Drew University
College of Liberal Arts

Protein Factors Affecting the Small RNA Regulation of the Mannitol Operon in *Vibrio cholerae*

A Thesis in Biochemistry and Molecular Biology

by

Robert Scheffler

Submitted in Partial Fulfillment
of the Requirements
for the Degree of
Bachelor in Arts
With Specialized Honors in Biochemistry and Molecular Biology

May 2014

Acknowledgments

I would like to thank Dr. Jane Liu for all her help throughout my four years of research. She has been the best advisor I could have ever asked for and I am glad to have worked with her. In addition, I would like to thank my advisory committee of Dr. Adam Cassano, Dr. Joanna Miller, and Prof. Audrey Evrard for all their help in the thesis process. I would also like to thank all my lab members at both Drew University and Pomona College for making working in the lab a great time. Finally, I'd like to thank my family and friends for their unending support for the last four years.

Abstract

Vibrio cholerae causes hundreds of thousands of deaths yearly and continues to reemerge around the world after natural disasters. In order to better combat the pathogen more must be learned about how it adapts to the differing environments of fresh or saltwater reservoirs and the human small intestine. One possible mechanism for the rapid adaptation to these differing environments is through the use of small RNAs. *V. cholerae* possess a small RNA, MtlS, that regulates the synthesis of the mannitol transporter protein, MtlA, which allows mannitol into the cells. Mannitol is important as it can be used by the pathogen as a carbon source or possibly as a compatible solute for dealing with osmotic stress. MtlS, is the focus of this thesis. Three questions were addressed: What is the role of Hfq in the system, what is the role of MtlR in the system, and how can we study MtlR further. The results suggest that MtlS works independent of Hfq, a widely utilized chaperone protein for small RNAs, to repress MtlA synthesis and that MtlR also represses MtlA synthesis. Detection of MtlR proved a challenge; we conclude that MtlR is very lowly expressed. Future studies of MtlR must be done better understand how it functions and how the system is regulated overall. If more can be learned about the ways pathogens such as *V. cholerae* adapt and survive, the better treatment and prevention methods that can be created to fight infectious diseases.

Table of Contents

Figures and Tables

Introduction

The Long Standing Threat of Infectious Diseases

Infectious diseases have long been a major cause of deaths globally, having only declined in recent decades due to scientific advancements including the discovery of penicillin, increased levels of sanitation, and more widespread vaccinations. However, despite these prevention and treatment innovations, in 2012, approximately 15 million people (about 25% of the total deaths globally) were caused by infectious diseases (Fauci and Morens 2012). While it may be expected that the number of deaths due to infectious diseases has been decreasing, over the past decade it has been seen that the number has remained fairly constant with about 15 million deaths (about 25% of total deaths) in both 2004 and 2008 (Morens et al. 2004; Morens et al. 2008). In addition, these numbers do not include those that become ill due to infectious diseases, which causes an even larger global burden in lost work hours and health care costs (Fonkwo 2008). This trend is rather jarring, and indicates the importance and need for research in the field in order to combat the pathogens that lead to disease.

Infectious diseases are not a new threat and have been well noted throughout history. One of the first well documented infections was the plague of Athens in 430-426 BC; infections have been described since, including the Black Death in 1347-50, the Spanish influenza in 1918-19, and the current HIV/AIDS epidemic that began in 1981 (Morens et al. 2008). The diseases listed are often thought as some of the most important infectious diseases to affect human health and are well documented for the number of deaths they caused worldwide. The Black Death killed approximately 34 million in

Europe, 16 million in Asia, and continues to threaten (Morens et al. 2008). The pathogen that causes Black Death, *Yersinia pestis*, was used in one of the earliest attempts at bioterrorism during World War II through the use of infected fleas (Morens et al. 2008). Similarly, Spanish influenza was responsible for 50-100 million deaths (Morens et al. 2008). This death count was about two times higher than the death count of World War I, which ended soon before the outbreak (Morens et al. 2008). Finally, the HIV/AIDS epidemic continues to rage on in modern day showing the continued threat of infectious diseases worldwide. Although infectious diseases are not a new hazard, new diseases continue to emerge and reemerge causing a constant health concern.

Three Categories of Infectious Disease

One way that infectious diseases can be distinguished is into one of three broad categories: established, newly emerging, or reemerging (Fauci and Morens 2012). All three of the categories lead to many deaths each year, and cause problems for the global population. Established diseases cause a predicted number of deaths each year and a lot of research goes into determining how to prevent or eradicate these diseases. Many of these diseases are common respiratory and diarrheal diseases caused by bacteria or viruses (Fauci and Morens 2012).

Newly Emerging diseases lead to a different set of challenges for researchers because they have yet to be discovered. Considerations such as determining where to find new diseases, and possible ways to combat these pathogens when little is known about them come into play with these newly emerging diseases (Fauci and Morens 2012). One

such newly emerging disease is Nipah, which is carried by pigs (Morens et al. 2004). Due to overcrowding of pigs, Nipah infected 257 handlers in Southeast Asia in 1998-99 (Morens et al. 2004).

Similar to newly emerging diseases, remerging diseases are investigated for their causes of emergence, and if new treatment or prevention methods are needed to stop the spread of the pathogen. Tuberculosis is one of the biggest threats to re-emerge in the past decade because of the increase in immunocompromised individuals due to HIV/AIDS (Morens et al. 2004). As the immune system is weakened, latent tuberculosis infections in the patients lead to the reemergence of the disease (Morens et al. 2004). Overall, the three broad categories have different needs in terms of research and thus make it unlikely that a "silver bullet" to cure all diseases will ever be found.

There are a number of factors which affect infectious diseases, and the three categories to different degrees. A few of these factors include climate change, pathogen adaptations, and host susceptibility (Morens et al. 2008). While some of the factors are manageable by human behavior, others are well outside our control, including microbial change (Morens et al. 2008). The inability to control all factors that affect diseases is a large part of why infectious diseases persist throughout time and cause the numbers of deaths they do worldwide.

Cholera Disease Worldwide

Cholera is a particularly dangerous disease that has caused many deaths throughout history. Cholera dates way back into the 5th century in India (Harris et al.

2012). One of the most well reported cases of cholera is the 1832 outbreak in Paris, which is said to have led to modern epidemiology (Morens et al. 2008; Harris et al. 2012). There are between 3-5 million cases of cholera yearly, which lead to about 100,000 deaths each year (Figure 1; WHO 2012). Cholera is defined as a reemerging disease because outbreaks continue to occur in new areas of the world. Diarrheal diseases, such as cholera, are the second highest global cause of death in children under the age of 5 (Nelson et al. 2009; Prüss-Üstün et al. 2008).

Cholera is a waterborne disease caused by the gram negative, rod-shaped bacterium *Vibrio cholerae.* Since 1961 we have been experiencing the seventh cholera

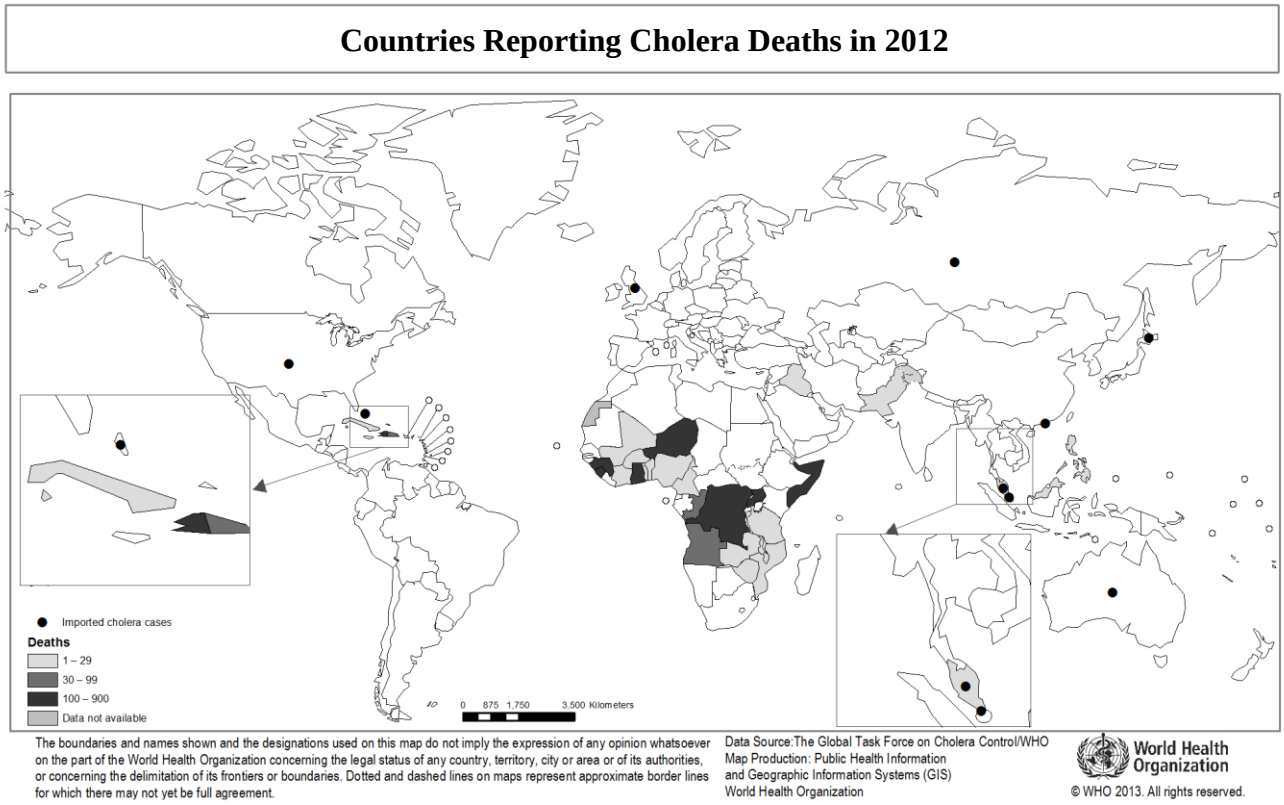

Figure 1. Map of deaths due to cholera worldwide. Deaths due to cholera remain prevalent in areas of Africa, Asia, and the Caribbean well after the natural disasters that initiated the outbreaks in previous years. The pattern of disease shown is this map is consistent with previous and subsequent years. Map is from WHO website (WHO, 2012)

pandemic and, as noted by recent outbreaks it does not appear as if the current pandemic will end soon (WHO 2012; Charles and Ryan 2011; Harris et al. 2012). In 2008-9, Zimbabwe experienced one of the largest outbreaks in recent history leading to more than 100,000 cases and 4,000 deaths (Nelson et al. 2009). Most cases of cholera occur in Asia and Africa but outbreaks after natural disasters such as the earthquake in Haiti in 2010 are well known (Figure 1) (Harris et al. 2012; Nelson et al. 2009; WHO 2012). The outbreak in Haiti has been ongoing since the earthquake and has so far resulted in 8,562 deaths and over 400,000 cases (Archibold and Sengupta 2014). These troubling trends indicate the need for new prevention methods to help stave off both current and future pandemics.

Changing Infectious Diseases

If infectious diseases, like cholera, are not a new concern, why do they continue to be a global health concern? One of the major reasons is that infectious diseases are caused by pathogens which can adapt and evolve (Morens et al. 2004). Infectious pathogens include bacteria, viruses, fungi, and parasites. Viruses and bacteria are the two that cause the highest number of diseases (Morens et al. 2004). Both viruses and bacteria generally take a shorter period of time to replicate and lead to a large number of offspring in that time (Campbell et al. 2008). This means that the process of evolution can occur more rapidly for the pathogen than for the host. More rapid evolution allows for the pathogens to be better equipped to infect individuals. Another important factor in the

evolution of pathogens is that they become resistant to our treatments, causing a constant need for new treatments (Fauci and Morens 2012).

In addition to the rapid evolution of the pathogens, many pathogens infect animals; these diseases are called zoonotic diseases (Campbell et al. 2008). Zoonotic diseases can be transferred from animal to human and sometimes back, which is called a reverse zoonotic disease (Campbell et al. 2008). Some animal carriers include rodents, poultry, and other livestock. These animals can be reservoirs for pathogens and can pass the disease directly to a human host (Campbell et al. 2008). Insects such as mosquitos and ticks can also carry the pathogen and transfer the pathogen from one human to another. Diseases such as malaria, Lyme, and dengue are transmitted by arthropods (Morens et al. 2004; Fauci and Morens 2012). Often, the pathogen does not cause disease in the animal host, making identification of infected animals difficult. Since it is hard to identify the infected animals, it becomes hard to completely eliminate the disease.

One reason for the continually high threat of cholera is the adaptations that *V. cholerae* makes to better infect the host. The current cholera epidemic is caused by the *V. cholerae* strain classified as O1 El Tor, which has replaced the "classical" strain in the last 20 years (Charles and Ryan 2011; Nelson et al. 2009). The main difference between El Tor and the classical strain is that El Tor may be better adapted to live in aquatic environments, which means that it could lead to higher amounts of infection, especially inapparent infections (Charles and Ryan 2011). Inapparent infections are when someone is infected with the pathogen but do not show symptoms of the disease, which can lead to the unintentional infection of other individuals (Campbell et al. 2008). In the past decade,

variations of the El Tor strain have been identified and noted to express the cholera toxin usually associated with the classical strain and at higher rate than is usual from typical El Tor strains (Charles and Ryan 2011). The increased levels of cholera toxin can lead to more severe disease, continuing the threat of cholera.

V. cholerae is also becoming increasingly more antibiotic resistant. Serotype O139, which was responsible for the outbreaks in the 1990's, as well as some O1 El Tor isolates, have gained an SXT element which provides resistance to sulfamethoxazole-trimethoprim and streptomycin (Charles and Ryan 2011; Nelson et al. 2009; Harris et al. 2012). More recent O1 El Tor *V. cholerae* isolates have shown tetracycline, erythromycin, and ciprofloxacin resistance (Charles and Ryan 2011; Nelson et al. 2009; Harris et al. 2012). As *V. cholerae* becomes increasingly more dangerous and our treatments become less efficient, the threat of cholerae increases dramatically.

The infection cycle of cholera also promotes the evolution of *V. cholerae*, *V. cholerae* enters the stomach and the majority of the ingested bacteria are killed by the gastric acid (Harris et al. 2012). The bacteria that survive the harsh conditions are the ones that are then able to reach the small intestine, where they settle and begin colonization. This process favors bacteria that are better adapted to human colonization survive and thus can proliferate and be transmitted to further hosts. This cycle leads to more infectious bacteria and thus leads to more dangerous pathogens faster. The adaptations of *V. cholerae* have made the pathogen and thus the disease an increasing global health threat.

Additionally, waterborne diseases, like cholera, can persist in water reservoirs and lead to future disease. Cholera can persist in fresh water supplies undetected (WHO 2012). Connected to the fact that *V. cholerae* persists in water is that it can also contaminate food (WHO 2012). With no good way to detect the pathogen's presence in either food or water, if it goes undetected, it has the potential to lead to disease in many individuals. All in all, infectious diseases continue to pose a threat to human health because they either avoid or adapt to our defenses against them.

Molecular Basis of Cholera Disease

One of the biggest factors that makes cholera so dangerous is the short infection period and ability to cause death quickly. Upon ingestion of contaminated food or water, *V. cholerae* colonizes in the small intestine, and incubates for 12 to 72 hours before leading to symptoms (Nelson et al. 2009). Depending on the severity of the infection, the host sheds the bacteria anywhere from 1- 14 days after incubation (WHO 2012; Nelson et al. 2009).

Shedding is the process where bacteria are expelled from the small intestine due to the release of cholera toxin from the *V. cholerae* (Harris et al. 2012). Cholera toxin (CT) is a protein exotoxin released by *V. cholerae* and is the main cause of the symptoms of cholera (Nelson et al. 2009; Harris et al. 2012). CT is comprised of a single A subunit and five B subunits which form a ring (Nelson et al. 2009; Harris et al. 2012). The B subunit rings binds the small intestine cell and the A subunit, which is an ADP-ribosyltransferase, enters the cell where it causes the constant activation of a G protein,

which leads to constantly activating adenylyl cyclase (Figure 2) (Bharati and Ganguly 2011). This activation increases cAMP levels, which leads to a signal transduction pathway that causes the secretion of chloride from the cells, leading to the watery diarrhea symptoms seen with the cholera disease (Harris et al. 2012). This also leads to the shedding of bacteria, which can then contaminate more water supplies and potentially infect new individuals. The loose watery diarrhea that occurs is often referred to "rice water stool" and releases anywhere from 10^{10} to 10^{12} vibrios per liter (Nelson et al. 2009).

The short incubation and time of infection, as well as the high possibility of causing additional infections due to the high number of bacteria released, especially from

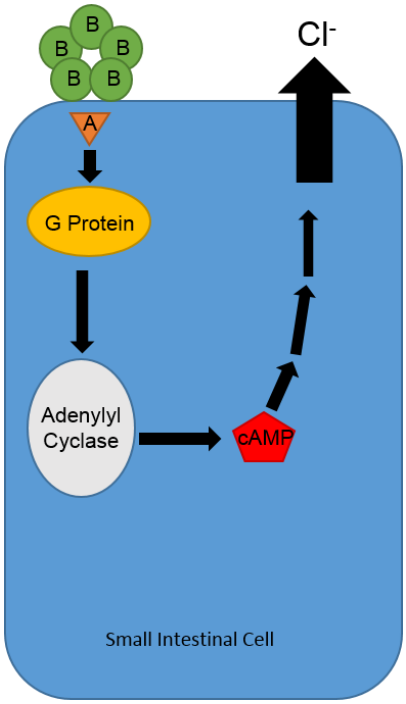

Figure 2. Molecular basis of cholera disease. The B subunits (B) of cholera toxin form a ring and bind to the small intestinal cell allowing subunit A (A) to enter the cell to activate a G protein which in turn activates adenylyl cyclase. An increase of cAMP then causes a signal transduction leading to the release of chloride ions. The release of chloride leads to the "rice water stool" symptom seen in patients.

severe patients, shows the necessity of early action to help treat cholera. Differences amongst individuals can also determine the severity of the infection. Individuals with type O blood tend to have more severe cholera symptoms (Harris et al. 2012; Nelson et al. 2009). Additionally, vitamin A (retinol) deficiency also seems to be paired with more severe symptoms (Harris et al. 2012, Nelson et al. 2009). These not entirely understood links between cholera and severity of symptoms indicates the need for a better understanding of the disease and pathogen.

Challenges of Preventing and Treating Cholera

The treatment of cholera is fairly simple, but rapid administration is required to ensure survival. Infected individuals can die in a few hours due to severe dehydration caused by the watery diarrhea; thus, oral rehydration and salts are required (WHO 2012). If done in a timely fashion, 80% of patients recover with less than 1% case fatality (WHO 2012). The use of intravenous fluids and antibiotics are only recommended in severe cases as the use of antibiotics can shorten the amount of shedding, but can lead to increased antibiotic resistance (WHO 2012). Although antibiotics are not widely recommended, *V. cholerae* has begun to show increased resistance to antimicrobial agents which could lead to more issues when trying to cure the more severe cases (Charles and Ryan 2011). While the treatment of cholera is fairly straightforward, it should not be assumed that it is always easy. Most patients get cholera from contaminated water, and it can be hard to find uncontaminated water required for proper treatment (Harris et al. 2012).

When thinking about infectious diseases, one must consider both treatment options as well as preventive measures such as vaccines. There is currently an oral cholera vaccine which has approximately 85% efficiency after 6 months, and has about 60-85% efficiency for about 2 years (Nelson et al. 2009; Harris et al. 2012). Although the vaccine provides fairly high protection, there are a several disadvantages. The first major issue is that it requires two dosages which must be seven days to six weeks apart (WHO 2012). Due to short course of infection, the vaccine cannot easily be given at the start of an outbreak to help mitigate the outbreak. The vaccine is useful, however, where cholera is endemic and thus could be administered to prevent a widespread outbreak. Recently, it was determined that the vaccine may be cost effective as is, but the question of who will pay for the production and distribution of the vaccines remains (Harris et al. 2012). New vaccines are being developed and administered to increase the vaccine efficiency as well as the cost efficiency to help better manage cholera outbreaks worldwide (Nelson et al. 2009; Harris et al. 2012). While the current vaccine provides good protection for a few years, it is currently a short term prevention method. In order to eliminate cholera, more effective vaccines, in terms of both cost and amount of protection, are needed.

Molecular Biology Primer

One way to understand how *V. cholerae* is adapting, surviving, and changing is to look into the molecular biology of the bacterium. In order to give context to the remainder of the thesis, it is worthwhile to review some molecular biology terminology and conventions that will be used throughout the thesis. The central dogma of molecular

biology is that DNA is transcribed into RNA which is translated into protein (Figure 3) (Campbell et al. 2008). Deoxyribonucleic acid (DNA) encodes for all the information of life in sequences referred to as genes. These genes are then transcribed into ribonucleic acid (RNA). In bacteria, genes can be found in operons where the genes are transcribed together in one long RNA transcript. RNA transcripts, or messenger RNAs (mRNA), can then be translated into proteins by ribosomes. Ribosomes direct translation by binding the ribosomal binding site and initiating translation at the start signal and terminating at the stop signal. Proteins serve a variety of functions both inside and outside the cell. DNA and RNA are both directional molecules with what are known as the 5' and 3' ends based on the free carbon of the sugar backbone of the nucleotide, which are the building blocks of both DNA and RNA. Genes encode information with a four base system (A,C,T,G in DNA, and A,C,U,G in RNA) with two sets of complimentary bases (A with T or U, and G with C) which allow for the gene to be transcribed and translated. There are a number of factors that affect both transcription and translation which are called regulators.

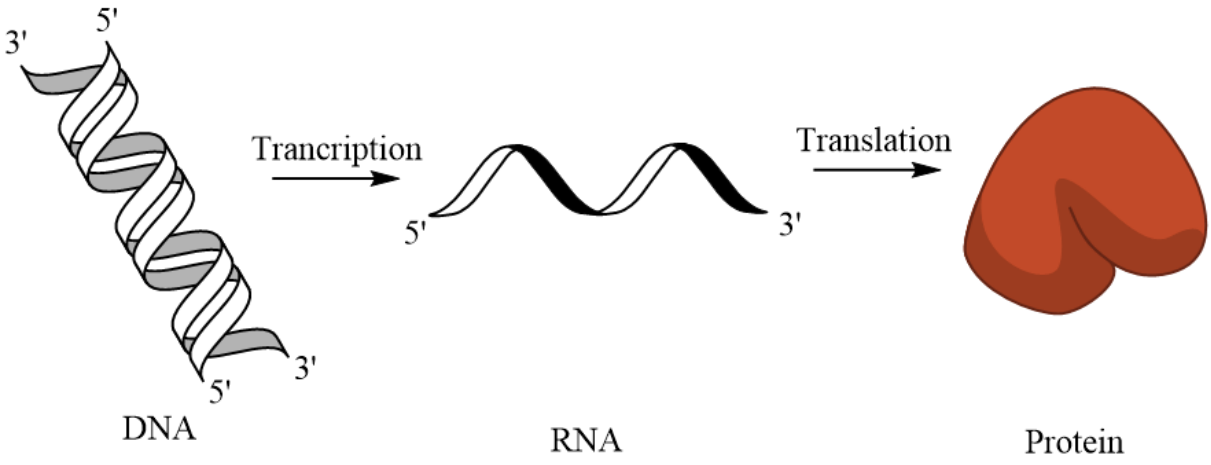

Figure 3. Central Dogma of Molecular Biology. DNA is transcribed into RNA which is translated into proteins. Regulation of gene expression can occur pre-transcription, pre-translation, or post-translation.

Regulators can either inhibit or activate the expression of a gene through a variety of mechanisms, some of which will be explored further in this thesis.

Rapid Adaptation through Small RNAs

Variable regulation of genes is crucial for pathogens to adapt to their environments, which is why there are so many mechanisms of regulation. In order to survive, particular genes have to be switched on to produce proteins that allow for a variety of functions. These same genes then have to be shut off in other environments so that the pathogen does not waste resources. One possible mechanism that the pathogen can utilize to quickly adapt to the new conditions, including changes in temperature and salinity, as is the case with *V. cholerae*, is through the use of small RNA mediated systems. Small RNAs (sRNAs) are short, 50-400 nucleotides in length, pieces of RNA that are transcribed but generally not translated (Fröhlich and Vogel 2009). sRNA systems can work in a variety of ways to help regulate a number of pathways ranging from metabolic processes to virulence factors, as has been seen in *V. cholerae*, and have shown both inhibitory and promoting effects (Fröhlich and Vogel 2009; Waters and Storz 2009). Since sRNAs are transcribed but do not need to be translated, it is possible for sRNAs to more rapidly affect the target system in the pathogen, than their regulatory protein counterparts. This rapid regulation helps with efficient adaptations to the host environment (Water and Storz 2009).

Most sRNA systems operate through base pairing between the target RNA and the sRNA (Waters and Storz 2009). Base pairing of the sRNA can be either *cis*-encoded,

meaning the sRNA gene is next to the target gene, including sometimes overlapping on the opposite strand, or *trans*-encoded, meaning the sRNA gene is transcribed from another location distant from the target gene (Fröhlich and Vogel 2009; Waters and Storz 2009). Similar types of regulation can occur in both *cis*-encoded systems and *trans*-encoded systems but may work slightly differently. Typically, *trans*-encoded regulation requires the chaperone protein Hfq, which both stabilizes and transports the sRNA to its target. *Trans*-encoded sRNAs typically pair with multiple mRNA targets (Prévost et al. 2007). This is most likely due to the fact that *trans*-encoded sRNAs make multiple discontinuous pairings with their target rather than a longer continuous stretch of pairing often seen in *cis*-encoded sRNAs (Waters and Storz 2009). Small RNA regulatory systems coupled with regulatory protein systems allow for precise regulation of gene expression in *V. cholerae*. Multiple regulation pathways allow for efficient adaptations to changing environments.

Mechanisms of Inhibitory Regulation via sRNAs

Although most sRNA regulatory systems involve base pairing, there are a number of ways that the regulation can occur from base pairing. The main method of inhibitory regulation is for the sRNA to bind the 5' untranslated region (UTR) of the mRNA and block the ribosome binding site, thereby stopping the translation of the mRNA (Figure 4A) (Waters and Storz, 2009). Moreover, the act of base pairing at the 5' UTR can sometimes lead to destabilization of the mRNA and lead to its destruction by ribonucleases like RNAse E, further reducing the expression of the gene (Figure 4A)

(Water and Storz 2009). This type of regulation has been seen in *Escherichia coli* with the sRNA RhyB, which is expressed when iron levels are low in order to limit the expression of iron storage protein (Massé et al. 2003). RhyB binds one of a number of target mRNAs, which all encode for iron storage proteins (Massé et al. 2003). The formation of the duplex then recruits RNAse E to degrade both the target and the sRNA (Massé et al. 2003). The total degradation of the complex seems counterintuitive because it also eliminates the sRNA so that it can no longer perform regulatory actions. It is thought that the total degradation might occur to ensure that the regulation cannot be reversed (Morita et al. 2006).

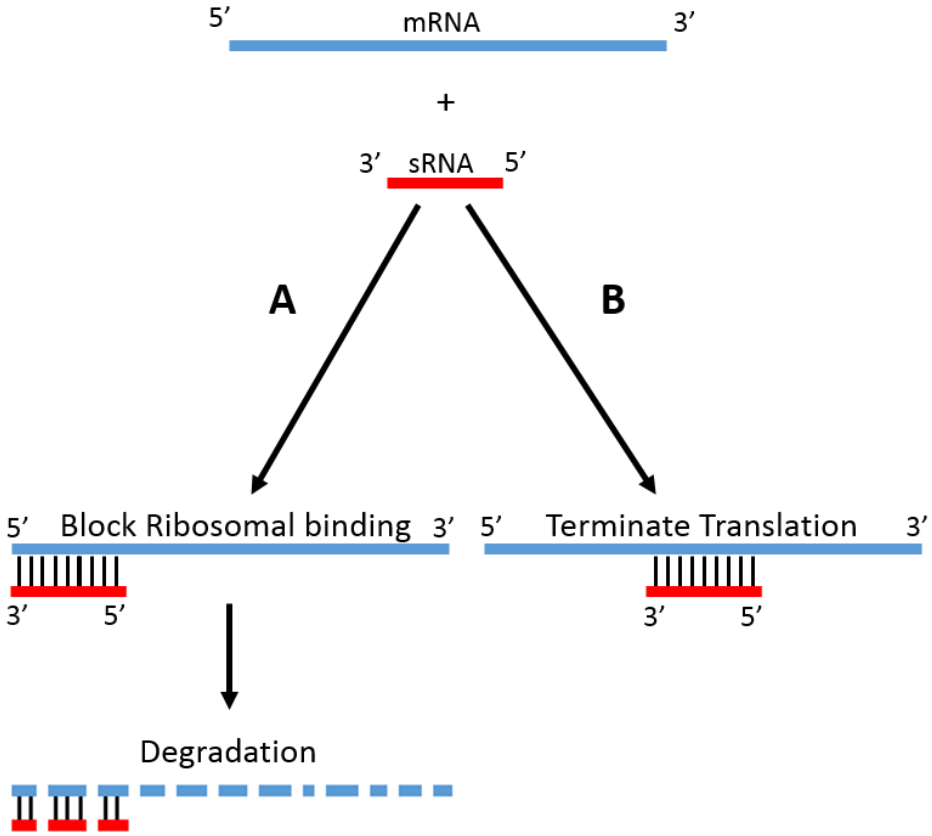

Figure 4. Inhibitory Effects of sRNAs. (A) sRNAs can bind at the 5' untranslated region blocking the ribosomal binding site which often lead to degradation of the complex. (B) Alternatively the sRNA can bind within the mRNA transcript terminating translation.

Another way that an sRNA can lead to inhibitory effects is it can bind in the middle of transcript which terminates translation early, leading to the down regulation of the gene expression (Figure 4B) (Waters and Storz, 2009). In cyanobacterium *Synechocystis*, the sRNA IsrR is translated antisense to *isiA* (Dühring et al. 2006). When iron is present, IsrR is transcribed in order to down regulate *isiA*, IsrR binds to the mRNA, terminating translation, and additionally leading to the degradation of the duplex (Dühring et al. 2006).

Mechanisms of Activating Effects of sRNAs

While in certain systems sRNAs play an inhibitory role, there are also systems where sRNAs have an activating effect. There are three main mechanisms by which an sRNA can activate a system, some of which are similar to the inhibitory mechanisms. If an sRNA is a *cis*-encoded antisense between two genes of an operon, this can lead to binding of the sRNA within the transcript (Waters and Storz, 2009). This binding leads to the cleavage of the complex, and increases the levels of transcripts of genes on either side of the cleavage (Figure 5A) (Waters and Storz 2009). This process occurs in *E. coli* with the *gadXW* mRNA and the GadY sRNA (Opdyke et al. 2004). GadY sRNA binds the transcript at the 3’ end of the *gadX* gene leading to cleavage of the duplex, releasing separate *gadX* and *gadW* transcripts (Opdyke et al. 2004). In addition, GadY also stabilizes the newly formed *gadX* mRNA resulting overall higher levels of the transcript (Opdyke et al. 2004).

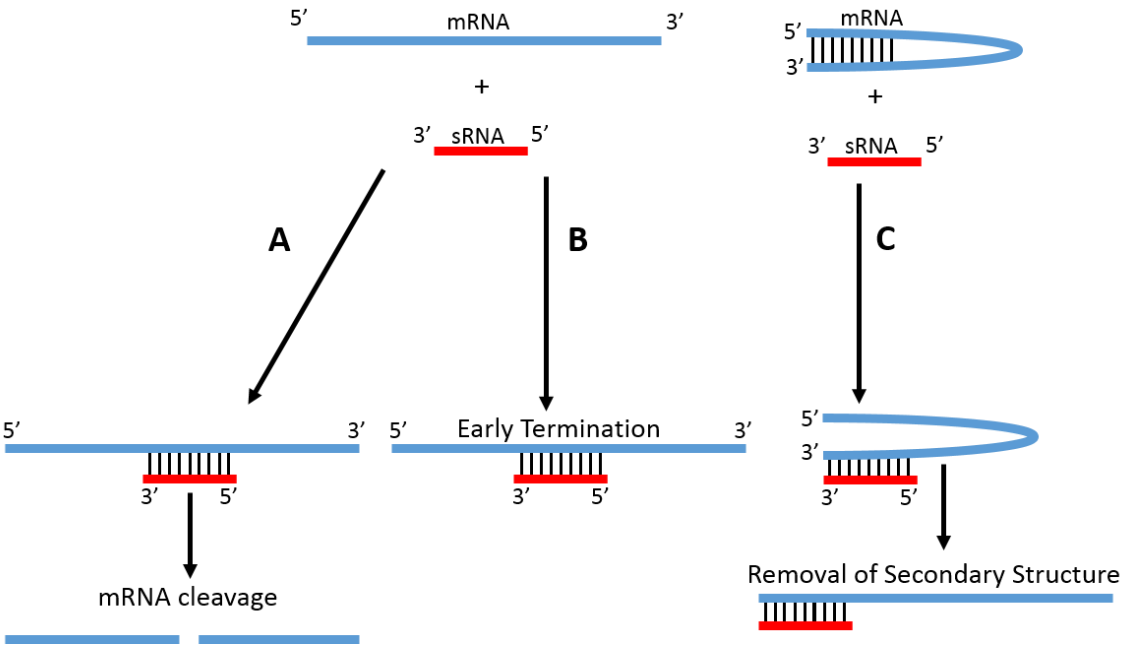

Figure 5. Activating effects of sRNAs. (A) sRNAs can bind within the mRNA transcript of an operon and cause the cleavage of the mRNA leading to the up regulation of the genes on either side of the cleavage. (B) Similarly sRNA can bind in the middle of the transcript and lead to early termination and an increase of the protein before the bound sRNA. (C) Alternatively the sRNA can bind the mRNA and remove the internal secondary structure of the mRNA.

An sRNA can also bind to an operon transcript and then terminate translation leading to the up-regulation of another part of the transcript (Figure 5B) (Waters and Storz 2009). This mechanism has been seen in *Vibrio anguillarum* with the *fatDCBAangRT* transcript and the sRNA RNAβ (Stork et al. 2007). The sRNA RNAβ binds antisense between *fatA* and *angR* resulting in the increased expression of *fatDCBA* mRNA since translation is terminated early meaning an increased rate of translation (Stork et al. 2007).

A different way that an sRNA can activate the expression of an mRNA is by binding to the mRNA so that the internal secondary structure of the mRNA is no longer present; thus, revealing the ribosomal binding site allowing for the translation of the

mRNA (Figure 5C) (Waters and Storz 2009). An example of this mechanism is found in *Staphylococcus aureus* where the sRNA RNAIII binds to the *hla* mRNA that encodes for the exoproteins that cause disease (Morfeldt et al. 1995). RNAIII is transcribed when the culture reaches the late exponential phase of growth and exoproteins are most highly expressed (Morfeldt et al. 1995). RNAIII and the *hla* mRNA form a duplex that competes with the internal secondary structure of the mRNA (Morfeldt et al. 1995). The binding event releases the Shine-Delgarno sequence allowing the ribosome to bind and expression of the gene to occur so that exoproteins are created (Morfeldt et al. 1995).

These studies show that sRNAs can bind, and sometimes destabilize mRNAs, and make the duplex a target for degradation. Other times, sRNAs can stabilize and activate expression of specific genes. This duality shows the versatility and specificity of sRNA systems.

The Role of the Chaperone Protein Hfq

One of the key factors to many sRNA systems, especially the *trans*-encoded ones, is the chaperone protein Hfq. Hfq is a ring shaped hexamer that can interact with U-rich portions of sRNAs (Valentin-Hansen et al. 2004). In certain cases, Hfq serves to protect the sRNA from RNAse E (Valentin-Hansen et al. 2004; Massé et al. 2003). The importance of Hfq can been seen when looking at *E. coli* where all *trans*-encoded sRNA systems require the help of the RNA chaperone (Waters and Storz 2009).

Additionally, Hfq may facilitate interactions of RNA-RNA binding, making it required for proper function of the sRNA (Valentin-Hansen et al. 2004). This has been

seen with OxyS, which represses *fhlA* (Zhang et al. 2002). In response to oxidative stress in *E. coli*, OxyS is transcribed to repress the expression of *fhlA* which encodes for a transcriptional activator (Zhang et al. 2002). Without Hfq, the sRNA cannot properly interact with the mRNA and repression does not occur (Zhang et al. 2002). It was noted that Hfq does not affect stability but instead is required for ternary structure formation (Zhang et al. 2002). Hfq is sometimes required to help with base pairing which correlates with the fact that *trans*-encoded systems utilizing multiple non-perfect binding areas to regulate expression.

While Hfq is important to many systems, it may not be required for all, as seen by a number of examples including the sRNA VrrA in *V. cholerae,* which does not require Hfq to serve its function in repression of *ompA* translation (Song et al. 2008). OmpA is a required for virulence of the bacteria and VrrA regulates its expression in order to regulate virulence (Song et al. 2008). However, due to Hfq's importance in many systems, it should be considered when investigating all sRNA systems.

Pathways Regulated by sRNAs

Studies in recent years suggest that nearly every aspect of cellular function is somehow regulated by sRNA systems. One important aspect that must be regulated when looking at infectious pathogens are virulence factors. In *V. cholerae,* the HapR protein, which regulates downstream factors affecting virulence and biofilm formation, is regulated by the Qrr1-4 sRNAs (Matson et al. 2007). The Qrr1-4 sRNAs are expressed when the colony senses that cell density is low via quorum sensing, which is how bacteria

determine the cell density (Matson et al. 2007). With the help of Hfq, the sRNAs efficiently reduce the amount of *hapR* mRNA by binding to the transcript and destabilizing it, leading to its degradation (Matson et al. 2007). The repression of *hapR* leads to decreased expression of the genes that cause virulence and biofilm formation (Matson et al. 2007). This regulation makes sense because at low cell density there are not enough bacteria to cause a response from the release of virulence factors or to form a biofilm. Regulating these two systems makes it possible for the pathogen to not waste resources. This system also shows the importance of Hfq and the usefulness of sRNAs to regulate systems quickly and efficiently.

If the pathways of virulence factors are better understood, then it becomes easier to fight the pathogen. In *Pseudomonas aeruginosa* PAO1, the sRNA NrsZ regulates virulence (Wenner et al. 2013). NrsZ activates the expression of *rhlA* which produces rhamnolipids (Wenner et al. 2013). Rhamnolipids are virulence factors that allow for swarming motility in the pathogen (Wenner et al. 2013). Swarming motility is a social behavior of the bacteria that is thought to be a virulence trait (Wenner et al. 2013). By understanding how the bacteria cause disease we can target those factors to stop disease.

Virulence factors are not the only important functions that pathogenic bacteria need to regulate. Some of the most important cellular functions are those involved in metabolism, especially the utilization of carbon. In *E. coli*, there are a number sRNAs that all operate on the galactose operon. One of these sRNAs is Spot 42, which was one of the first discovered sRNAs (Ikemura and Dahlberg, 1973). The transcription of s*pf* gene, that encodes Spot42, is activated by the uptake of glucose (Görke and Vogel,

2008). The sRNA regulates *galK*, which is involved in the metabolism of galactose (Görke and Vogel, 2008). Spot42, in conjunction with Hfq, binds to the *galETKM* transcript to repress the expression of *galK* by blocking translation of the *galK* mRNA (Görke and Vogel, 2008). This makes sense because when glucose is present, *galK* is not needed.

Perhaps the most well studied and important carbon source to most cells is glucose. In *E. coli*, the uptake of glucose is in part regulated by sRNAs. The glucose transporter IICB is regulated by the sRNA SgrS (Negrete et al. 2010). The Hfq-dependent sRNA SgrS is expressed when the cell is in "phosphosugar stress" and binds *ptsG* mRNA, the gene that encodes for the IICB glucose transporter, leading to the degradation of the RNA duplex (Görke and Vogel 2008). "Phosphosugar stress" is due to an accumulation of glucose-6-phosphate, an intermediate of glycolysis (Görke and Vogel 2008). The action of SgrS thus helps alleviate the stress by decreasing the expression of the glucose transporter and thus decreasing the amount of glucose entering the cell.

In different strains of *E. coli*, it was seen that SgrS expression was different in the same conditions (Negrete et al. 2010). In *E. coli* strain K-12, at high glucose concentrations, there is decreased expression of *sgrS*, which leads to higher levels of glucose in the cell (Negrete et al. 2010). Under the same growth conditions, BL-21 has increased *sgrS* expression leading to lower levels of glucose (Negrete et al. 2010). This differential expression in different strains is an interesting phenomenon and shows the versatility and complexity of sRNA regulated systems in bacteria. This high level of

regulation suggests the importance of regulating carbon intake and utilization systems and highlights how bacterial systems apply sRNAs in many ways.

Mannitol and the Mannitol Operon

One of the many carbon source uptake operons that is shared by a number species is the mannitol operon. Mannitol is a six carbon sugar alcohol that can accumulate in the human small intestine after consuming mannitol-containing fruits and vegetables (Wang and van Eys 1981). Mannitol is also the main product of photosynthesis by brown algae and accumulates in the waters around brown algae (Ymele-Leki et al. 2013). Many species, including *E. coli, V. cholerae,* and *Bacillus subtilis* have the operon expressing mannitol metabolism genes. The conservation of this operon across species indicates its probable importance to survival. Mannitol can be utilized as a carbon source by bacteria, however, it is not a preferred carbon source (Behrens et al. 2001). Mannitol may act as a compatible solute to help with osmoregulation (Kets et al. 1996). Compatible solutes are important to counteract the effects of osmotic stress by restoring turgor pressure, protecting enzymes, and stabilizing membranes (Kets et al. 1996).

The mannitol operon encodes for an EII protein allowing for the uptake of mannitol (Postma et al. 1993). The mannitol class EII protein is part of the phosphoenolpyruvate-dependent phosphotransferase systems (PTS), and was the first of its class to be sequenced (Postma et al. 1993; Honeyman and Curtiss 2000; Figge et al. 1994). There are three major components of the PTS, two of which are shared across systems, EI an HPr, and the other, EII, is carbohydrate specific (Postma et al. 1993;

Honeyman and Curtiss 2000). EII is the transporter protein and can be broken into three sections EIIA, EIIB, and EIIC (Postma et al. 1993; Honeyman and Curtiss 2000). These three pieces can either be separate proteins or parts of the same protein (Postma et al. 1993; Honeyman and Curtiss 2000). Typically, the mannitol operon consists of three genes which encode for three separate proteins: MtlA, MtlD, and MtlR. MtlA is the EIIABC protein (Figge et al. 1994). MtlD is a dehydrogenase that converts mannitol-1-phosphate to fructose-6-phosphate (Rambhatla et al. 2011). Finally, MtlR is a putative regulator protein of the mannitol operon and has been shown to have varying effects in different species (Honeyman and Curtiss 2000; Figge et al. 1994; Joyet et al. 2013; Tan et al. 2009). Overall, the mannitol operon is a complex operon that has been studied in many species, but remains to be fully characterized and studied in *V. cholerae*.

MtlR in Different Species

While the mannitol operon is highly conserved across species, it is still interesting to study the differences between the operons, especially the differences in the function of MtlR. Part of the differences arise due to the difference between gram-negative and gram-positive strains. In *E. coli*, a classic gram-negative species, the operon is arranged *mtlADR,* which is the same for *V. cholerae*, where *mtlA* encodes for the EIICBA protein in one large polypeptide (Postma et al. 1993; Figge et al. 1994). When it was first described in *E. coli*, MtlR was determined to be a transcriptional repressor, thus it was named the mannitol repressor protein, MtlR (Figge et al. 1994). It was later determined that MtlR in *Vibrio parahaemolyticus* may belong to a new class of transcriptional

regulators, due to the fact that the structure of MtlR revealed a number of negative residues that do not facilitate DNA binding (Tan et al. 2009). It is most likely that MtlR must work in conjunction with another protein in order to regulate transcription (Tan et al. 2009).

When MtlR has been studied in gram-positive species such as *B. subtilis,* it was seen to be a transcriptional activator (Joyet et al. 2013). The difference in the way MtlR regulates the system in *B. subtilis* is possibly due to the fact that the biology of gram-positive and gram-negative bacteria varies greatly, and thus the regulation of the pathways varies. Interestingly, MtlA and MtlD of *B. subtilis* have high homology with the *E. coli* orthologs for about the first 400 amino acids despite the difference in MtlR function (Akagawa et al. 1995).

In the gram-positive species *Streptococcus mutans,* the function of MtlR is currently unknown, but it was noted that MtlR is not needed for mannitol utilization or expression of the operon (Honeyman and Curtiss 2000). Another gram-positive bacteria, *Clostridium acetobutylicum,* also contains the mannitol operon, arranged *mtlARDF,* where *mtlF* encodes for the mannitol EIIA (Behrens et al. 2001). In *C. acetobutylicum* scientists noted that MtlR did not contain the standard helix-turn-helix motif associated with DNA binding and thus may act in a different manner (Behrens et al. 2001). Although MtlR has different functions in different species, the conservation of the regulatory protein in addition to the conservation of the entire operon indicates the importance of these genes and the regulation of the mannitol operon.

There is little known about the exact way in which MtlR functions in different species. A BLAST search of MtlR from *V. cholerae* does not reveal any known conserved functional domains. A sequence alignment between MtlR from *V. parahaemolyticus* and *V. cholerae* (Figure 6A) shows that the two are 68% identical in

A

```
V. cholerae          1   MSRAVTRSRLNSTTDQFMAEKINESDILERLNQTHTVRGF 40
V. parahaemolyticus  1   -----------------MADNINETEIIERLNSAPSVRGF 23

V. cholerae          41  FITTVDVLTEAIDALMQRIFRKDNFAVKSVVEPLLHDTGP 80
V. parahaemolyticus  24  FIATVDVFNESIDGLIQRIFRKDNFAVQSVVGPLLQDSGP 63

V. cholerae          81  LGDLTVRLKLLFGLGVIPDEVFHDIEHLIKLRNQLNHDAT 120
V. parahaemolyticus  64  LGDLSVRLKLLFGLGVLPDDIYHDIEDIIKLKNQLNSDAS 103

V. cholerae          121 EYQFTDPQILAPIKALNLVKKMGMLHLNVVEPDDDIDLSF 160
V. parahaemolyticus  104 DYEFTDPNILEPIKKLHLVKKMGMVQLEVNEPDDDIDLEF 143

V. cholerae          161 YHLQLQRQQQVIKSGLSLAIIQICNALNKDSPF 193
V. parahaemolyticus  144 YQLQLQRQQQIIKSGLSLAIVEICNELGKDSPF 176
```

B

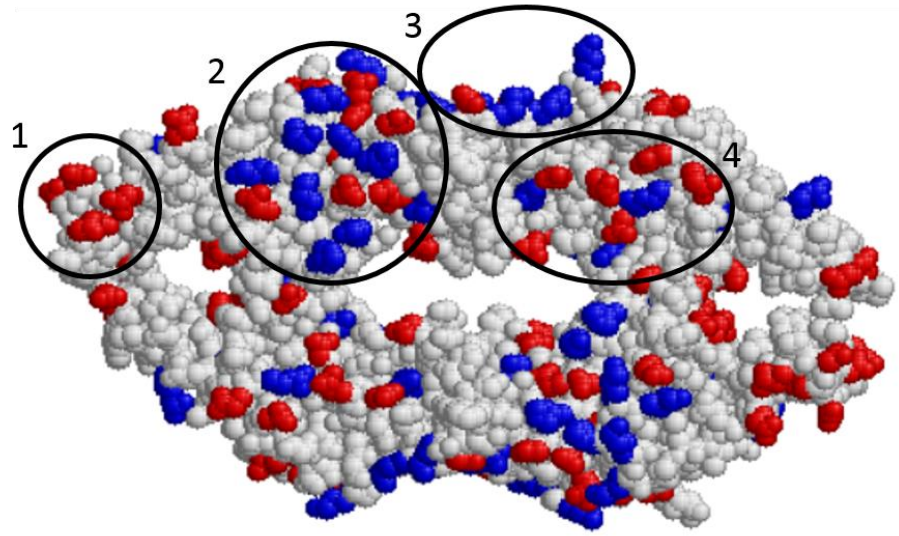

Figure 6. Sequence alignment of MtlR, and crystal structure of MtlR in *V. parahaemolyticus*. (A) Protein sequences of MtlR from *V. cholerae* and *V. parahaemolyticus* were aligned using COBALT (NCBI). Underlined sequences are identical, blue amino acids are basic, and red are acidic. (B) MtlR in *V. parahaemolyticus* is made up of 4 chains which forms a dimer (Tan et al. 2009). Areas 1 and 4 are highly negatively charged areas where RNA is unlikely to bind while area 3 is a highly positively charged area where RNA is more likely to bind. In area 2, there is still a fair amount of positive charge, but also a good amount of negative charge which makes this area uncertain about the binding properties, but it is possible that nucleic acids could bind there. Structure was analyzed using Rasmol.

protein sequence. When looking at the crystal structure of MtlR in *V. parahaemolyticus*, there are a few areas with a high density of positive change that may bind DNA, but there are also areas of negative charge that would not interact well with a nucleic acid backbone (Figure 6B). This ambiguity does not gives little insight into how MtlR functions. Overall, little is known about the biochemical properties of MtlR, thus studies of both its molecular and biochemical functions are required.

Mannitol Operon in V. cholerae

It is highly possible that mannitol is being used by *V. cholerae* that live near brown algae, which produce mannitol, as an osmoregulator giving it an advantage in a marine environment (Ymele-Leki et al. 2013). Additionally, mannitol has been seen to activate biofilm formation of *V. cholerae*, which provides additional advantage in marine environments (Ymele-Leki et al. 2013).

V. cholerae contains the *mtlADR* genes, where MtlA is the only mannitol specific transporter protein in the species (Kumar et al. 2011). Overall, however, little is known about the operon (Kumar et al. 2011). The difference between non *Vibrio* species and *V. cholerae* is that the latter has an sRNA, MtlS, which is antisense to the 5' UTR of the *mtlA* gene and has 70 base pairs of perfect complementarity (Liu et al. 2009). Unlike other sRNAs, such as SgrS that regulates carbon uptake in *trans*, MtlS regulates the mannitol transporter protein in *cis* (Liu et al. 2009; Negrete et al. 2010). Previous studies by the Liu Lab have revealed much about the mannitol operon in *V. cholerae*, especially about the relationship between MtlA and MtlS. It has been seen that *mtlA* is only

expressed in the presence of mannitol whereas MtlS is expressed in the presence of all other carbon sources (Mustachio et al. 2012). This inverse relationship suggests an inhibitory effect of MtlS but does not yet provide insight into how the mechanism operates. When the bacteria are switched from media containing mannitol to glucose, the levels of mannitol transporter protein can no longer be detected after 45 minutes and the levels of sRNA reach the amount found in the glucose control at about 60 minutes (Mustachio et al. 2012). These observations reemphasize the inverse relationship between the expression of the sRNA and the MtlA protein, and the inhibitory action of the sRNA. When the system is also tested to see if MtlS could repress in *trans*, and it is observed that MtlS represses the expression of MtlA in *trans* in the same way as *cis*, indicating a direct role of MtlS to repress expression (Mustachio et al. 2012).

Another interesting feature of MtlS is that unlike many sRNAs which destabilize the mRNA, MtlS does not seem to cause the degradation of the *mtlA* mRNA it binds to, but instead seems to only block the ribosomal binding site (Mustachio et al. 2012). In addition to MtlS, the mannitol system in *V. cholerae* is regulated by MtlR; however, its exact effect in *V. cholerae* is unknown as of yet (Mustachio et al. 2012). Currently, it seems that MtlR does not affect the expression of MtlS, but may affect other areas of the system (Mustachio et al. 2012).

Further elucidation of regulation of the mannitol operon in *V. cholerae*, is the subject of this thesis. There were two main goals of this research. The first was to investigate the role of the sRNA MtlS in the mannitol system and the second was to investigate the protein factors involved in the system. These goals led to the following

three questions: What was the role of Hfq in the system; How was MtlR involved in the system; and, finally, How can we further study MtlR in *V. cholerae*? Overall, the mannitol operon is highly conserved across species indicating its importance to the survival of those species.

Results

MtlS Regulates the Expression of mtlA Independent of Hfq

To further understand the mechanism by which MtlS regulates MtlA, the Hfq dependence of MtlS was studied. Since Hfq is essential for the functioning of many sRNA mediated systems, it was possible that MtlS was also Hfq-dependent. We hypothesized that Hfq was not required for regulation of the mannitol operon because the system has been seen to work in *cis*, which generally does not require the help of Hfq. In order to test if Hfq was required for MtlS moderated regulation of *mtlA*, MtlA protein, *mtlA* mRNA, and MtlS sRNA levels were determined in both a wild-type "WT" (JL2) and an *Δhfq* mutant strain (JL54). MtlA levels remained unchanged when the WT and the mutant were grown in glucose or mannitol containing media (Figure 7A). Levels of *mtlA* mRNA also remained consistent in both strains in both growth conditions (Figure 7B). Finally, the levels of MtlS were the same across the conditions tested regardless of the presence or absence Hfq (Figure 7C). These results suggest that MtlS works in an Hfq-independent manner. This result is interesting since so many sRNAs require Hfq; Hfq-independence indicates that MtlS is stable and can easily bind to *mtlA* mRNA.

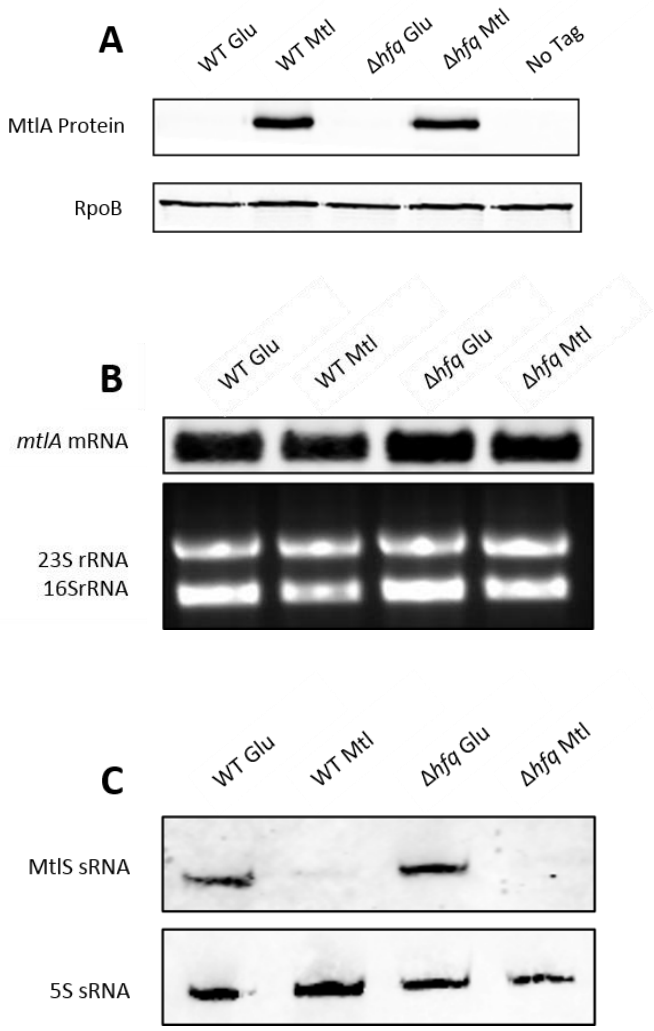

Figure 7. MtlS regulates *mtlA* expression independent of Hfq. Wild-type *V. cholerae* and Δ*hfq* were grown in glucose (Glu) or mannitol (Mtl) containing media until mid-log phase at 37 ºC. (A) Western blotting was done with full cell lysates, and anti-FLAG and anti-RpoB antibodies to detect MtlA and RpoB, respectively. (B,C) 2 μg total RNA was used for northern blotting and probed with *mtlA* mRNA or MtlS sRNA specific riboprobes. The same total RNA samples were used for *mtlA* mRNA and MtlS sRNA blotting. RpoB, 16 and 23 rRNA, and 5S sRNA were used as loading controls for their respective blots. The data presented are representative of at least three separate experiments.

In order to further study any possible effects of Hfq on MtlS, the stability of MtlS was tested in both the wild-type and the Δ*hfq* mutant. Hfq is known to stabilize sRNAs by blocking the action of RNAse E which degrades sRNA-mRNA duplexes (Valentin-Hansen et al. 2004; Massé et al. 2003). MtlS appears to have a half-life of about 20 minutes (Figure 8A), which is standard for a sRNA (Majdalani et al. 2001). In the absence of Hfq, the half-life of MtlS remains at about 20 minutes (Figure 8B). These results suggest that Hfq is not required for the stability of MtlS, which further indicates that MtlS regulates *mtlA* expression independently of Hfq.

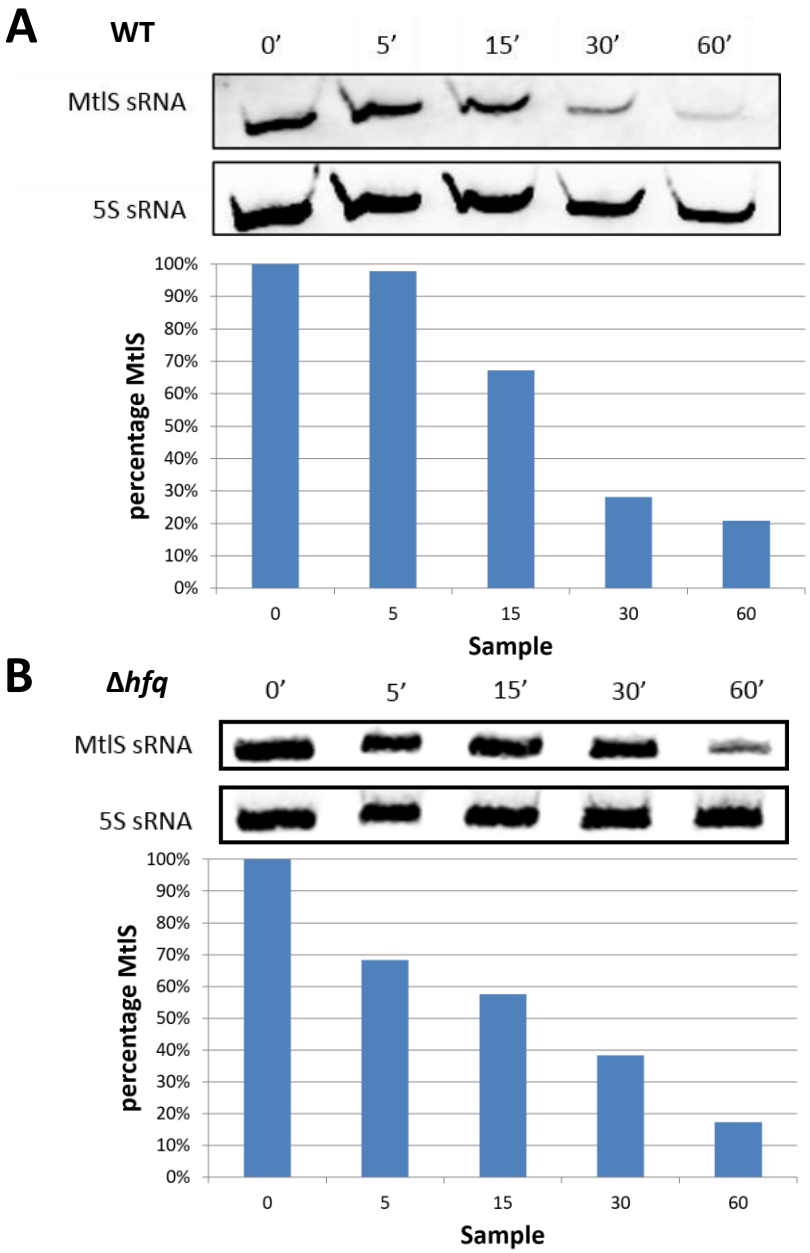

Figure 8. MtlS has a half-life of 20 minutes, independent of Hfq. Wild-type *V. cholerae* and Δ*hfq* were grown in glucose (Glu) containing medium until mid-log phase at 37 ºC. Rifampicin was added (100 µg/mL) and time points over the course of an hour were taken. 2 µg total RNA was used for northern blotting and probed with MtlS specific riboprobe. 5S sRNA was used as loading control. Half-life was determined by comparing the MtlS concentration at that time compared to time 0. The data presented are representative of at least three separate experiments. The faint band in the Mtl lane (B) is the result of spill over from the Glu lane. The data presented are representative of at least two separate experiments

MtlR Represses the Expression of mtlA

Once Hfq independence had been determined, attention was focused on the putative mannitol operon regulatory protein MtlR. Based on the fact that MtlR was a repressor in the gram-negative species *E. coli*, we hypothesized that MtlR in *V. cholerae,* was also a repressor. In order to study the function of MtlR, a wild-type and a Δ*mtlR* mutant strain (JL55) were grown in either glucose or mannitol containing media. Levels of the MtlA protein, *mtlA* mRNA, and MtlS sRNA were all analyzed to determine if there were differences between the WT and the mutant. When MtlR is knocked out, MtlA is synthesized when the cells are grown in glucose medium (Figure 9A). The levels of MtlA protein when the mutant strain is grown in glucose medium are not fully equivalent to when the bacteria were grown in mannitol. We believe the presence of MtlS is contributing to down-regulation of *mtlA* when the cells are grown in glucose. The levels of *mtlA* mRNA and MtlS sRNA do not change in the mutant compared to the wild-type (Figure 9B and 8C). These results suggest that MtlR represses the expression of *mtlA* in conjunction with MtlS. This also indicates a high level of regulation of the mannitol operon, suggesting the importance of the operon.

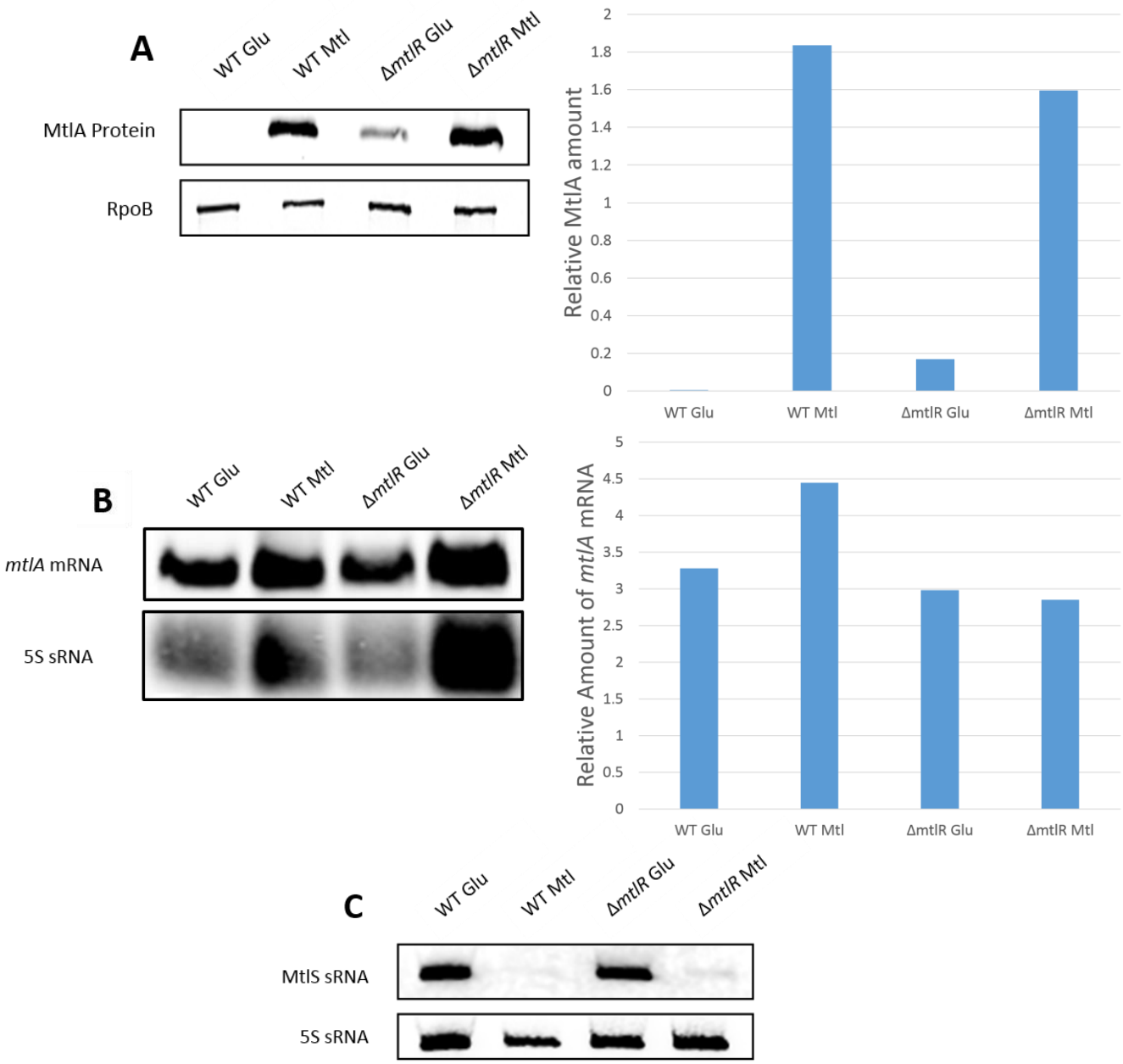

Figure 9. MtlR represses MtlA synthesis. Wild-type *V. cholerae* and Δ*mtlR* were grown in glucose (Glu) or mannitol (Mtl) containing media until mid-log phase at 37ºC. (A) Western blotting was done with full cell lysates, with anti-FLAG and anti-RpoB antibodies to detect MtlA and RpoB respectively. Normalization of protein showed a 1/8 fold increase in Δ*mtlR* Glu compared to Δ*mtlR* Mtl (B,C) 2 µg total RNA was used for northern blotting and probed with MtlS or *mtlA* mRNA specific riboprobes. Normalization of *mtlA* mRNA showed near equal levels in all conditions. The same total RNA samples were used for MtlS sRNA and *mtlA* mRNA blotting. 5S sRNA, 16 and 23 rRNA, and RpoB were used as loading controls for their respective blots. The data presented are representative of at least three separate experiments.

His_6-MtlR is not Detected Using Western Blotting Analysis

In order to further study MtlR, we set out to analyze MtlR levels in bacteria grown in glucose versus mannitol. We also hypothesized that if we could isolate MtlR we could further study its function. The genome of *V. cholerae* was modified to express MtlR with a His_6-tag. An N-terminal His_6 tagged MtlR was constructed as had been done in *V. parahaemolyticus* (Tan et al. 2009). When using western blotting analysis, tagged MtlR could not be observed when the bacteria were grown in either glucose or mannitol media (data not shown). The α-His_6 antibody did detect His_6-tagged GFP, thus the antibody was working properly (data not shown). We speculated that the positively charged histidine groups from the epitope tag might be interacting with the negatively charged amino acids of the MtlR protein, causing it to be unstable and not detectable via western blot. If MtlR was being inactivated, however, it should show a similar pattern of MtlA expression to the Δ*mtlR* strain. When compared side by side, it was seen that this was not the case (Figure 10). Thus, the N-terminal tag does not appear to affect MtlR function with respect to *mtlA* expression.

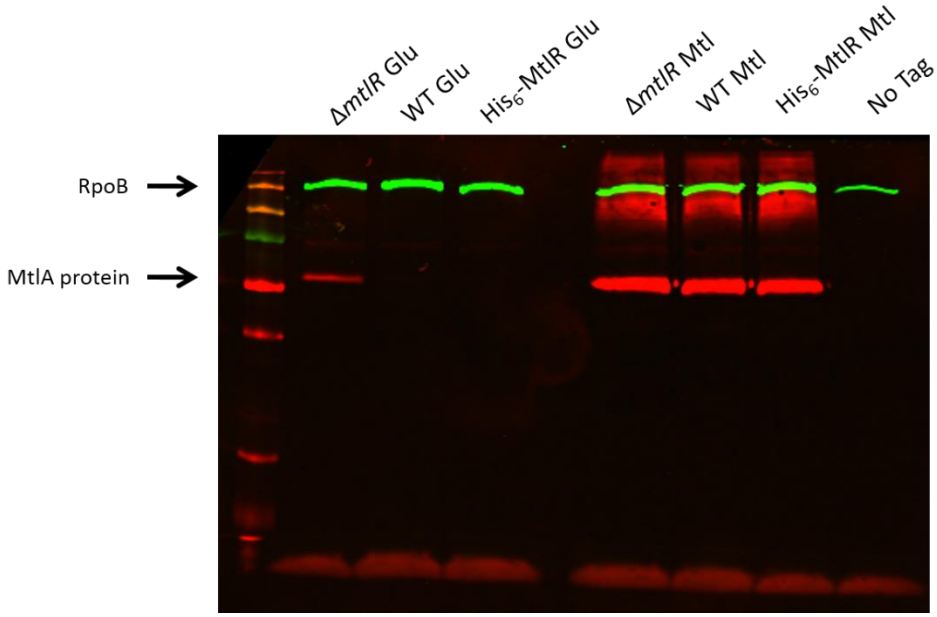

Figure 10. His_6-MtlR MtlA expression does not look like Δ*mtlR.* Wild-type *V. cholerae*, His_6-MtlR, Δ*mtlR*, and Wild-type with no tag on either MtlR or MtlA were grown in glucose (Glu) or mannitol (Mtl) containing media until mid-log phase at 37 ºC. Western blotting was done with full cell lysates and anti-FLAG (to detect MtlA), anti-RpoB, and anti-His_6 antibodies. RpoB was used as a loading control.

Overexpression of MtlR Revealed MtlR in E. coli

Since His_6-MtlR could not be detected via western blotting, we were unsure whether the tagged protein was unstable or lowly expressed. A TOPO cloning system was utilized to highly overexpress MtlR to determine if a His_6-tagged MtlR could be created. Using TOPO cloning, MtlR constructs were labeled with His_6 on either the N-terminus or the C-terminus to observe if there was a distinguishable difference depending on the location of the tag. MtlR was seen when overexpressed in *E. coli* (Figure 11). More specifically, both N-terminally tagged and C-terminally tagged MtlR was seen and at the correct size, while no MtlR was seen in *V. cholerae*. This result means that the His_6 tagged coding sequence can lead to a stable protein, at least in *E. coli.* Also, this result

suggests that MtlR is either lowly expressed or rapidly degraded in *V. cholerae*, thus causing the expression of *mtlR* to not be observed. Interestingly, levels of N-terminally labeled MtlR were lower than C-terminally labeled MtlR, which may have also made it harder to see MtlR with the N-terminal His_6-MtlR in the *V. cholerae.*

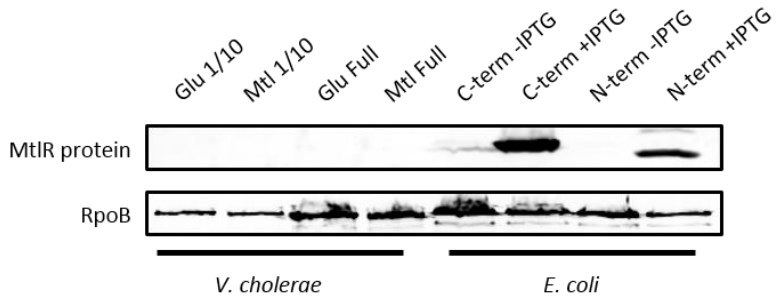

Figure 11. MtlR is seen when overexpressed in *E. coli* and not seen when normally expressed in *V. cholerae*. His_6-MtlR *V. cholerae* was grown in glucose (Glu) or mannitol (Mtl) containing media and BL21 (DE3) *E. coli* containing pET101::C-terminally or N-terminally His_6 labeled MtlR were grown in LB until mid-log phase at 37 ºC. Western blotting was done with 1/10 or full cell lysates, and anti-RpoB and anti-His_6 antibodies. RpoB was used as loading controls. IPTG was added to induce the expression of the pET101 plasmid. The data presented are representative of at least two separate experiments. The faint band in the C-term –IPTG lane is due to spillover.

Additional Epitope Tags

After the His_6-tagged MtlR could not be detected in *V. cholerae* using western blotting, additional epitope tags were used in an attempt to detect MtlR. A StrepII-tag was attached to the C-terminus, but MtlR could not be detected and the use of streptavidin only lead to non-specific binding (data not shown). In a separate experiment, an HA-tag was attached to the C-terminal; however, MtlR could still not be detected (data not shown). It is likely that MtlR synthesis in *V. cholerae* is extremely low and thus using western blotting to detect the presence of MtlR is not a sensitive enough method.

mtlR mRNA not Seen Using Northern Blotting

We also investigated *mtlR* mRNA levels. When using northern blotting, *mtlR* mRNA could not be viewed (Figure 12). It is possible that the mRNA is lowly expressed, thus leading to the low expression of protein. Double the amount of RNA was also tested and the same result was produced (data not shown). *mtlR* mRNA, like MtlR protein, is either expressed at a low level naturally, or it is highly unstable and thus degraded.

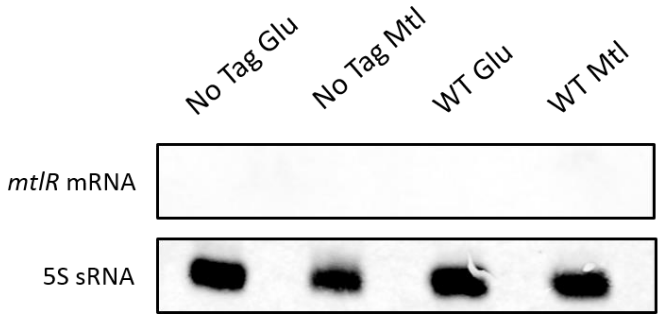

Figure 12. *mtlR* mRNA levels are not visible. Wild-type *V. cholerae* and non-epitope tagged MtlA and MtlR (no tag) were grown in glucose (Glu) or mannitol (Mtl) containing media until mid-log phase at 37 ºC. 2 µg total RNA was used for northern blotting and probed with *mtlR* mRNA specific riboprobe. 5S sRNA were used as loading control. The data presented are representative of at least two separate experiments.

mtlR mRNA is Present and Seen when Using qRT-PCR

Although northern blotting did not detect *mtlR* mRNA, the mRNA must be present in order to produce MtlR. We know the *mtlR* gene product is made because when *mtlR* is knocked out, MtlA expression increased when the cells were grown in glucose medium. Due to the very low levels of *mtlR* mRNA, a more sensitive method was used in order to detect its presence. Using quantitative reverse transcription polymerase chain reaction (qRT-PCR), the presence of *mtlR* mRNA could be quantified (Figure 13). The data suggest that the levels of *mtlR* mRNA are present in samples grown in both glucose

and mannitol containing media, but are too low for detection via northern blotting. This also could indicate why the levels of MtlR protein are low. The results also indicate that there is about a 4-fold increase in *mtlR* mRNA when the bacteria are grown in mannitol media over when grown in glucose media, however, the levels in all cases are still low. This result is consistent with the idea that the *mtlR* is part of the operon which is more highly transcribed when in the presence of mannitol. A possible reason that increased *mtlA* mRNA transcription is not seen is because northern blotting is not sensitive enough to detect the small increase.

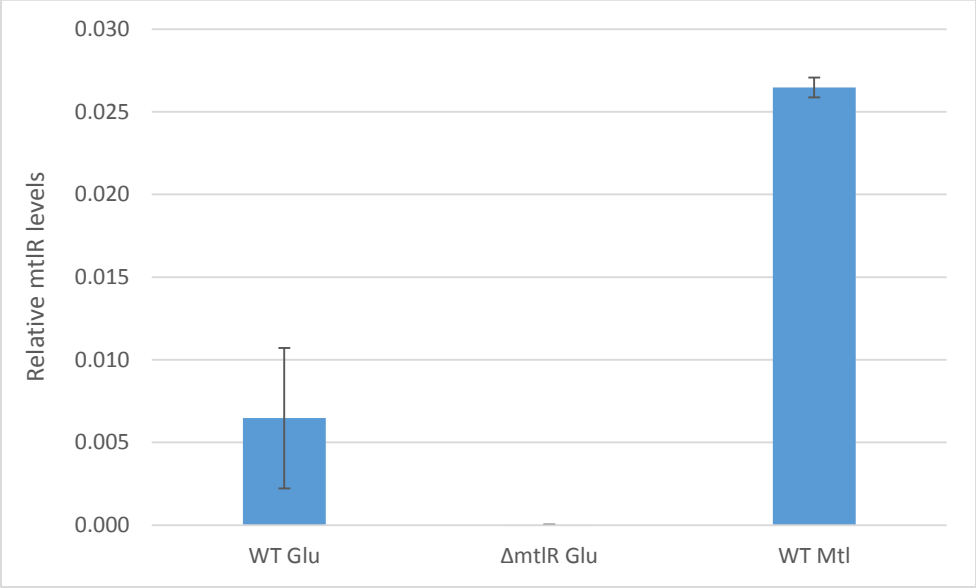

Figure 13. *mtlR* mRNA present when grown in both glucose and mannitol, but not in Δ*mtlR*. The negative expression indicates an absence of *mtlR* mRNA. Wild-type *V. cholerae* and Δ*mtlR* were grown in glucose (Glu) or mannitol (Mtl) containing media until mid-log phase at 37 ºC. 200 ng total RNA was used for RT and primers flanking *mtlR* gene were used for PCR. Error bars represent the standard deviation of the triplicate trials done in the experiment. These data are representative of at least two separate experiments.

Discussion and Conclusions

The three questions that this thesis set out to investigate were: What is the role of Hfq in MtlS regulation of MtlA; What role does MtlR play in the system; and How can

MtlR be studied in more depth. The data indicate that MtlS works independently of Hfq. This conclusion was determined through analyzing the patterns of synthesis of MtlS, *mtlA* mRNA, and MtlA protein of the wild type and *Δhfq* (Figure 7). Since the levels of all three factors remained constant regardless of the presence of Hfq, MtlS regulates *mtlA* expression in an Hfq-independent manner. The stability of MtlS was also analyzed by determining the half-life with and without Hfq. In both cases, the half-life of MtlS was about 20 minutes re-indicating that Hfq is not required to stabilize MtlS (Figure 8). We predicted that Hfq was most likely not involved in the system due to the *cis* nature of MtlS as well as the 70 nucleotides of perfect complementarity. Due to this long stretch of perfect complementarity, when MtlS is transcribed, it can easily bind to the *mtlA* mRNA transcript, thus regulating the system. Since MtlS can bind to *mtlA* mRNA quickly and easily, the functions of Hfq, which is known to stabilize and allow for RNA binding, are not required. Although the finding was not necessarily surprising, the result is still interesting because of the high percentage of Hfq dependent sRNA systems.

The next area studied was the role of MtlR. MtlR was shown to act as a repressor in the system. This was determined through analysis of MtlS, *mtlA* mRNA, and MtlA protein in the wild type and *ΔmtlR* strains, revealing the presence of MtlA protein when the mutant was grown in glucose (Figure 9). This result was also expected based on the literature (Figge et al. 1994; Tan et al. 2009). Since *V. cholerae* is a gram-negative species, we expected that MtlR acts similarly to MtlR in other gram-negative species like *E. coli* (Figge et al. 1994).

After the general function of MtlR as a repressor was determined, a more in depth study is needed to know the way in which it functions. However, the study of MtlR was plagued with issues that did not allow for further study but did give insight into what to do next. Multiple attempts at tagging MtlR on either terminus proved unsuccessful. We hypothesized that the His_6 tag was interfering with MtlR through the attraction of the positive histidine residues to the negative charge of MtlR, which made the His_6 tag inaccessible for binding. This was proven to be incorrect through when a comparison of the knockout to the tagged MtlR was done, and no MtlA was seen, meaning His_6-tagged MtlR was functioning properly (Figure 10). Heterologous overexpression of His_6-tagged MtlR in *E. coli* revealed the presence of MtlR, indicating that a stable protein product with a His_6-tag could be made (Figure 11).

Due to the difficulties in tagging and detecting MtlR, further studies of the MtlR protein were not be completed. However, the experiments did provide the insight that MtlR is lowly expressed at both the mRNA and protein levels in *V. cholerae*. Based on the results, in order to study MtlR protein it should be overexpressed. If MtlR is overexpressed in *V. cholerae*, it could be possible to determine what MtlR is binding to in *V. cholerae* through use of a co-immunoprecipitation. In order to study the native expression of *mtlR* mRNA in *V. cholerae*, more sensitive methods such as quantitative reverse transcription PCR should be utilized.

Now that MtlR is known to repress expression of *mtlA*, the question remains how it does so. Due to the high number of negative charges on MtlR (Tan et al. 2009), it is unlikely that it binds DNA or RNA directly, but rather acts with an additional factor or

factors to repress translation (Figure 14A). While MtlR is functioning, MtlS is also repressing expression by binding the 5' untranslated region of the *mtlA* mRNA and thus blocking the ribosome from binding (Figure 14A).

Additionally, it appears that MtlR repression is a post-transcriptional event because the levels of *mtlA* mRNA remain the same in both the wild type and the mutant. It is also possible that MtlR and MtlS form a complex that causes the repression of MtlA, however there may still be an additional factor to facilitate binding as MtlR has no known conserved RNA-binding domains (Figure 14B).

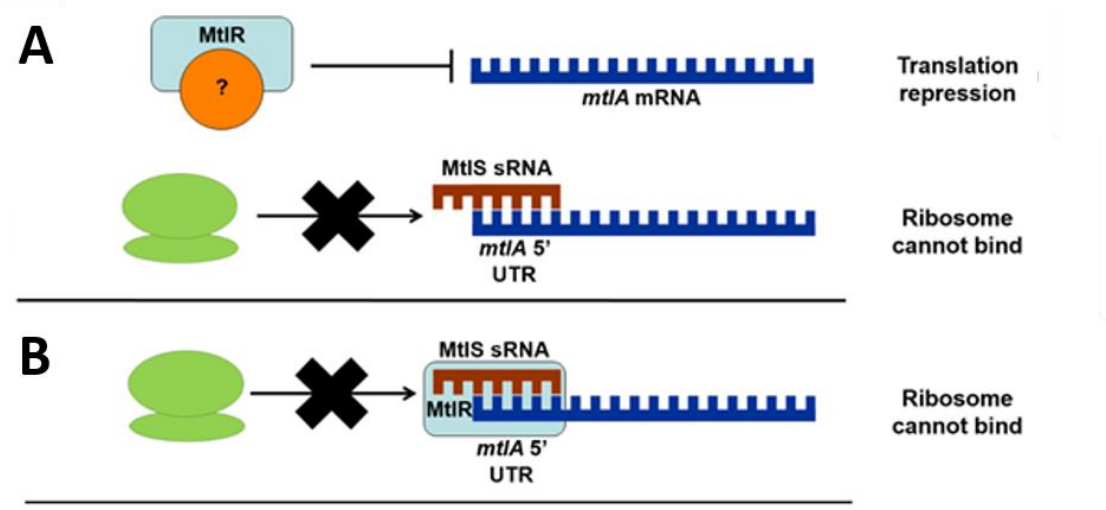

Figure 14. Working hypotheses for the repression of the synthesis of MtlA protein. (A) MtlR (blue rectangle) may be working with an additional unknown factor (orange circle) to repress translation of the *mtlA* mRNA. Additionally, MtlS may act separately from MtlR to block ribosomal binding and cause repression. (B) MtlR may also work in conjunction with MtlS to cause full repression.

In order to test whether MtlR is directly binding *mtlA* or if there is an additional factor needed, an electrophoretic mobility shift assay (EMSA) could be performed. This would be done by purifying MtlR and seeing if it could directly bind *mtlA* mRNA. If there was shift in the band from pure MtlR sample, this would indicate that MtlR is

involved in direct binding, providing evidence for Model A without the additional factor. If there is no shift then it would support the way Model A is shown with an additional factor.

Since MtlR can be tagged and detected via western blotting in *E. coli*, additional studies such as a pull down assay can be done. Since we hypothesized that MtlR may be binding to another protein factor, a pull down assay using MtlR should be completed. A pull down assay would involve using a nickel column to bind the His_6 tag of the tagged MtlR which will have been purified from the overexpression system. Purification must be done so that MtlR can be isolated from anything it may have bound in the *E. coli* system it was overexpressed in. Then, full cell lysate of *V. cholerae* can be run through the column and whatever MtlR binds to, if anything, can then be coprecipitated out. This would support the idea that an additional factor is needed to bind the RNA which adds complexity to both models. Overexpression of MtlR in *V. cholerae* could also make it possible to do an immunoprecipitation in the native environment.

Tests could also be done to look at the relationship of MtlR and MtlS. The stability of MtlS in a *ΔmtlR* strain could be tested in the same way it was tested in the *Δhfq* strain. If MtlS is less stable without MtlR than it would help support Model B where the two factors, and perhaps additional factors, are involved in a complex (Figure 14B). While there are no known RNA binding motifs in the MtlR sequence it is still possibility that MtlR may be helping with MtlS stability or protection from RNAse E. This is particularly possible because the system is Hfq-independent (Figure 7).

Another set of tests that could be done with MtlR involves looking at MtlA without MtlR or when MtlR is overexpressed. The half-life of MtlA in a *ΔmtlR* strain could be determined through the addition of chloramphenicol to stop protein production. This would also help us understand how MtlR affects the system and whether it is affecting MtlA stability or the translation of MtlA. If the half-life of MtlA is the same regardless of MtlR, then that would provide evidence towards MtlR acting as a translational repressor, which is believe to be the case in both models (Figure 14).

The mannitol system in *V. cholerae* is so highly regulated, the importance of the mannitol operon and mannitol must be explored. Mannitol is an interesting sugar for a few reasons. The first is that it can be utilized as a carbon source which provides an advantage when other carbon sources are not available. Perhaps more importantly however, is mannitol's role as a compatible solute. A compatible solute is a small molecule that can be taken up by the cell in order for the cell to deal with osmotic stress (Kets et al. 1996). This is particularly important to bacteria that encounter high salt conditions that would cause the cells to lose their water if they do not uptake compatible solutes to counter the stress of the salt. *V cholerae* is a species that has to deal with very differing environments from the human small intestine to freshwater reservoirs to saltwater reservoirs (Harris et al. 2012). In order to survive in these environments, it has to be able to regulate pathways that allow for quick adaptations to the environment. Since mannitol can be utilized as a compatible solute and is found around brown algae (Ymele-Leki et al. 2013), where *V. cholerae* can also be found, it makes sense that the mannitol operon would be so highly regulated. When the bacteria enter an area of high salt and

mannitol is present, they turn on the mannitol operon so that they can survive. This is possible in both a saltwater environment where the brown algae live or perhaps in the small intestine if mannitol is around. When the conditions become less harsh and mannitol is no longer present, the bacteria need to rapidly shut off the system so that energy is not wasted in making the transporter protein.

Because of the importance of mannitol and the mannitol operon to *V. cholerae,* as denoted by the high levels of regulation, one study that could be completed would be testing the compatible solute properties of mannitol. Previous studies (not shown) have shown that glucose is the preferred carbon source of *V. cholerae;* thus, it is reasonable to hypothesize that mannitol may be used more for osmoregulation than as a carbon source. Since mannitol can accumulate in the human small intestine to small extent, or more importantly, when *V. cholerae* are in an aquatic environment near brown algae beds, it

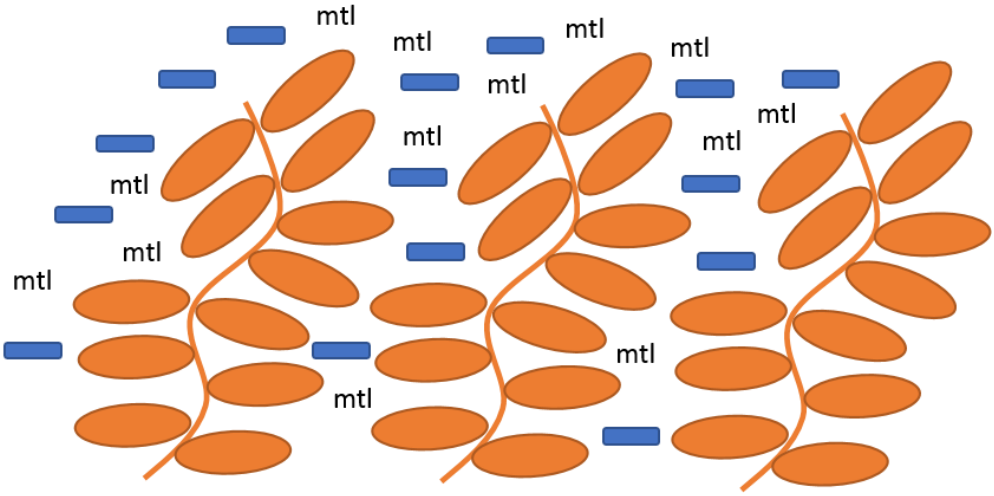

Figure 15. *V. cholerae* uses mannitol as a compatible solute to survive in an aquatic environment. *V. cholerae* (blue rods) can live in aquatic environments near brown algae (orange brown structures) which produce mannitol (mtl). Mannitol can be utilized by the bacteria in order to combat osmotic stress thus giving them an advantage in the aquatic environment. Since mannitol allows for this substantial advantage, it is important for *V. cholerae* to highly regulate the mannitol operon. Figure adapted from Ymele-Leki et al. 2013

would be useful to see if the bacteria are using mannitol to adapt to their surroundings (Wang and van Eys 1981; Ymele-Leki et al. 2013). If it is true that *V. cholerae* is utilizing mannitol as a compatible solute, then it should be able to better survive higher salt conditions when mannitol is present than in the absence of mannitol. In order to do this test, a growth curve could be set up with conditions of varying salt concentrations from low to high. The bacteria could be grown in glucose media, and with/without additional mannitol added. Glucose would need to be in high enough concentration so that the mannitol would not be used as a carbon source. The cultures would be allowed to grow at 37 ºC, taking regular time points to track the growth of the cultures. The growth curves would then indicate if *V. cholerae* is utilizing the mannitol as a compatible solute for osmoregulation. Cultures that had mannitol should show larger amounts of growth in high salt concentration or even all salt concentration indicating they could survive better in these conditions with mannitol. Without mannitol, it should be observed that the culture could not grow in high salt conditions and thus the growth curve would show little to no growth. This study would indicate whether or not mannitol was being utilized as a compatible solute.

In conclusion, as *V. cholerae* continues to threaten many areas around the world, studying the ways *V. cholerae* adapts to differing environments remains an area of interest. Small RNA regulation is one way that cells can rapidly control gene expression in a variety of pathways. The pathways that sRNAs regulate are not limited to helping with adapting to new carbon sources but are also involved in causing disease. The more knowledge that can be learned about infectious pathogens, the more methods of treating

or preventing the disease we can create in the long range future. This statement is especially true when thinking about *V. cholerae,* which continues to cause wide spread disease. Studying the mannitol operon in *V. cholerae* is just one step toward better understanding the pathogen. It may be possible that targeting regulation of the mannitol operon could help treat or prevent the disease in the distant future.

Materials and Methods

Bacterial Strains and Growth Conditions

V. cholerae strain N16961 is an El Tor strain, and all *V. cholerae* strains used in this study had the Δ*tcpA* mutation, which is attenuated for virulence for safety purposes (Mustachio et al. 2012). All mutations were made through the use of plasmid pCVD442 which allows for allelic exchange with chromosomal DNA (Donnenberg and Kaper, 1991; Thelin and Taylor, 1996). The plasmid was propagated in *E. coli* strain DH5αλpir and transferred to *E. coli* SM10αλpir for conjugation into *V. cholerae.* The mutation of choice was created using splicing by overlapping extensions (SOE) – PCR and in some cases Gibson Assembly. Briefly, DNA fragments about 500bp upstream and downstream the mutation were created via PCR from *V. cholerae* genomic DNA. The two segments were annealed together via sequence complimentary and amplified by PCR. The product was digested with SacI and SphI restriction enzymes and ligated into the plasmid. The plasmid with the mutation was transformed into *E. coli* DH5αλpir via electroporation, and transformed into *E. coli* SM10αλpir which was conjugated on an LB plate with *V. cholerae*. Once the plasmid was in *V. cholerae,* sucrose-resistant colonies were selected

and confirmed via PCR

E. coli strains used for TOPO cloning were from Invitrogen and were either TOP10 for propagation or BL21 Star^{Tm} for expression. Bacterial strains were grown in either aerated LB broth or M9 minimal media with added 0.1% trace metals (5% $MgSO_4$, 0.5% $MnCl_2 \cdot 4H_2O$, 0.5% $FeCl_3$, 0.4% nitrilotriacetic acid) and 0.4% carbon source (glucose or mannitol). Strains grown on solid media were grown on LB agar plates with the necessary antibiotics. All strains were grown at 37 ºC. When IPTG was required for

Table 1. Strain List Used in this Thesis

Strain	**Relevant genotype**
V. cholerae	
JL1	N16961 Δ*tcpA* (VC0828) Sm^r
JL2	N16961 Δ*tcpA mtlA*-FLAG Sm^r
JL30	N16961 Δ*tcpA* Δ*mltR* pTrc99A Sm^r Cb^r
JL31	N16961 Δ*tcpA* Δ*mltR* pTrc99A::*mtlR* Sm^r Cb^r
JL54	NI6961 ΔtcpA Δ*hfq mtlA*-FLAG Sm^r
JL55	N16961 Δ*tcpA* Δ*mtlR mtlA*-FLAG Sm^r
JL130	N16961 Δ*tcpA* His$_6$-*mtlR* Sm^r
JL134	N16961 Δ*tcpA* His$_6$-*mtlR mtlA*-FLAG Sm^r
JL139	N16961 Δ*tcpA mtlR*-StrepII *mtlA*-FLAG Sm^r
JL154	N16961 Δ*tcpA mtlR*-HA *mtlA*-FLAG Sm^r
E. coli	
DH5αλpir	F$^-$ (*lacZYA-argF*) *U169 recA1 end A1 hsdR17 supE44 thi-1 gyrA96 relA1* λ::*pir*
SM10αλpir	*thi recA thr leu tonA lacY supE* RP4-2-Tc::*Mu* λ::*pir*
JL155	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80*lacZ*ΔM15 Δ*lacX*74 *recA*1 *araD*139 Δ(*ara-leu*)7697 *galU galK rpsL* (Str^r) *endA*1 *nupG* pET101::*mtlR*-His$_6$ Ap^r
JL156	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80*lacZ*ΔM15 Δ*lacX*74 *recA*1 *araD*139 Δ(*ara-leu*)7697 *galU galK rpsL* (Str^r) *endA*1 *nupG* pET101::His$_6$-*mtlR* Ap^r
JL157	F$^-$ *ompT hsdS*$_B$ ($r_B^-m_B^-$) *gal dcm rne*131 (DE3) pET101::*mtlR*-His$_6$ Ap^r
JL158	F$^-$ *ompT hsdS*$_B$ ($r_B^-m_B^-$) *gal dcm rne*131 (DE3) pET101:: His$_6$-*mtlR* Ap^r

gene expression from a plasmid, IPTG was added to a concentration of 1 mM. Antibiotics were added based on the strain to the following concentrations: streptomycin 100 μg/mL, ampicillin 100 μg/mL, and carbenicillin 100 μg/mL. All strains are listed in Table 1.

Northern Blotting Analysis

Total RNA was isolated from cultures that had reached mid-log phase (about 10^8 cells). When rifampicin was used, it was added at mid-log phase at a concentration of 100 μg/mL, and samples were taken over the course of an hour. In the Phase Lock Gel Acid Phenol/Chloroform technique, cells were lysed by lysis solution (40 mM NaAcetate (pH 5.0), 1% SDS, 2 mM EDTA). An equal volume of acid phenol/chloroform (Ambion) was added to the cell lysate and incubated at 65 ºC for 5 minutes, vortexing after every minute. The mixture was centrifuged at 16,000 x g for 10 minutes. The top layer was transferred to a Phase Lock Gel (Eppendorf) to which an equal volume of Acid Phenol/Chloroform was added, and centrifuged at the same speed for 5 minutes. The top layer was removed, transferred to a new tube, and an equal volume of isopropanol was added to precipitate the RNA. For qRT-PCR, RNA was purified using a Zymo DirectZol RNA Miniprep Kit. TRI Reagent (Zymo) was used to resuspend the pellet of cells at mid-log phase determined via OD_{600}. An equal part of ethanol was added to the mixture before purification using Zymo DirectZol RNA Miniprep Kit. Purified RNA was stored at -20 ºC and used for analysis.

For sRNA northern blots, Loading Buffer II (Ambion) was added to the RNA sample and heated for 5 minutes at 85 ºC. The sample was run on a 10% denaturing TBE-Urea polyacrylamide gel in 1x Tris-borate-EDTA (TBE) at 200 V for 45-60 minutes in a

Mini-Protean Tetra Cell tank (Bio-Rad). The gel was transferred to a nylon membrane (Amersham) using a wet transfer apparatus (Bio-Rad) at 200 mA for 1 hour. The blot was washed with 6x Saline-Sodium Citrate (SSC), cross-linked using the autocross link function of UV crosslinker, and washed with 1x SSC.

For mRNA northern blots, the RNA sample was mixed with 6x Loading Buffer (0.4% bromophenol blue, 0.4% xylene cyanol, 50% glycerol) and loaded into a 1% agarose gel. The gel was run at 120 V for 40 minutes and using the NorthernMax system (Ambion) transferred to a nylon membrane (Amersham). The membrane was crosslinked three times in UV crosslinker using the auto crosslink setting.

After crosslinking, the method for northern blotting detection and imaging is the same for both sRNA and mRNA blots. The membrane was pre-hybridized with ULTRAhyb-Oligo (Ambion) at 65 ºC for 30 minutes. Riboprobe specific for the RNA of interest was added to the buffer and hybridized overnight at 65 ºC. Riboprobe was created using a PCR generated DNA template with the T7 sequence attached to the 5' end, which could then be used with biotinylated UTP, rNTPs, DTT, 1x buffer (Promega) and T7 polymerase (Promega). After the membrane was hybridized, it was washed with low stringency buffer followed by high stringency buffer (Ambion). The membrane was blocked with blocking buffer (LiCor) and 1% SDS. Blocking buffer and 1% SDS containing 1:10,000 Streptavidin-IRDye 680 was added and incubated at room temperature for 1 hour. The blot was washed with 1x Phosphate Buffered Saline with 0.1% Tween (PBST) and imaged on a LiCor Odyssey detector. Additionally, the blots

were probed for 5S RNA as a loading control using a 5S RNA 5' end labelled with IRDye 800 (IDT). Quantification was done via LiCor Odyssey software.

Western Blotting Analysis

Full cell lysates (about 10^7 cells) were mixed with 5x sample buffer (250 mM Tris-Cl (pH 6.8), 10% sodium dodecyl sulfate (SDS), 50% glycerol, 10% β-mercaptoethanol, 0.5% orange G) and heated to 95 ºC for 10 minutes. The samples were run on a 10% or 12% SDS polyacrylamide gel. The gel was run at 200 V for 45 minutes in a Mini-Protean Tetra Cell tank (Bio-Rad) in 1 x Tris Glycine SDS Buffer (LiCor). The proteins were transferred to a nitrocellulose membrane (Licor) using a wet-transfer apparatus (Bio-Rad) at 300 mA for 60 minutes in Transfer Buffer (25 mM Tris, 192 mM glycine, 20% methanol). The membrane was blocked with 5% milk in PBS and washed with 1 x PBST, before incubation with 1:3,000 dilution of anti-FLAG antibody (Abcam) or 1:5,000 of anti-His_6 (Abcam), and 1:10,000 anti-rpoB (Abcam). After washing with 1 x PBST the blot was incubated with 1:10,000 anti-mouse or anti-rabbit secondary antibodies which were bound to IRDyes (Licor) so that the blot could be imaged using an Licor Odyssey instrument. When detecting StrepII labeled proteins, 1:10,000 Streptavidin-680 IRDye (LiCor) in blocking buffer was used instead of the dual antibody system.

qRT-PCR

In order to do qRT-PCR, total RNA was isolated using the TRI Reagent Zymo DirectZol RNA Miniprep Kit. The purified RNA was treated with rDNAse (Life technologies) twice, and the DNA-free RNA was collected. Using a reverse primer that

flanked the gene of interest and M-MuLV Reverse Transcriptase (NEB), a reverse transcription reaction was carried out at 42 ºC for 1 hour. qPCR was done by mixing the reverse transcription reaction, and water for a no transcription control, or gDNA with iTaq Universal SYBR Green Supermix (BioRad), and forward and reverse primers that flanked the gene of interest. PCR was run with the following parameters: 50 °C, 2 min; 95 °C, 5 min; 40 cycles of 95 °C, 15 seconds; 60 °C, 1 minute.

Bibliography

Akagawa E, Kurita K, Sugawara T, Kouji N, Kasahara Y, Ogasawara N, Yamane K. 1995. Determination of a 17484 bp nucleotide sequence around the 39º region of the *Bacillus subtilis* chromosome and similarity analysis of the products of putative ORFs. Microbiology. 141:3241–5.

Archibold RC, Sengupta S. 2014 April 19. U.N. Struggles to Stem Haiti Cholera Epidemic. New York Times.

Behrens S, Mitchell W, Bahl H. 2001. Molecular analysis of the mannitol operon of *Clostridium acetobutylicum* encoding a phosphotransferase system and a putative PTS-modulated regulator. Microbiology.147:75–86.

Bharati K, Ganguly NK. 2011. Cholera toxin: a paradigm of a multifunctional protein. The Indian journal of medical research. 133:179–87.

Campbell NA, Reece JB, Urry LA, Cain ML, Wasserman SA, Minorsky PV, Jackson RB. 2008. Biology 8th Edition.

Charles RC, Ryan ET. 2011. Cholera in the 21st century. Current Opinion in Infectious Diseases. 24(5):472–7.

Donnenberg MS, Kaper JB. 1991. Construction of an eae deletion mutant of enteropathogenic *Escherichia coli* by using a positive-selection suicide vector. Infection and Immunity. 59(12):4310–7.

Dühring U, Axmann IM, Hess WR, Wilde A. 2006. An internal antisense RNA regulates expression of the photosynthesis gene *isiA*. Proceedings of the National Academy of Sciences of the United States of America. 103(18):7054–8.

Fauci AS, Morens DM. 2012. The perpetual challenge of infectious diseases. The New England Journal of Medicine. 366(5):454–61.

Figge RM, Ramseier TM, Saier MH. 1994. The mannitol repressor (MtlR) of *Escherichia coli*. Journal of Bacteriology. 176(3):840–7.

Fonkwo PN. 2008. Pricing infectious disease. EMBO reports. 9:S13–7.

Fröhlich KS, Vogel J. 2009. Activation of gene expression by small RNA. Current Opinion in Microbiology. 12(6):674–82.

Görke B, Vogel J. 2008. Noncoding RNA control of the making and breaking of sugars. Genes & Development. 22(21):2914–25.

Harris JB, LaRocque RC, Qadri F, Ryan ET, Calderwood SB. 2012. Cholera. Lancet. 379(9835):2466–76.

Honeyman a L, Curtiss R. 2000. The mannitol-specific enzyme II (mtlA) gene and the mtlR gene of the PTS of *Streptococcus mutans*. Microbiology. 146 (Pt 7:1565–72.

Ikemura T, Dahlberg JE. 1973. Small Ribonucleic Acids of *Escherichia coli*: I. Characterization by polyacrylamide gel electrophoresis and fingerprint analysis. Journal of Biological Chemistry. 248(14):5024–32.

Joyet P, Bouraoui H, Aké FMD, Derkaoui M, Zébré AC, Cao TN, Ventroux M, Nessler S, Noirot-Gros M-F, Deutscher J, et al. 2013. Transcription regulators controlled by interaction with enzyme IIB components of the phosphoenolpyruvate: sugar phosphotransferase system. Biochimica et Biophysica Acta. 1834(7):1415–24.

Kets EP, Galinski EA, de Wit M, de Bont JA, Heipieper HJ. 1996. Mannitol, a novel bacterial compatible solute in *Pseudomonas putida* S12. Journal of Bacteriology. 178(23):6665–70.

Kumar S, Smith KP, Floyd JL, Varela MF. 2011. Cloning and Molecular Analysis of a Mannitol Operon of Phosphoenolpyruvate-dependent Phosphotransferase (PTS) type From *Vibrio Cholerae* O395. Archives of Microbiology. 193(3):201–208.

Liu JM, Livny J, Lawrence MS, Kimball MD, Waldor MK, Camilli A. 2009. Experimental discovery of sRNAs in *Vibrio cholerae* by direct cloning, 5S/tRNA depletion and parallel sequencing. Nucleic Acids Research. 37(6):e46.

Majdalani N, Chen S, Murrow J, St John K, Gottesman S. 2001. Regulation of RpoS by a novel small RNA: the characterization of RprA. Molecular Microbiology. 39(5):1382–94.

Massé E, Escorcia FE, Gottesman S. 2003. Coupled degradation of a small regulatory RNA and its mRNA targets in *Escherichia coli*. Genes & Development. 17(19):2374–83.

Matson JS, Withey JH, DiRita VJ. 2007. Regulatory networks controlling *Vibrio cholerae* virulence gene expression. Infection and Immunity. 75(12):5542–9.

Morens DM, Folkers GK, Fauci AS. 2004. The challenge of emerging and re-emerging infectious diseases. Nature. 430(6996):242–9.

Morens DM, Folkers GK, Fauci AS. 2008. Emerging infections: a perpetual challenge. The Lancet Infectious Diseases. 8(11):710–719.

Morfeldt E, Taylor D, von Gabain A, Arvidson S. 1995. Activation of alpha-toxin translation in *Staphylococcus aureus* by the trans-encoded antisense RNA, RNAIII. European Molecular Biology Organization. 14(18):4569–77.

Morita T, Mochizuki Y, Aiba H. 2006. Translational repression is sufficient for gene silencing by bacterial small noncoding RNAs in the absence of mRNA destruction. Proceedings of the National Academy of Sciences of the United States of America. 103(13):4858–63.

Mustachio LM, Aksit S, Mistry RH, Scheffler R, Yamada A, Liu JM. 2012. The *Vibrio cholerae* mannitol transporter is regulated post transcriptionally by the MtlS small regulatory RNA. Journal of Bacteriology. 194(3):598–606.

Negrete A, Ng W-I, Shiloach J. 2010. Glucose uptake regulation in *E. coli* by the small RNA SgrS: comparative analysis of *E. coli* K-12 (JM109 and MG1655) and *E. coli* B (BL21). Microbial Cell Factories. 9:75.

Nelson EJ, Harris JB, Morris JGJ, Calderwood SB, Camilli A. 2009. Cholera transmission: the host, pathogen and bacteriophage dynamic. Nature Review Microbiology. 7(10):1–24.

Opdyke JA, Kang J, Storz G. 2004. GadY, a Small-RNA Regulator of Acid Response Genes in *Escherichia coli*. Journal of Bacteriology. 186(20):6698–6705.

Postma PW, Lengeler JW, Jacobson GR. 1993. Phosphoenolpyruvate:carbohydrate phosphotransferase systems of bacteria. Microbiological Reviews. 57(3):543–94.

Prévost K, Salvail H, Desnoyers G, Jacques J-F, Phaneuf E, Massé E. 2007. The small RNA RyhB activates the translation of shiA mRNA encoding a permease of shikimate, a compound involved in siderophore synthesis. Molecular Microbiology. 64(5):1260–73.

Prüss-Üstün A, Bos R, Gore F, Bartram J. 2008. Safer Water, Better Health. World Health Organization. Geneva, Switzerland.

Rambhatla P, Kumar S, Floyd JT, Varela MF. 2011. Molecular Cloning and Characterization of Mannitol-1-phosphate dehydrogenase from *Vibrio cholerae*. Journal of Microbiology and Biotechnology. 21(9):914–920.

Song T, Mika F, Lindmark B, Liu Z, Schild S, Bishop A, Zhu J, Camilli A, Johansson J, Vogel J, et al. 2008. A new *Vibrio cholerae* sRNA modulates colonization and affects release of outer membrane vesicles. Molecular microbiology. 70(1):100–11.

Stork M, Di Lorenzo M, Welch TJ, Crosa JH. 2007. Transcription termination within the iron transport-biosynthesis operon of *Vibrio anguillarum* requires an antisense RNA. Journal of Bacteriology. 189(9):3479–88.

Tan K, Clancy S, Borovilos M, Zhou M, Hörer S, Moy S, Volkart LL, Sassoon J, Baumann U, Joachimiak A. 2009. The mannitol operon repressor MtlR belongs to a new class of transcription regulators in bacteria. The Journal of Biological Chemistry. 284(52):36670–9.

Thelin KH, Taylor RK. 1996. Toxin-coregulated pilus, but not mannose-sensitive hemagglutinin, is required for colonization by *Vibrio cholerae* O1 El Tor biotype and O139 strains. Infection and Immunity. 64(7):2853–6.

Valentin-Hansen P, Eriksen M, Udeseen C. 2004. The bacterial Sm-like protein Hfq : a key player in RNA transactions. Molecular Microbiology. 51(6):1525–33.

Wang YM, van Eys J. 1981. Nutritional significance of fructose and sugar alcohols. Annual Review Nutrition 1:437– 475.

Waters LS, Storz G. 2009. Regulatory RNAs in bacteria. Cell. 136(4):615–28.

Wenner N, Maes A, Cotado-Sampayo M, Lapouge K. 2013. NrsZ: a novel, processed, nitrogen-dependent, small non-coding RNA that regulates *Pseudomonas aeruginosa* PAO1 virulence. Environmental Microbiology. 4:1–16.

WHO (World Health Organization). Cholera. 2012 http://www.who.int/mediacentre/factsheets/fs107/en/index.html

Ymele-Leki P, Houot L, Watnick PI. 2013. Mannitol and the mannitol-specific enzyme IIB subunit activate *Vibrio cholerae* biofilm formation. Applied and Environmental Microbiology. 79(15):4675–83.

Zhang A, Wassarman KM, Ortega J, Steven AC, Storz G. 2002. The Sm-like Hfq protein increases OxyS RNA interaction with target mRNAs. Molecular Cell. 9(1):11–22.