mL of culture were added to 0.75 mL 5X StayRNA. Next, the cells were pelleted and resuspended in 357.3 µL Diethylpyrocarbonate (DEPC) treated ddH₂O,
40 μL 10% SDS, and 2.67 μL 3M sodium acetate (pH 5.2). The mixture was vortexed for 15 seconds. Next, cells were heated at 90°C for 7 minutes. After, 1 mL Trizol was added to the cells. The new mixture was transferred into a Phase-lock tube and incubated at room temperature for 5 minutes. Then, 250 μL of chloroform were added to each sample. Samples were shaken for 1 minute. After, samples incubated at room temperature for 2 minutes. Then, samples were centrifuged at 14,000 rpm for 2 minutes. The resulting upper phase was transferred to a new tube, and 1 mL 100% ethanol was added. Samples were precipitated at -80°C overnight. Precipitated RNA was centrifuged at 13,200 rpm for 15 minutes. The ethanol was aspirated, and samples were dried in a vaccufuge. Samples were dried until no liquid was seen, which took approximately 3 to 7 minutes. RNA was resuspended in 50 μL DEPC treated ddH₂O.

Next, samples were divided in half. Three μL of Ambion Turbo® DNA-free 10X buffer and 1 μL Turbo® DNA-free enzyme were added to each half sample. Samples were incubated at 37°C for 90 minutes. Next, 4 μL DNase inactivation reagent were added to each sample. Samples incubated at room temperature for 5 minutes. Then, samples were centrifuged at 14,800 rpm for 2 minutes. Supernatant was collected and tested for DNA contamination using a screening PCR. Each PCR reaction consisted of 14 μL ddH₂O, 2 μL 10X Taq buffer, 0.2 μL primer virB9, 0.2 μL primer virB10, 0.2 μL Taq polymerase, and 1 μL template. A scraping of wild type *Shigella* was used at a positive control and RNase-free H₂O was used as a negative control. Reactions were heated to 95°C for 5 minutes. Then, samples began a cycling through the following steps 30 times: 95°C for 30 seconds, 48°C for 30 seconds, and
72°C for 1 minute. Finally, the samples were heated to 72°C for 5 minutes. These samples were run out on a 2.0% agarose gel. If bands appeared in the sample wells, DNase reactions were performed again. This screening continued until all samples were free of DNA. Next, samples were used to make cDNA. One µL of clean RNA was mixed with 4 µL of 5X Iscript® buffer, 1 µL reverse transcriptase, and 14 µL RNase-free H₂O. Samples were heated to 25°C for 5 minutes, 42°C for 30 minutes, and 83°C for 5 minutes. Diluted cDNA samples were used for qPCR analysis.

2.4.6 Quantitative Real Time PCR

_Shigella_ was cultured to early logarithmic phase at 25°C and 37°C, and _ompA_ mRNA levels were be measured using quantitative real time PCR (qPCR) analysis as previously implemented in Dr. Murphy’s lab [30-32]. qPCR is a method of quantifying the relative amount of a specific mRNA molecule.

_S. dysenteriae_ _ompA_ mRNA levels were analyzed by Real-time PCR following growth of the organism at 25°C and 37°C. _ompA_ levels were normalized to that of _rrsA_ in each sample and are expressed relative to the amount measured in the 25°C (ΔΔCT). Data was analyzed using averages and student’s t-tests.

2.5 Discussion

As shown by Figure 14, _S. dysenteriae_ grown to early logarithmic phase at 25°C has more _ompA_ mRNA than _S. dysenteriae_ grown at 37°C. According to Figure
15, S. dysenteriae grown to early logarithmic phase at grown 25°C and 37°C have similar levels of protein. Since S. dysenteriae grown at 37°C has similar OmpA protein levels but less ompA mRNA as compared to S. dysenteriae grown at 25°C, translation of ompA mRNA is more efficient at 37°C as compared to 25°C. In other words, at 37°C, S. dysenteriae is making similar amounts of OmpA protein with less ompA mRNA as compared to those of S. dysenteriae grown at 25°C. Increased translational efficiency of ompA at 37°C as compared to that at 25°C is consistent with the hypothesis of an RNA thermometer mediating regulation. As the temperature rises, the thermometer melts into its permissible structure, allowing ribosomal binding. Consequently, as the temperature rises, more ompA mRNA can be translated into protein.

2.5.1 Future Directions: Quantifying Western Blot Analysis

Western blot analysis will be repeated in triplicate. Data will be analyzed with densitometry.

2.5.2 Future Directions: Mutations

After stabilized and destabilized mutant Shigella are constructed, Western blot and RNA analyses will be performed. The analyses will be performed in the same manner as previously described.

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 protein levels as compared to those in wild-type following growth of each strain at the permissive temperature of 37°C.

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 at 25°C and 37°C. 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.

2.5.3 Future Directions: Virulence Assays

Additionally, virulence assays will be performed using mutant and wild type Shigella. 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 destabilized mutants will be better able to form plaques.

2.5.4 Future Directions: Protein Stability

While data collected is consistent with the hypothesis of an RNA thermometer mediating temperature-dependent regulation of ompA, other explanations must be
considered and eliminated. One such explanation involves protein stability. While less likely, it is possible that OmpA protein from *S. dysenteriae* grown at 25°C is less stable than OmpA protein from *S. dysenteriae* grown at 37°C. Usually, protein is more stable at cooler temperatures, but there may be unknown factors affecting the protein levels. Protein stability can be tested using a protein stability assay. This assay would expose the OmpA protein to a substance that degrades the protein. Protein levels would be assessed at different time points, and those from bacteria grown at 25°C would be compared to those from bacteria grown at 37°C. If OmpA protein is less stable at 25°C, protein from bacteria grown at 25°C would degrade faster than protein from bacteria grown at 37°C. If it were determined that protein from *S. dysenteriae* grown at 25°C is less stable than protein from *S. dysenteriae* grown at 37°C, the RNA thermometer hypothesis would no longer be supported with this data.

### 2.5.5 Future Directions: Toe Printing Assays

Toe printing assays could help to confirm the location of the RBS within the RNA thermometer. RNA is mixed with the 30s subunit of the ribosome, a synthetic DNA primer that attached downstream of the binding site initiator tRNA, deoxynucleotide triphosphates, and reverse transcriptase (RT). RT extends the primer toward the 5' end of the RNA until the ribosome is reached. The ribosome physically inhibits RT from continuing along the RNA. Resulting cDNA is visualized on gel, and the length of the cDNA can be determined. The location of the ribosome’s attachment, or the RBS, can be inferred from the cDNA’s length.
Glossary

**Bacillus**: rod-shaped

**Cis-acting regulatory elements**: regulatory elements which mediate the regulation of nearby genes

**Congo Red**: red chemical dye

**ddH₂O**: double distilled water

**Epithelium**: tissue that forms an outer protective membrane.

**Facultative anaerobe**: an organism that survives with aerobic respiration when oxygen is present but can switch to fermentation or anaerobic respiration when ample oxygen is not available

**Gram-negative**: bacteria with a thin peptidoglycan layer

**HeLa cells**: an immortal cell line of human cervical carcinoma epithelial cells.

**In silico**: done on the computer.

**Lumen**: the cavity inside of a tubular structure.

**Lyse**: to burst and kill a cell.

**Microfold cells (M cells)**: cells that sample intestinal contents to find foreign invaders, which they then deliver to antigen-presenting cells.

**Monolayer**: a layer that is one cell thick.

**Polymerase**: an enzyme that copies DNA or RNA

**Primer**: a string of nucleic acids that act as a starting point for DNA synthesis.
**RNA thermometer**: a temperature-sensitive non-coding RNA molecule that regulates gene expression, usually by changing its secondary structure.

**Rrsa**: a housekeeping gene whose expression levels are not affected by experimental conditions used in these experiments.

**Sub mucosa**: dense irregular connective tissue beneath a mucous membrane.

**Virulence**: the ability to cause disease.
Bibliography


