Functional Characterization of Regulators of Bacterial Pathogenicity and Metabolism

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By
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ABSTRACT

Functional Characterization of Regulators of Bacterial Pathogenicity and Metabolism

A thesis presented to the Biochemistry Department

Graduate School of Arts and Sciences
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By Richard Stefan Isaac

Regulatory proteins from pathogenic bacteria have the potential to serve as novel therapeutic targets. This study discusses the characterization of three regulatory bacterial proteins. First, the HlyU protein of *Vibrio vulnificus* belongs to the SmtB/ArsR family of metal ion binding repressors and is a global regulator of virulence factors. It has been shown to directly bind a DNA sequence upstream of the transcription start site in the *rtxA1* operon and to compete with the repressor H-NS. In this study, a homology model was constructed using the crystal structure of the metallo repressor SmtB from *Synechococcus elongatus* PCC7942. A high-throughput assay was utilized to monitor DNA binding *in vitro*. The model structure, metal analysis, and denaturation assays all support the hypothesis that HlyU lacks the key metal ion binding residues of other members of the SmtB/ArsR family. Future studies will determine key residues in DNA recognition and binding and will determine how HlyU is regulated. Second, the Diphtheria toxin repressor (DtxR) is the best characterized member of a family of metal ion-activated repressors involved in metal ion homeostasis, virulence factor expression, and stress response in bacteria. This study presents evidence that DtxR may be involved in an oxidative stress related response system through the oxidation of the metal ion ligand C102. Future studies will determine how, structurally, oxidation of C102 abolishes repressor activity. Third, propionate kinase (PK) from
the highly infectious bacterial pathogen *Francisella tularensis* is a key enzyme in the metabolism of serine and threonine. Previous studies determined a 2.2 Å structure of the apo-enzyme. In this study, structural studies were performed to cocrystallize PK with its substrate in order to better understand the mechanism of phosphate transfer. While crystals with substrate were not obtained, a novel crystallization condition of the apo-enzyme was determined. Future studies will reveal the structure of propionate kinase bound to its substrate.
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<tr>
<td>ACD</td>
<td>Actin Cross-Linking Domain</td>
</tr>
<tr>
<td>ADPR</td>
<td>Adenosine Diphosphate Ribose</td>
</tr>
<tr>
<td>Amp</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>βME</td>
<td>β-mercaptoethanol</td>
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<tr>
<td>bp</td>
<td>Base Pair</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic Acid</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CDC</td>
<td>Center for Disease Control</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony Forming Unit</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese Hamster Ovary</td>
</tr>
<tr>
<td>CHP</td>
<td>Cumene Hydroperoxide</td>
</tr>
<tr>
<td>CPD</td>
<td>Autocatalytic Cysteine Protease Domain</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5′-dithiobis-(2-nitrobenzoic acid)</td>
</tr>
<tr>
<td>DtxR</td>
<td>Diphtheria Toxin Repressor</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetate</td>
</tr>
<tr>
<td>EF-2</td>
<td>Elongation Factor 2</td>
</tr>
<tr>
<td>EMSA</td>
<td>Electrophoretic Mobility Shift Assay</td>
</tr>
<tr>
<td>Fur</td>
<td>Ferric Uptake Regulator</td>
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<tr>
<td>Hlat</td>
<td>Hemolysin Aroyltransferase</td>
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<td>HTH</td>
<td>Helix-turn-helix domain</td>
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<tr>
<td>IC</td>
<td>Intensive-care</td>
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<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-thiogalactosidase</td>
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<tr>
<td>IV</td>
<td>Intravenous</td>
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<tr>
<td>IVIAT</td>
<td><em>in vivo</em>-induced antigen technology</td>
</tr>
<tr>
<td>ive</td>
<td><em>in vivo</em> expressed</td>
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<tr>
<td>Kan</td>
<td>Kanamycin</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani Media</td>
</tr>
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<td>LD₅₀</td>
<td>Median Lethal Dose</td>
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<td>MARTX</td>
<td>Multicomponent Autoprocessing Repeat-in-Toxin</td>
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<td>MME</td>
<td>Monomethyl Ether</td>
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<td>MnSOD</td>
<td>Manganese Superoxide Dismutase</td>
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<tr>
<td>NAD</td>
<td>Nicotinamide Adenine Dinucleotide</td>
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<td>Ni-NTA</td>
<td>Nickel-nitrilotriacetic acid</td>
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<tr>
<td>OD</td>
<td>Optical Density</td>
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<td>ONPG</td>
<td>o-nitrophenol-β-D-galactosidases</td>
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<td>ORF</td>
<td>Open Reading Frame</td>
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<td>PAGE</td>
<td>Polyacrylamide Gel Electrophoresis</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<td>Polyethylene Glycol</td>
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<td>Rho GTPase Inactivation Domain</td>
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<td>Ribonuclease A</td>
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<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<td>RPA</td>
<td>Ribonuclease Protection Assay</td>
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<tr>
<td>RT-PCR</td>
<td>Real-time Polymerase Chain Reaction</td>
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<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<td>SEC</td>
<td>Size Exclusion Chromatography</td>
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<tr>
<td>T1SS</td>
<td>Type 1 Secretion System</td>
</tr>
<tr>
<td>TSBS</td>
<td>Trypticase Soy Broth</td>
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<tr>
<td>VNBC</td>
<td>Viable But Nonculturable</td>
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Chapter 1: Functional Characterization and *in silico* Modeling of HlyU

Introduction

*Vibrio vulnificus* is an opportunistic, invasive, and highly lethal gram-negative bacillus. Like all members of the *Vibrio* genus, *V. vulnificus* is a motile, curved rod bacterium. It also possesses a single, polar flagellum to aid in movement [1, 2]. This species is distinguished from other *Vibrios* by its ability to ferment lactose [1]. *V. vulnificus* was first isolated by the Center for Disease Control (CDC, www.cdc.org) in 1964 when it was mistakenly identified as a strain of *Vibrio parahaemolyticus*. The first recent report of infection was in 1970. It was given its current name in 1979; the word “vulnificus” comes from the Latin “wounding” which describes the species’ ability to cause secondary lesions in infected individuals [1, 2].

While the number of people infected globally by *Vibrio vulnificus* is low in comparison with other *Vibrio* species, it is responsible for a significant percentage of *Vibrio*-related illnesses in the United States [1]. In addition, it is responsible for the majority of seafood related deaths in the United States, and, on average, there are around 50 confirmed cases per year. Infections usually present themselves in one of three syndromes: primary septicemia, wound infection, and gastroenteritis [1-3].

Epidemiological studies have identified a number of trends related to infections. The peak incidence of cases is in the summer months, but the range is from March to November, likely due to elevated water temperatures [2]. *V. vulnificus* primarily infects men rather than women; the male-to-female ratio is approximately 4-to-1. In a review of 459 reported cases between 1992 and 2007, 85.6% of the infected individuals were male [3]. A number of conditions and diseases are considered risk factors for *V. vulnificus* infection. In a review of 180
cases between 2002 and 2007, 92.8% of individuals had consumed raw oysters or seafood prior to the onset of symptoms, and 95.3% had a preexisting disease or condition. Liver disease and other diseases with possible hepatic involvement or elevated serum iron levels have been linked to an increased probability of infection. These include cirrhosis, alcoholism, hepatitis or a history of hepatitis, metastatic cancer, liver transplantation, hemochromatosis, and thalassemia major [1, 2]. Individuals with therapeutically induced or naturally low gastric acid, such as those affected by achlorhydria, are also at risk [2]. Finally, individuals with compromised immune systems are especially at risk. This group includes individuals with AIDS or AIDS-related complexes and chemotherapy patients. It is reported that an individual is up to 80% more likely to develop septicemia if in an immunocompromised state [2]. Other linked conditions include diabetes mellitus, renal disease, chronic intestinal disease, and steroid dependency.

Of the three clinical syndromes, primary septicemia is considered the most common and the most deadly, with mortality rates exceeding 50% [2]. Of 422 cases documented from 1988 to 1996, 43% were classified as primary septicemia, 45% were classified as wound infections, and only 5% were classified as gastroenteritis [3]. Primary septicemia is characterized by the abrupt onset of fever and chills, the development of secondary lesions within the first 24 hours, intense lower extremity pain, vomiting, diarrhea, abdominal pain, nausea, and hypotensive septic shock. The secondary lesions are presented as cellulitis, bullae, and ecchymosis. These lesions can quickly become necrotic, requiring surgical debridement or amputation. Patients who become hypotensive within the first 12 hours are twice as likely to die from infection than those with a delay in the development of hypotension [1-3]. Individuals who suffer from wound infection usually have had direct contact with seawater on open wounds. This syndrome is characterized
by chills, fever, and inflammation at the wound site which can advance to cellulitis. Similar to primary septicemia, the wound can quickly become necrotic [2]. Mortality rates for those with wound infections are much lower, around 20-30%. There are far fewer cases of gastroenteritis, which is characterized by vomiting, diarrhea, and abdominal pain, and this syndrome is rarely fatal [2].

Regarding treatment, it is vital that patients receive medical care as soon as possible after infection. Supportive care with intravenous (IV) fluids and intensive-care (IC) units are essential. Surgical debridement, and sometimes amputation, is necessary to treat lesions and wounds. Early antibiotic treatment has shown some efficacy. *Vibrio vulnificus* is susceptible to a wide variety of antibiotics *in vitro* including tetracycline, erythromycin, ampicillin, cephalosporin, chloramphenicol, norfloxacin, and gentamicin [2]. The most effective treatment appears to be tetracycline in combination with gentamicin or chloramphenicol. Despite some efficacy, the associated mortality with primary septicemia is high.

Elevated serum iron levels are directly correlated with the infectious dose, and there are several theories regarding how iron confers an increased risk for infection. One theory is that iron enhances the growth of *V. vulnificus*, and it was shown that excess serum iron increased the growth rates of this pathogen in mice [3]. A second theory suggests that excess serum iron results in decreased neutrophil activity in the host organism. Individuals with chronic liver disease were shown to have an overall decrease in neutrophil activity. Elevated serum iron levels may play multiple roles, and these two theories are not mutually exclusive.

*Vibrio vulnificus* has been shown to have a number of adaptations to help it invade and survive in the host organism. Once it passes through the digestive tract, *V. vulnificus* enters the
bloodstream and encounters the host’s immune response. Neutrophils are usually the first responders, but, as previously discussed, high risk individuals already show decreased neutrophil activity. In fact, survival of this pathogen is inversely correlated with phagocytosis by neutrophils [3]. In addition to neutrophils, the bacteria also encounter macrophages and other leukocytes. Clinical strains of *V. vulnificus* have been shown to induce macrophage apoptosis, and in a mouse model, neutrophils alone were not able to clear an infection in the absence of macrophages.

*Vibrio vulnificus* is known to possess a number of virulence factors including a hemolysin, an extracellular protease, a phospholipase, siderophores, and capsular polysaccharides. An *in vivo*-induced antigen technology (IVIAT) method was used to identify *V. vulnificus* genes induced *in vivo* [4]. Several classes were identified including genes involved in chemotaxis, signaling, biosynthesis, secretion, and transcriptional activation. Mutations in these genes were made and cytotoxicity was determined in HeLa cells using log-phase cultures. Three genes showed significant decreases in cytotoxicity when mutated: two genes involved in biosynthesis and metabolism (*pyrH* and *purH*) and a gene involved in transcription, *hlyU*. The effect of these on lethality was determined using a mouse model. The LD$_{50}$ increased 53-fold with the Δ*hlyU* strain, 14-fold with the Δ*pyrH* strain, and 22-fold with the Δ*purH* strain.

However, as previously discussed, individuals with higher iron serum levels are more likely to suffer from a *Vibrio vulnificus* infection. Kim et al. used an iron-normal mouse model to determine the LD$_{50}$ of the Δ*hlyU* mutant. Therefore, to determine the LD$_{50}$ in a more appropriate system, an iron-overloaded mouse model was utilized [5]. The LD$_{50}$ increased from fewer than 10 colony forming units (CFU) for the wild-type to approximately 2.4x10$^5$ CFU for
the ΔhlyU mutant, establishing the hlyU gene product as a major virulence factor in both iron-normal and iron-overloaded systems.

The hlyU gene of Vibrio vulnificus is homologous to the hlyU gene of Vibrio cholerae which encodes the transcriptional upregulator HlyU [4]. In Vibrio cholerae, this protein upregulates the hemolysin HlyA. HlyU was classified into a small regulatory family including NoIR of Rhizobium meliloti, SmtB of Synechococcus elongatus sp. strain PCC7942, and ArsR of Staphylococcus aureus, and these proteins contain a conserved DNA binding helix-turn-helix (HTH) motif. In addition, Vibrio vulnificus produces the hemolysin VvhA which shares a homologous domain with HlyA. The Vibrio vulnificus ΔhlyU mutant did not produce VvhA. Thus, HlyU was implicated as a potential master regulator.

However, further studies brought to question the role of VvhA in virulence. For one, it is known that both virulent and avirulent strains produce VvhA. Several different animal models were used, and ΔvvhA mutants did not show a decrease in pathogenicity when compared to the wild-type [6]. However, VvhA lysed mammalian erythrocytes from 17 different species, was toxic to Chinese hamster ovary (CHO) cells, and was lethal for mice [7]. In addition, VvhA directly caused hypotension and tachycardia in rats [8]. Therefore, while VvhA may not be a major virulence factor, it is possible that it could play a role in the pathogenesis of hypotensive shock.

Due to the increase in the LD_{50} in ΔhlyU mutants, it was thought that HlyU likely regulates the expression of other virulence-related genes. A microarray analysis of wild-type and ΔhlyU strains growing in trypticase soy broth (TSBS) was used by Liu et al. to identity significant differences in transcription patterns [5]. A gene cluster with a decrease in expression
in the ΔhlyU strain when compared to wild-type was located on chromosome II and includes three open reading frames (ORFs): VV20479, VV20480, and VV20481. VV20479 possesses a high degree of similarity (89%) with the MARTX toxin RtxA of *Vibrio cholerae*, and was renamed *rtxA1*. VV20480 and VV20481 encode a predicted hemolysin acyltransferase and a predicted peptide chain release factor 1, respectively. To determine if this gene cluster is regulated by HlyU, RT-PCR was performed on VV20481 and *rtxA1*; both were significantly downregulated in the ΔhlyU mutant.

The cytotoxicity of each of these genes was determined by incubating the wild-type and mutant strains with HeLa cells [5]. Deletions of either *hlyU* or *rtxA1* resulted in an abolishment of cytotoxicity. However, deletion of the putative hemolysin acyltransferase and peptide chain release factor I did not result in a change in cytotoxicity when compared to wild-type. The virulence of these mutant strains was determined using an iron-overloaded mouse model. Compared to the LD$_{50}$ of the wild-type strain, fewer than 10 CFU, the Δ*rtxA1* strain had an increase in the LD$_{50}$ to 2.5x10$^3$ CFU, and the ΔVV20480-VV20481 only had an increase to 184 CFU.

While it appeared that HlyU directly regulates this operon and *vvhA*, it was not clear how HlyU was regulating the system. A β-galactosidase assay was performed in order to monitor expression *in vivo* [5]. An 803-bp fragment, spanning from 750 bp upstream to 53 bp downstream of the VV20481 start codon, was cloned upstream of the *lacZ* gene and conjugated into a *Vibrio vulnificus ΔlacZ* mutant. With HlyU present, the level of β-galactosidase activity was approximately 8,369 Miller units. In the ΔhlyU strain, however, this activity reached only half of that level, a value of approximately 3,397 Miller units. When the ΔhlyU strain was
complemented with the wild-type \textit{hlyU} gene, \(\beta\)-galactosidase activity was restored to the wild-type level, demonstrating that HlyU regulates expression at the transcriptional level.

In order to determine whether HlyU directly binds to DNA, electrophoretic mobility shift assays (EMSA) were performed using purified HlyU-His\textsubscript{6} protein and various DNA sequences upstream of the VV20481 start codon. HlyU was found to bind the probe containing a 246-bp fragment between base pairs 514 and 750 upstream of the VV20481 start codon. In the presence of unlabeled, nonspecific competitor DNA, a more pronounced shift was seen as the concentration of labeled, specific DNA was added. This could be undermined by adding unlabeled, specific probe DNA. This experiment showed that HlyU directly binds DNA in a sequence-specific fashion.

To further understand HlyU function, the transcription start site was identified using an RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) assay to be 227 base pairs upstream of the putative start codon [9]. These data, combined with the previous EMSA data, limited the possible HlyU binding site to a region between 260 and 523 base pairs upstream of the transcription site. This distance from the transcription site is larger than that of most conventional positive regulators. A ribonuclease protection assay was used to determine whether any other transcripts initiated between the HlyU region and the transcription start site, but no other transcripts were identified [9]. Finally, a DNase 1 footprinting assay was used to determine the nucleotide sequence bound by HlyU. Protection was seen between 376 and 417 base pairs upstream of the transcription start site. This protected sequence is AT-rich and is an imperfect palindrome, implying that more than one HlyU molecule may be binding to the region.
These data, however, do not explain how HlyU might be regulating this operon. Progressively shorter regions upstream of the promoter region were analyzed using β-galactosidase assays with and without HlyU [9]. Using the full-length region, the β-galactosidase activity was higher in hlyU+ strains than in hlyU− strains, consistent with the hypothesis that HlyU is a positive regulator. A 95 base pair deletion that did not affect the HlyU binding site showed a 1.6-fold increase in β-galactosidase activity in hlyU+ background and a 4.1-fold increase in the hlyU− background. In fact, the β-galactosidase activities were now similar in both hlyU+ and hlyU− strains. Furthermore, activity remained approximately the same when the HlyU protected site was deleted. However, when the remainder of the sequence was deleted, leaving only the -35 and -10 RNAP binding sites, a defect in promoter activity was observed. An alternate theory was needed to explain these seemingly contradictory data. It was known that HlyU positively regulates the operon, but an increase in transcription levels was seen independent of HlyU when the region upstream of the promoter was deleted. It was hypothesized, therefore, that HlyU might be countering the activity of a repressor protein rather than directly activating transcription.

In an attempt to identify a repressor may that be involved in the regulation of the rtxA1 operon, Liu et al. [9] discovered several homologues of H-NS, a known repressor in many different bacterial species. The two H-NS homologues were identified in Vibrio vulnificus as VV12923 and VV10374. The ΔVV12923 mutant showed an increase in transcription levels of rtxA1 and VV20481 when compared to the wild-type while the ΔVV10374 mutant did not. Therefore, VV12923 was designated as the hns gene.
To determine the regulatory nature of the *hns* gene product, β-galactosidase assays were used to monitor *in vivo* expression [9]. β-galactosidase activity was measured to be 16,230 Miller units in *hns* strains but approximately 7,558 Miller units in *hns* strains, indicating that H-NS is involved in negative regulation. β-galactosidase assays were then performed with a plasmid containing multiple copies of *hlyU*. In *hns* strains, the multicopy *hlyU* increased the expression of β-galactosidase by a factor 2. However, in the *hns* strains, the increased amount of HlyU had no effect on expression, demonstrating that HlyU works by negating the function of a repressor rather than by directly augmenting transcription.

To determine the mechanism by which H-NS represses transcription of the *rtxA1* operon, gel shift assays were performed with DNA probes upstream and downstream of the transcription start site [9]. These assays indicated that H-NS is able to bind multiple regions of DNA. A DNase I footprinting assay using the 264 base pair fragment known to bind HlyU showed protection across the entire sequence except for a few GC-rich regions. In fact, two of the protection sites overlap with the HlyU protection site. In one possible mechanism, H-NS could mediate DNA bridging, tying up the sequence into loops. Competitive gel shift assays and footprinting assays were performed by adding either H-NS or HlyU to pre-bound DNA complexes of the other protein. It was found that low concentrations of HlyU were able to affect the binding of H-NS, and higher concentrations of H-NS were needed to substitute HlyU. It is unclear how exactly HlyU negates the repressive activity of H-NS, but HlyU binding may destabilize the H-NS::DNA complex or HlyU binding might block the H-NS polymerization process.
It is clear that HlyU is important in the regulation of several key virulence factors, making it an ideal drug target. In this work, I determined both expression and purification protocols for the His-tagged HlyU construct. Using size-exclusion chromatography, I demonstrated that HlyU exists as a dimer in solution. Some conditions of the apo-protein and protein-DNA complex have been found to produce crystals, and they can be optimized for future structure determination. A cleavable His-tag construct was also cloned in an attempt to crystallize HlyU. I constructed a homology model based on the related SmtB metallorepressor. This model is not conclusive about HlyU’s metal ion binding capability, although metal analysis results do not show metal ion ligands in a significant quantity. Thermal denaturation assays do not indicate that HlyU is stabilized by the presence of divalent metal ions. DNA binding in varying conditions was monitored using a thermal denaturation assay. In addition, a mutant construct was made based upon the homology model to determine key amino acid residues involved in DNA binding.

Recently, a different group published the crystal structure of HlyU at a resolution of 2 Å [10]. The conclusions made from this structure are consistent with the data presented in this work, and many new questions arise based on this information.
Materials and Methods

Bacterial strains, plasmids, and primers

The bacterial strains, plasmids, and primers used in this work are listed in Table 1.1. Primers were synthesized by Eurofins Operon. Bacteria were grown in Luria-Bertani broth (LB) supplemented with appropriate antibiotics. Antibiotics were used at concentrations of 100 µg/mL ampicillin and 50 µg/mL kanamycin.

<table>
<thead>
<tr>
<th>Strain or Plasmid</th>
<th>Characteristics</th>
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<tr>
<td>DH5a</td>
<td>F- Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rk-, mk+) phoA supE44 λ- thi-1 gyrA96 relA1</td>
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<td>XL-10 Gold</td>
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<td>F-</td>
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<tr>
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<td>F-ompT hsdS8 (rB-mB') gal dcm (DE3)</td>
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<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pET200</td>
<td>A protein expression vector with N-terminal His6 tag; Km</td>
</tr>
<tr>
<td>pProEX HT</td>
<td>A protein expression vector with N-terminal His6 tag; Km</td>
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<td>pET200-HlyU</td>
<td>pET200/D derivative containing the hlyU gene</td>
</tr>
<tr>
<td>pET200-HlyU (S54A)</td>
<td>pET200/D derivative containing mutant hlyU gene</td>
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<tr>
<td>pProEX HT-HlyU</td>
<td>pProEX derivative containing the hlyU gene</td>
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Table 1.1 Bacterial Strains and Plasmids

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<th>Sequence</th>
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Table 1.2 Primers
Site-directed mutagenesis of HlyU

The pet200c-hlyU construct was mutated using the Quik Change site-directed mutagenesis kit using primers HlyU_S54A and HlyU_S54A_r. Samples were prepared and cycled according to the manufacturer’s instructions. Original template DNA was digested by incubating with DpnI for 1 hour at 37°C. Mutated plasmids were transformed into XL-10 Gold Ultracompetent cells and plated on LB/Kan agar. Plasmid DNA was purified from overnight liquid cultures using the QIAprep® Spin Miniprep Kit (Qiagen, Valencia, CA), and the mutation was confirmed by sequencing (Genewiz, La Jolla, CA).

Construction of HlyU His-tag cleavable construct

The hlyU gene was PCR amplified using primers HlyU_C and HlyU_C_r; the former containing an overhang with both Precision and TEV protease cut sites. 5 µL of Pfu buffer, 5 µL of 10 mM dNTPs, 1 µL of 20 ng/µL DNA template, 1 µL of 20 mM forward primer, 1 µL of 20 mM reverse primer, 1 µL of Pfu, and 36 uL of ddH20 were combined for a total reaction volume of 50 µL. The presence of the 400 bp amplified region was confirmed using agarose gel electrophoresis. The DNA was purified from the agarose gel using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Orange, CA).

Both the PCR amplified product and the empty pProEX HT plasmid were double digested with BamHI and PstI. 10 µL of DNA, 2 µL of BSA, 2 µL of NEBuffer 3, 1 µL BamHI, 1 µL PstI, and 4 µL ddH2O were combined for a total reaction volume of 20 µL. The reaction was carried out at 37°C for approximately 4 hours. The resulting product was further purified using the DNA Clean and Concentrator™ Kit (Zymo Research), and the DNA concentration was determined using UV-Vis spectroscopy.
The digested HlyU amplified region and proEX HT plasmid were ligated by combining 5 μL of plasmid, 3 μL of insert, 1 μL of 10X ligation buffer, and 1 μL of T4 ligase. The reaction was allowed to proceed overnight at 16°C.

The ligated product was transformed into Top10 cells and plated on LB/Amp plates. DNA was purified from overnight liquid cultures using the QIAprep® Spin Miniprep Kit (Qiagen), and the DNA was sequenced to confirm the presence of the insert (Genewiz).

SDM was performed using primers HlyU_C_SDM and HlyU_C_SDM_r to reinsert two missing base pairs and restore the reading frame. Samples were prepared and cycled according to the manufacturer’s instructions. Original template DNA was digested by incubating with Dpn1 for 1 hour at 37°C. Plasmids were transformed into XL-10 Gold Ultracompetent cells and plated on LB/Amp. Plasmid DNA was purified from overnight liquid cultures using the QIAprep® Spin Miniprep Kit (Qiagen), and the insertion was confirmed by sequencing (Genewiz).

**Overexpression and purification of the HlyU protein**

The six-His tag expression plasmid pET200 encoding HlyU with an N-terminal fusion tag was obtained from Jorge Crosa (Oregon Health and Science University, Portland, Oregon). Five milliliters of overnight bacterial culture growing at 37°C in Luria-Bertani broth supplemented with kanamycin were inoculated in 1 L of the same fresh medium. When the OD$_{600}$ reached 0.6, 1 mM IPTG was added to induce the expression of the HlyU protein, and the bacteria were allowed to grow overnight at room temperature. The cells were collected and stored at -80°C.
Cells were resuspended in approximately 60 milliliters of 2X Phosphate buffered saline (PBS) containing 10 mM Imidazole and sonicated using the Sonic Dismembrator Model 500 (Fisher Scientific) at intervals of 15 seconds on and 30 seconds off for a total time of 3 minutes. Insoluble material was separated out by centrifuging at 13,000 RPM for a total time of 45 minutes.

The resulting lysate was applied to 10 mL of TALON Metal Affinity Resin (Clontech, Mountain View, CA) and allowed to incubate in batch while rocking overnight at 4°C. The flow through was collected and saved for gel analysis. The column was washed with 10 column volumes of 2X PBS containing 20 mM Imidazole, 3 column volumes containing 50 mM Imidazole, 2 column volumes containing 100 mM Imidazole, 3 column volumes containing 300 mM Imidazole, and 2 column volumes containing 500 mM Imidazole.

Fractions were analyzed using SDS-PAGE and samples containing HlyU were pooled and concentrated using Amicon® Ultra-15 Centrifugal Units with Ultracel-3 membranes (Millipore) to a final volume of 2 mL. This sample was further purified using size exclusion chromatography using a Hiprep™ 16/60 Sephacryl™ S-200 High Resolution size exclusion column (GE Healthcare). Protein was eluted using 2X PBS or Tris Buffer at a flow rate of 0.25 mL/min. Results were analyzed using UV-Vis spectroscopy and SDS-PAGE. The concentration of the purified HlyU protein was determined using the Bradford protein assay kit (Bio-Rad, Philadelphia, PA). The protein solution was concentrated to a final concentration of 20 mg/mL and stored with 15% glycerol at -80°C.
**Analytical size exclusion chromatography**

A Superdex 200 5/150 GL column (GE Healthcare, Piscataway, NJ) was used to perform analytical size exclusion chromatography. The column was calibrated using the GE Low Molecular Weight Gel Filtration Calibration Kit (GE Healthcare) containing ribonuclease A (MW 13,700 Da), Chymotrypsinogen A (MW 25,000 Da), Ovalbumin (MW 43,000 Da), Albumin (MW 67,000 Da), and Blue Dextran (MW 2,000,000 Da). Standards were reconstituted in 2X PBS. The size exclusion column (SEC) was equilibrated using the same buffer. Each sample was run 3 times to determine the elution volume. The molecular weight of HlyU was calculated using the formula:

\[ K_{av} = \frac{(V_e - V_O)}{(V_T - V_O)} \]

where \( V_e \) is the elution volume, \( V_O \) is the dead volume, and \( V_T \) is the total column volume. The \( \log_{10} \) of each molecular weight standard was determined, and the \( K_{av} \) was plotted against the \( \log_{10} \) values. From this, the \( \log_{10} \) value was determined for the HlyU sample, and the original molecular weight was calculated.

**Fluorescence-based thermal denaturation assay**

Protein stability was measured using a thermal denaturation assay with the fluorescent dye SYPRO® Orange Protein Stain (Invitrogen, Carlsbad, CA). Dye was diluted from the stock concentration of 5,000X using ddH₂O. 20 µL samples were prepared in MicroAmp® Fast Optical 96-Well Reaction Plates (Applied Biosciences, Foster City, CA). Plates were covered with MicroAmp® Optical Adhesion Film (Applied Biosciences) and analyzed in a StepOnePlus™ Real-Time PCR System (Applied Biosciences) using a temperature gradient of 25ºC to 95ºC with an increment of 0.3ºC per minute.
Initial calibrations were made by varying protein and SYPRO concentrations. For subsequent runs, a final concentrations of 12 µM protein and 12X dye were used. DNA concentrations were varied from 0 µM to 22 µM, and metal ion concentrations varied from 0 µM to 500 µM.

**Crystallization**

Multiple screens were utilized to find and optimize crystal conditions for HlyU including the Crystal screen, Index screen, SaltRX screen, Natrix screen (Hampton Research, Aliso Viejo, CA), Wizard I screen, Wizard II screen, and Wizard III screen (Emerald Biosystems, Bainbridge Island, WA). Screens were set up using the sitting drop method with the Phoenix™ liquid handling robot (Rigaku, The Woodlands, Texas), setting up 0.3 µL drops with a sample to mother liquor ratio of 1:1 and a mother liquor volume of 50 µL. Optimization screens were set up using the hanging drop method. Temperature, drop to mother liquor ratio, protein to DNA ratio, and condition concentrations were altered in an attempt to obtain and optimize crystallization conditions.

**Fold recognition and sequence alignment**

Sequence searches of the non-redundant (nr) database were carried out at NCBI using PSI-BLAST, using the *Vibrio vulnificus* HlyU (Vv-HlyU) sequence as a query [11]. Secondary structure prediction and tertiary fold-recognition were carried out using the GeneSilico meta-server gateway [12], and Prime. Multiple alignments were generated using the T-coffee server [13].
**Homology model construction**

A homology model was generated using Prime [14] through the Maestro graphical user interface [15] of the Schrödinger Suite Software Package. The crystal structure of SmtB from *Synechococcus elongatus* PCC7942 (PDB entry: 1R22) [16] was used as a template. The HlyU sequence was used as an input sequence, and the secondary structure elements of SmtB were used with slight modifications. Structures were modeled using the PyMol Graphics System ([http://pymol.org](http://pymol.org)) and Chimera [17].

A second homology model was generated using the Swiss-Model server [18-20] using the *Vibrio vulnificus* HlyU sequence and the structure of SmtB from *Synechococcus elongatus* PCC7942 (PDB entry: 1R22) [16] as a template. Structures were modeled and images made using the PyMol Molecular Graphics System ([http://pymol.org](http://pymol.org)) and Chimera [17].

**Metal analysis**

Samples were sent to Applied Speciation and Consulting, LLC for metal analysis. A blank of 2X PBS was used. Samples were received by the company in 15 mL centrifuge tubes and stored at 0.4°C. All samples for trace metal quantification were preserved with 10% HNO3 (v/v) prior to analysis via inductively coupled plasma dynamic reaction cell mass spectrometry (ICP-DRC-MS).

All samples were prepared in laminar flow clean hoods free from trace metals contamination. All applied water for dilutions and sample preservatives are monitored for contamination for any biases associated with the sample results.

All samples for trace metal quantification were analyzed by inductively coupled plasma dynamic reaction cell mass spectrometry (ICP-DRC-MS) on June 23, 2009. Aliquots of each
sample are introduced into a radio frequency (RF) plasma where energy transfer processes cause desolvation, atomization, and ionization. The ions are extracted from the plasma through a differentially-pumped vacuum interface and travel through a pressurized chamber (DRC) containing a specific reaction gas which preferentially reacts with interfering ions of the same target mass to charge rations (m/z). A solid-state detector detects ions transmitted through the mass analyzer, on the basis of their mass-to-charge ratio (m/z), and the resulting current is processed by a data handling system.
Results

Overexpression and purification of the V. vulnificus HlyU protein

The His-tagged HlyU protein was purified using TALON metal affinity resin and the resulting fractions were analyzed by SDS-PAGE. As seen in figure 1.1, several other bands are present along with the band at 13.5 kDa corresponding to the HlyU protein. A second purification with a size-exclusion column was used to isolate this protein, and figure 1.1 shows no other bands present. Later purification attempts used 10 mM imidazole in the wash buffer and resulted in fewer impurities in the elutions. Once purified, the samples were concentrated to 20 mg/mL and stored at -80°C. It was noted that the protein would precipitate out of solution unless stored in a high salt buffer (500 mM NaCl).

Analytical size exclusion chromatography

Protein size standards and HlyU were run on a size exclusion column to determine retention volumes. Although all samples were at a concentration of 10 mg/mL, the absorbance peak of HlyU appears significantly lower than those of the protein standards. This is likely due to HlyU’s small size and low abundance of aromatic side chains.

First, $K_{av}$ values were calculated for each sample using the equation:

$$K_{av} = \frac{(V_e - V_O)}{(V_T - V_O)}$$

The value for $V_T$, the total column volume, was 3 mL. The value used for $V_O$, the dead volume, was the elution volume of the Blue Dextran protein. $K_{av}$ values were plotted against the log values of the molecular weights of the protein standards, and a linear regression was performed. The resulting equation, $y=-0.4327x+2.5185$ was used to determine the molecular weight of HlyU.
by substituting the calculated $K_{av}$ value. This equation yields a calculated molecular weight of approximately 27 kDa, twice the value of the known monomeric molecular weight, 13.5 kDa.

**Crystallization of HlyU and the DNA-Bound complex**

Crystallization screens were set up using both the sitting drop and hanging drop methods. Crystals were obtained from drops containing HlyU and from drops containing both HlyU and the 42 base pair oligonucleotide. Morphologically, crystals were either asymmetrical or hexagonal in shape. Diffraction patterns obtained from the hexagonal crystals were not characteristic of salt. Rings were also observed in the pattern characteristic of DNA. However, these crystals did not diffract to a high enough resolution to index or to determine the structure.

Hexagonal crystals were obtained from several different conditions. One condition contained 36% MPD, 0.1 M sodium cacodylate, and 0.15 magnesium acetate. A protein to DNA concentration of 2:1 was used. A second condition contained 20% PEG 1000, 0.1 M imidazole, pH 8.0, and 0.2 M Ca(OAc)$_2$. Attempts to optimize these conditions were made, but these conditions were not always successfully replicated.

**Construction of HlyU cleavable construct**

Following ligation into the pProEX HT plasmid, products were transformed into Top10 cells. Plasmid purified from liquid cultures was sequenced. Sequencing results indicated a frame shift had occurred due to the deletion of 2 base pairs upstream of the protein coding sequence in the region coding for the cleavable His-tag. Site-directed mutagenesis was performed to reinsert the missing two base pairs to restore the reading frame.
Homology model of HlyU

As a crystal structure of HlyU could not be obtained, a homology model was built with Maestro and Prime using the HlyU sequence and the template structure of SmtB from *Synechococcus elongatus* PCC7942 (PDB entry: 1R22), a zinc-binding metallorepressor that shares 40% identity and 62% similarity with HlyU (Fig 1.5).

DNA binding assay

Figure 1.5 shows that HlyU is predicted to be a winged helix-turn-helix DNA-binding protein. Each monomer contains a helix-turn-helix motif followed by a beta hairpin “wing.” The helix-turn-helix motif consists of $\alpha_3$-turn-$\alpha_4$, where $\alpha_4$ is considered the recognition helix and likely interacts with the major groove of DNA.

Electrophoretic mobility shift assays performed by Liu *et al.* showed that HlyU was able to bind a DNA region upstream of the transcription start site [5]. DNase I footprinting assays performed by the same group determined a 42 base pair region protected by HlyU. Denaturation assays were performed to determine the overall stability of the HlyU protein. A double stranded 42 base pair synthesized oligonucleotide of the binding region was used as substrate. The melting temperature of HlyU was found to increase with the concentration of DNA up to a point where it leveled off. In total, samples with the highest concentration of DNA used showed a 5°C shift above the melting temperature of samples without DNA.

Sequence alignment with homologues of HlyU reveals several conserved residues located in the $\alpha_4$ helix. S54 is conserved in 4 related proteins, and may be involved in DNA recognition. A construct was made in which this amino acid was mutated to an alanine through site-directed mutagenesis, and this mutant will be characterized in future studies.
**Metal analysis**

HlyU belongs to a family of metallopressors, but it was not known whether HlyU is able to sense or bind metal ions itself. The three homologues analyzed, SmtB, CzrA, and CadC contain two metal ion binding sites. The first site is formed from α3 and several residues from the N-terminus. The second site is formed by the α5 helix from both monomers. Sequence alignments reveal that HlyU lacks the N-terminal residues needed for the α3N site, and is therefore unlikely to bind a metal ion there. Samples were sent for metal analysis by inductively coupled plasma dynamic reaction cell mass spectrometry (ICP-DRC-MS), but these results do not indicate the presence of significant quantities of metal ion.

Thermal denaturation assays were performed using several divalent metal ions. The melting temperature of HlyU did not vary with most metal ions. However, the melting temperature showed a consistent and sharp decrease when Cu(II) was present in solution.
Fig 1.1 SDS-PAGE analysis comparing the elutions of protein from the TALON resin with the elution purified from the size-exclusion column following TALON purification. The 50 mM imidazole elution, lane 4, shows the overexpressed HlyU protein at 13.5 kDa along with a number of other protein contaminants. Two peaks were observed in the chromatograph from the SEC. The first peak, lane 2, corresponds to larger molecular weight contaminant proteins, and the second peak, lane 3, corresponds to HlyU.
Fig 1.2 Analytical Size Exclusion Chromatograph of Standards and HlyU

Fig 1.2 Each standard was run three times. Blue Dextran (2,000 kDa) indicates the void volume of the column. Additional standards included albumin (MW 67 kDa), ovalbumin (MW 43 kDa), chymotrypsinogen A (MW 25 kDa), and ribonuclease A (MW 13.7 kDa). HlyU eluted at a volume of 2.34 mL, between ovalbumin and chymotrypsinogen A, estimating the molecular weight to be between 25 kDa and 43 kDa.

Fig 1.3 Analytical Size Exclusion Standards and Calculations

Fig 1.3 Plot of the calculated $K_a$ values for ribonuclease A, chymotrypsinogen A, ovalbumin, and albumin versus the Log$_{10}$ of their respective molecular weights. The resulting linear trend line was used to calculate the molecular weight of HlyU based on the calculated $K_a$, and the molecular weight was determined to be 27 kDa. The molecular weight of the denatured protein was previously known to be 13.5 kDa.
Fig 1.4 Crystals of HlyU-DNA Complex

Fig 1.4 Crystals obtained of HlyU with the 42 bp oligonucleotide. A monomer to DNA ratio of 2 to 1 was used. (A) Crystal obtained from 36% MPD, 0.1 M sodium cacodylate, and 0.15 magnesium acetate. (B,C) Crystals obtained from 20% PEG 1000, 0.1 M imidazole pH 8.0, and 0.2 M Ca(OAc)$_2$. Crystals were grown at 20°C.
Fig 1.5 Homology Model of HlyU

(A) Ribbon diagram of the monomer showing the α1-α2-α3-α4-β1-β2-α5 fold. The α3-turn-α4 helix-turn-helix motif is characteristic of DNA binding proteins, and the α4 helix is thought to be the primary recognition element. (B) Ribbon diagram of the HlyU dimer showing secondary structure. All images were made with Chimera [17].
Fig 1.6 Sequence Alignment of α4 Recognition Helix

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B

Fig 1.6 Sequences of HlyU and homologues were aligned using T-coffee server [13] and the homology model was visualized using PyMol (PyMol, [www.pymol.org](http://www.pymol.org)) (a) Sequence alignment of VvHlyU with SmtB from *Synechococcus elongatus* PCC7942, CzrA from *Staphylococcus aureus*, CadC from *Staphylococcus aureus* p1258, and HlyU from *Vibrio cholerae*. One secondary structure element, alpha helix α4, is shown and is thought to be the recognition helix of the major groove of DNA as part of the helix-turn-helix motif. VvHlyU S54 is completely conserved, and may be a vital residue in DNA recognition and binding. (b) Homology model of VvHlyU depicting the helix-turn-helix motif. S54 and Q55 extend outward from the helix and may be important in recognizing the major groove of DNA.
Fig 1.7 Thermal denaturation assay performed using 12 µM protein, 12X SYPRO Orange Dye, and varying concentrations of HlyU_42 oligonucleotide DNA from 0 µM to 6 µM. Data were normalized, and the melting temperature was defined as when the fraction unfolded is equal to 0.5. Melting temperature increased with an increase in DNA concentration. For concentrations of 0, 1, 2, 3, and 6 µM DNA, the melting temperatures were 63.1°C, 64.3°C, 65.5°C, 66.7°C, and 67.6°C respectively. From 0 µM to 6 µM DNA, a total melting temperature shift of 4.5°C was observed.
Fig 1.8 Sequence Alignment of Metal Ion Binding Sites

A

<table>
<thead>
<tr>
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<th>a3N Site</th>
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<tr>
<td>SmtB</td>
<td>mtkpvlqgdetvvggtlaaiaselq-aiapevaqlaeffavladpnrlrlslsll-ars</td>
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<tr>
<td>CzrA</td>
<td>mseqy--s------------------------eintdtlerveifkalgdynririmeq-svs</td>
</tr>
<tr>
<td>CadC</td>
<td>mkkkdte-ze-if--yydeekvngdqlgtvdisgsvsilkaiadenakityacqde</td>
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<td>vCHlyU</td>
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<td>vVHlyU</td>
<td>m--------------------------nlk-dmeqnsak-avvllkamanerrlqilcml-hnq</td>
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<th>a5 Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>SmtB</td>
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</tr>
<tr>
<td>CzrA</td>
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B

Fig 1.8 (A) Sequence alignment of VvHlyU with SmtB, CzrA, CadC, and VcHlyU. Although VvHlyU has a similar ELCVGD motif, but lacks the N-terminal residues involved in the a3N metal ion binding site. While the sequence is similar in the a5 metal ion binding site, the homology model does not necessarily indicate that HlyU is able to bind metal ions. Several possible metal ion ligands are present, but H92 is pointing away from the site. Image was made using PyMol (PyMol, www.pymol.org).
Table 1.3 Metal Analysis of HlyU

<table>
<thead>
<tr>
<th>Analyte</th>
<th>HlyU (mg/L)</th>
<th>Blank (mg/L)</th>
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</thead>
<tbody>
<tr>
<td>Mg</td>
<td>0.013</td>
<td>0.015</td>
</tr>
<tr>
<td>Ca</td>
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<td>ND</td>
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<tr>
<td>Mn</td>
<td>ND</td>
<td>ND</td>
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<td>Fe</td>
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</tr>
<tr>
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<td>ND</td>
</tr>
<tr>
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</tr>
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</tr>
<tr>
<td>Zn</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Table 1.3 Metal analysis was performed by ICP-DRC-MS for a number of possible analytes. None of the analytes were present in significant quantities over the blank buffer. ND indicates that an analyte was not detected.

**Fig 1.9 Thermal Denaturation Assay for Metal Ion Binding**

Fig 1.9 Thermal denaturation assay performed using 12 µM protein, 12x SYPRO Orange Dye, and 50 µM concentrations of several divalent cations. Data were normalized, and the melting temperature is defined as when the fraction unfolded is equal to 0.5. Melting temperature did not vary from samples with no metal ions when calcium, cobalt, zinc, or iron were added. Addition of copper shows a decrease in melting temperature of 4°C, indicating that copper may be destabilizing the protein.
Discussion

Purification of His-tagged HlyU

His-tagged HlyU was overexpressed using BL21(DE3) E. coli cells and purified using TALON metal affinity resin. A second purification step was performed by running the TALON purified protein over a size exclusion column, and this resulted in a high level of purity. As seen using SDS-PAGE analysis in figure 1.1, an additional faint band is observed at approximately 27 kDa and could either be contamination or a population of dimeric HlyU.

Size exclusion chromatography indicates that HlyU is a dimer

HlyU belongs to the SmtB/ArsR family of DNA-binding proteins that bind metal ions and DNA as a dimer. It was expected that HlyU would also be present in solution as a dimer, but this had not been shown. In addition, the His-tagged HlyU from Vibrio cholerae was shown to be a monomer in solution but dimerized upon His-tag cleavage [21]. Analytical size exclusion chromatography was used to determine if HlyU is a dimer in solution. Figure 1.2 shows the chromatographs of each standard and of HlyU. This alone estimated the size of HlyU to be between the molecular weight of chymotrypsinogen A (25,000 kDa) and ovalbumin (43,000). Calculations from the elution volumes of each standard and HlyU determined the molecular weight to be 27,000 Da, exactly twice the of the known molecular weight of the monomer. This experiment indicates that HlyU is a dimer in solution and that the N-terminal His-tag does not seem to interfere with dimerization.

Crystallization of HlyU and the DNA-bound complex

Crystallization was attempted with the apo-protein and with 42 and 43 base pair double stranded oligonucleotides of the sequence known to be protected by HlyU in DNase I
footprinting assays [5]. Hampton Research screens were used to determine possible crystallization conditions using varied temperatures, protein to mother liquor ratios, and protein to DNA ratios. As seen in figure 1.4, small crystals were obtained; some were asymmetric in shape while others were hexagonal. Diffraction patterns of the hexagonal crystals was not characteristic of salt crystals, but these crystals did not diffract to a high enough resolution to index the data or determine the structure. Rings characteristic of DNA were observed in the diffraction images.

The N-terminal His-tag, although it does not interfere with dimerization, could interfere with crystallization. Therefore, a construct was cloned with both Precision and TEV protease sites to remove the His-tag from the purified recombinant protein.

**Homology modeling**

As a crystal structure was not obtained, a homology model was built using the HlyU sequence and the template structure of SmtB from *Synechococcus elongatus* PCC7942 (PDB Entry: 1R22). Similar to the homology model built by Saha *et al.* of the *Vibrio cholerae* homologue [22], HlyU of *Vibrio vulnificus* exhibits a winged helix-turn-helix motif. As seen in figure 1.6, following the DNA-binding helix-turn-helix motif of \( \alpha_3 \)-turn-\( \alpha_4 \), there is a \( \beta \)-hairpin “wing.” Helix \( \alpha_4 \) is thought to be the primary recognition element and is likely to recognize the major groove of a DNA molecule. Sequence alignments with SmtB, CatR, CadC, and VcHlyU show that there are several highly conserved residues along this helix including S54, which may be key residues involved in DNA recognition (Fig 1.6). Figure 1.6(B) shows both S54 and Q55 protruding from \( \alpha_4 \), and these could possibly interact with DNA nucleotides through hydrogen bonding.
Monitoring of DNA binding using a high-throughput assay

A thermal denaturation assay was performed using the SYPRO orange protein binding dye. This dye binds to hydrophobic residues. As a protein unfolds, more hydrophobic residues, normally packed away from water, will be exposed. As the dye binds these residues, it emits a higher fluorescence signal. By normalizing the data, the melting temperature can be determined as the point when half of the protein is unfolded. Stabilization of a protein should lead to an increase in melting temperature, while destabilization would lead to a decrease in melting temperature. It is thought that binding of substrate would accompany a stabilization and thus an increase in melting temperature.

Initially, the system was calibrated by testing a range of protein and dye concentrations to empirically determine optimal conditions. Protein and dye concentrations of 12 µM and 12X (diluted down from the stock concentration of 5,000X using water), respectively, were used in subsequent assays. Figure 1.7 shows a denaturation assay using a range of DNA concentrations from 0 µM to 6 µM. An increase in melting temperature is observed as DNA concentration increases, showing that HlyU is stabilized by the DNA substrate. The melting temperature shifted from 63.1°C without DNA was present to 67.6°C with 6 µM DNA, a total shift of 4.5°C. The denaturation assay can therefore be used to test a number of DNA binding conditions at a time.

The homology model structure and sequence alignments were analyzed to determine possible key DNA binding residues. S54 is conserved among VvHlyU, VcHlyU, SmtB, CzrA, and CadC, and may be an important residue in DNA binding. S54 was then mutated to an alanine to determine if a decrease in DNA binding is observed. This construct has been made,
and future experiments will determine differences in DNA binding due to this serine to alanine substitution.

HlyU does not bind divalent cations

Belonging to a family of metal ion binding repressors, it was unclear whether HlyU is able to bind metal ions. Saha et al. compared two known metal ion binding sites with VcHlyU [22]. SmtB and CadC both possess the α3N binding site formed by several N-terminus ligands and a conserved ELCVG/CD motif. CzrA lacks the N-terminal ligands and does not bind metal ion in the α3N site. Similarly, VvHlyU, although it contains a somewhat conserved ELSVGE version of the motif, does not have the N-terminal ligands. Therefore, it is unlikely that HlyU binds metal ions in this site. SmtB also coordinates a metal ion in the α5 site using an aspartate, a glutamate, and two histidines, and this site is formed by residues from each monomer.

Sequence alignments show that HlyU has a serine, a glutamate, a histidine, and a tyrosine nearby in the sequence. An examination of the homology model shows that C96 is also nearby, and could be positioned to coordinate metal ion. In fact, Y95 is pointing away from the other residues: S81, E83, C96, and H92. H92 is also pointed away, but this could be an artifact of the homology model as this is not an actual crystal structure. In total, this model cannot eliminate the possibility of HlyU coordinating metal ion.

A fluorescent thermal denaturation assay was performed to determine whether the melting temperature would shift with the addition of metal ions with respect to the apo-protein. 50 µM divalent cations were added, but the melting temperature did not vary from wild-type with the addition of calcium, cobalt, zinc, or iron. A decrease in melting temperature of approximately 4°C was observed with the addition of 50 µM copper. However, the mechanism
of destabilization is not revealed by this assay. One possible model is that copper(II) ions favor the monomeric form of HlyU, resulting in destabilization and a drop of melting temperature. However, as the buffer is slightly alkaline at a pH of 7.5, the results could be explained by the biuret reaction in which peptides react with cupric copper and reduce it to cuprous copper. This reaction could destabilize HlyU, consistent with the observed decrease in melting temperature.

Closing remarks and future work

Liu et al. [5] previously showed using electrophoretic mobility shift assays that HlyU binds a region of DNA upstream of the transcription start site. This region was further narrowed down to a 42 base pair region using DNase I footprinting assays [9]. Here, I showed that a high-throughput denaturation assay can be used to measure DNA binding to the 42 base pair region. This assay can be used to further determine the conditions necessary to bind DNA. One mutation, S54A, was made using site-directed mutagenesis. The denaturation assay, along with EMSA gels, could show whether the mutant construct displays a lower affinity for DNA. The importance of other residues along the helix-turn-helix motif could also be determined by using mutant constructs.

Sequence alignments and the homology model show that HlyU does not contain the α3N metal ion binding site, but these results were not conclusive concerning the α5 site. Thermal denaturation assays showed that HlyU is not stabilized by metal ions, further indicating that HlyU might not bind metal ion. Metal analysis by mass spectrometry did not show a significant amount of any metal ions above the standard buffer. In addition, HlyU was active and able to bind DNA without the addition of metal ion in the thermal denaturation assays. HlyU was able to still bind DNA in the presence of the chelator EDTA in electrophoretic mobility shift assays.
HlyU does not appear to be regulated by metal ion, but its regulation remains unclear. Future experiments could determine at what level, transcriptional, translational, or posttranslational, HlyU is regulated.

Although a structure was not determined in this work, a 2 Å crystal structure was recently determined. This structure shows a similar overall fold to that of the homology model. In addition, this structure is consistent with the hypothesis that HlyU is unable to bind metal ions. However, a structure of the HlyU:DNA bound complex has yet to be determined. Determining the structure of this complex could reveal how HlyU recognizes DNA and why it has a higher binding affinity for the DNA sequence than the repressor H-NS.

A better understanding of the structure and function of HlyU will help elucidate how *Vibrio vulnificus* controls the production of virulence factors including the MARTX extracellular toxin. Small molecule inhibitors of HlyU, if developed, could be vital treatment for individuals infected with *V. vulnificus*, a disease with a mortality rate exceeding 50%.
Chapter 2: Oxidation of the Diphtheria Toxin Repressor Inhibits Activity

Introduction

The diphtheria toxin repressor (DtxR) is a well-studied, iron-dependent global regulatory protein from *Corynebacterium diphtheriae*, and it is also a prototype for iron-dependent repressor proteins in gram-positive bacteria. Here, evidence is provided that indicates DtxR may sense oxidative species, which in turn could trigger the upregulation of oxidative stress related factors.

Diphtheria is an acute, communicable disease caused by the gram-positive bacterium *Corynebacterium diphtheriae*. The bacterium usually localizes to the upper respiratory tract where it causes the formation of an inflammatory pseudomembrane. Diphtheria can be transmitted between individuals by direct contact, sneezing, and coughing. *C. diphtheriae* is able to affect any age group, but nonimmunized children are usually affected before the age of 5 [23].

The major virulence factor produced by *C. diphtheriae* is a 58 kDa extracellular toxin known as the diphtheria toxin [24]. It has been shown to inhibit protein synthesis in eukaryotic cells and cell-free extracts in a nicotinamide adenine dinucleotide (NAD) dependent fashion. The toxin transfers the adenosine diphosphate ribose (ADPR) moiety from NAD to elongation factor 2 (EF-2), a key member of the proteosome, inhibiting its activity and ultimately that of the proteosome. [24]. The toxin is absorbed into the circulatory system where it is able to damage remote organs and potentially cause death [23]. It has been known that toxin activity is regulated by iron; as iron levels deplete, toxin levels increase. This is not surprising as there are a number of other iron-regulated toxins including the shiga toxin from *Shigella dysenteriae* and exotoxin A from *Pseudomonas aeruginosa* [25].
A 25 kDa protein, the diphtheria toxin repressor, was found to bind the promoter region of the tox gene and repress in an iron-dependent manner [25, 26]. DtxR is able to bind a number of divalent cations other than iron including cadmium, cobalt, manganese, nickel, and zinc, and metal ion binding stabilizes dimer formation and activates the repressor, allowing it to bind DNA [24]. DNase protection assays identified a 30 base pair region protected by the binding of DtxR [25].

The structure of DtxR revealed three domains. The N-terminal domain contains a helix-turn-helix DNA-binding motif. The second domain contains the dimerization interface and two metal ion binding sites. The third domain is primarily disordered. Its function is unknown, but it possesses an SH3-like fold [24].

The two metal ion binding sites in each monomer are not identical, and therefore they were not expected to bind metal ions with the same affinity. The first binding site coordinates a metal ion using H79, E83, and H98, and solvent molecule or ion. The second site binds metal ion using C102, E105, H106, and a solvent molecule. Structures showed that the occupancy of the first site is high, while site 2 is often unoccupied [26]. Any mutation in C102 results in an inactive mutant, except a change to an aspartic acid residue. DtxR was first purified as a disulfide-linked dimer, and reduction was necessary to activate DtxR. It was also found that, upon exposure to air, DtxR forms inactive dimers. The distance between the two sulfur atoms is approximately 23 Å, but a dimer with an alternate conformation could be formed. The structure of the oxidized protein is currently unknown [25].

It is possible that this system could be used to sense oxidative species and promote the expression of repair and antioxidant systems. The presence and formation of oxidative species
inside the cytosol is unfavorable. Therefore, an oxidative stress mechanism would need to use molecules whose oxidation is favorable. There are a number of examples of bacterial proteins that form disulfide bonds as a result of chemistry with reactive oxygen species (ROS) including RsrA [27] and CatR [28] from *E. coelicolor*, PerR from *B. subtilis* [29], and OxyR from *E. coli* [30, 31]. Other transcription factors are known to form sulfenic and sulfonic acid derivatives as a response to oxidative stress including OhrR from *B. subtilis* [32] and MgrA from *S. aureus* [33].

Here, I present evidence that the oxidation of C102, located in the second metal ion binding site, abolishes the ability of DtxR to bind DNA and increases transcription levels *in vivo* of genes under the repressive control of DtxR. This suggests a role of C102 in sensing oxidative stress.
Materials and Methods

Protein expression and purification

Protein expression was induced by a 1.0 mM final concentration of isopropyl-β-D-thiogalactosidase (IPTG) at an O.D. of 0.6 after transformation of the plasmids pET-11c\textit{dtxr} and pET-11c\textit{dtxr(C102D)}, containing the sequences for DtxR and DtxR(C102D), into BL21-CodonPlus(DE3)-RIL cells at 37ºC. Following induction, the cells were allowed to express for 3 hours. Cells were centrifuged and the resulting pellet was stored at -80ºC.

The cell pellet was resuspended in 50 mL of the lysis buffer containing 10 mM Tris-HCl (pH 7.5), 300 mM NaCl, and 2 mM β-mercaptoethanol (βME) and sonicated using the Sonic Dismembrator Model 500 (Fisher Scientific). The lysate was centrifuged at 13,000g for 45 minutes at 4ºC. The supernatant was loaded onto a nickel-nitrilotriacetic acid (Ni-NTA) affinity column (Qiagen) equilibrated with lysis buffer. The column was washed with 100 mL of lysis buffer. The protein was eluted with 50 mM imidazole in the lysis buffer at a flow rate of 2 mL/min. Samples were analyzed using SDS-PAGE, pooled, and dialyzed with 10 mM Tris-HCl (pH 7.5) and 2 mM βME to remove imidazole and NaCl.

Reduced and oxidized sample preparation

Reduced DtxR samples were obtained by removing the reducing agent, βME, by passing the stored samples through a desalting PD-10 column (GE Healthcare). Removal of the reducing agent was tested using the DTNB sulfhydryl determination assay (described below) on aliquots of the flow-through. The oxidized sample was generated by dialyzing with 10 mM Tris-HCl (pH 7.5) to remove βME, followed by incubating the sample with an excess of H2O2 for one hour. The sample was then dialyzed with 10 mM Tris-HCl (pH 7.5) to remove excess oxidizing agent.
**Free sulfhydryl determination**

The DTNB assay was performed as described by Riddles et al. [34]. The working solution was prepared by mixing 8.4 mL of water, 1 mL of 1 M Tris-HCl (pH 8.0), and 0.5 mL of DTNB (5,5’-dithiobis-(2-nitrobenzoic acid)) solution (2 mM DTNB in 50 mM Sodium Acetate). 495 µL of working solution was mixed with 5 µL of sample. The absorbance was measured at 412 nm to determine the amount of free thiol in solution.

**β-Galactosidase assay**

pRDA-\textit{dtxr}(C102D) was subcloned from pRDA-\textit{dtxr} (citation) using the QuikChange mutagenesis kit (Stratagene, La Joya, CA) using the primer 5’-GGTTCACGATGAGCCGAGCCGCTGGGAACACGTTATG-3’ and its reverse complement. Mutagenic oligonucleotide primers were synthesized by Operon Technologies (Huntsville, AL). The plasmid was sequences to verify that nucleotide changes. The plasmids containing wild-type DtxR and DtxR(C102D) sequences were transformed into \textit{E. coli} DH5α:λRS45toxPO/lacZ. Cultures were incubated overnight at 37°C. Cultures were diluted 200 fold, and 250 µL of diluted culture was applied to individual wells in a 96 well plate. The samples were read in a Bioscreen C (Oy Growth Curves Ab Ltd, Helsinki, Finland) with maximum shaking at 37°C. When the OD$_{600}$ reached the appropriate value, 2.5 µL of oxidizing agent were added to the wells. The plate was further incubated until the OD$_{600}$ reached 0.9. At that point, 5 µL of lysis buffer (1:1:1:5 toluene/MnSO$_4$/10% SDS/BME) was added and the samples were allowed to shake for an additional 5 minutes. 100 µL of each sample were added to plastic disposable cuvettes containing 900 µL of Z Buffer (0.06 M Na$_2$HPO$_4$, 0.04 NaH$_2$PO$_4$, 0.01 M KCl, 0.001 M MgSO$_4$, 0.05 βME, pH 7.0). 200 µL of o-nitrophenol-β-D-galactosidase (ONPG) were added to
each cuvette. The reaction was allowed to proceed and was quenched by the addition of 500 µL of Na$_2$CO$_3$. The absorbance of the samples was determined spectrophotometrically at 420 and 550 nm.
Results

Protein expression and purification

Wild-type DtxR was purified using Ni-NTA resin exploiting the repressors’ natural affinity for metal ions as the construct does not contain a His tag fusion. A large band was observed in the 50 mM imidazole elution corresponding to approximately 25 kDa. Several other bands were also present in this elution (Fig 2.1). Later purification attempts using a gradient from 0 to 10 mM imidazole showed fewer additional protein bands by SDS-PAGE analysis (Fig 2.2). The eluted volume was dialyzed to remove imidazole and the concentration was determined to be 3.12 mg/mL using the bicinchoninic acid (BCA) protein assay (Fig 2.3).

Oxidation of thiol groups can be measured using DTNB

After preparing both reduced and oxidized samples of wild-type DtxR and DtxR(C102D), the oxidation state of the only cysteine was measured using the DTNB assay. The concentration of the product formed by the reaction of free thiols with DTNB can be calculated using a modified version of the Beer-Lambert law:

\[
Concentration = \frac{A_{412} \times Dil.}{N \times 13,600}
\]

Where \(A_{412}\) is the absorbance at 412 nm, Dil is the dilution factor, \(N\) is the number of reactive thiols, and 13,600 is the extinction coefficient of the product.

\textbf{B-Galactosidase in vivo assay}

\textit{In vivo} \(\beta\)-galactosidase assays were used to determine the \textit{in vivo} levels of repression by DtxR in the presence of three reactive oxidative species: hydrogen peroxide, cumene hydroperoxide (CHP), and paraquat (PQ). The \(OD_{600}\) was measured as the cells grew; and the
A\textsubscript{420} and A\textsubscript{550} were determined spectroscopically following the addition of the artificial substrate ONPG. The activity was calculated in terms of Miller Units using the formula:

\[
\text{MillerUnits} = 1000 \times \frac{(A\textsubscript{420} - (1.75 \times A\textsubscript{550}))}{(t \times v \times OD\textsubscript{600})}
\]

Where A\textsubscript{420} is the absorbance of the yellow o-nitrophenol, A\textsubscript{550} is the scatter from cell debris, t is the reaction time in minutes, v is the volume of culture assayed in milliliters, and the OD\textsubscript{600} reflects cell density.

Figure 2.4 shows activity as a percent increase from the base value when no reactive oxidative species was added. Figure 2.4(A) shows cultures that were exposed to H\textsubscript{2}O\textsubscript{2} at an OD\textsubscript{600} of 0.4. As H\textsubscript{2}O\textsubscript{2} increases from 0 to 25 mM, the β-galactosidase activity increases by 2400% with wild-type DtxR, but only by 100% with C102D. In cultures exposed at an OD\textsubscript{600} of 0.7, the wild-type cultures show an increase in β-galactosidase activity of 900%, whereas the cultures with C102D increase by 50%. Similar results are seen when CHP and PQ are added to the live cultures. As CHP concentrations reach 1 mM, the activity in cells containing wild-type DtxR increases by 3500% but only by 100% in cells containing C102D. Using paraquat, concentrations of 50 mM result in an increase of 200% with wild-type DtxR, and 50% with C102D.
**Fig 2.1 SDS-PAGE Analysis of Ni-NTA Purified DtxR**

Fig 2.1 SDS-PAGE analysis of purified lysate from BL21(DE3) *E. coli* cells overexpressing DtxR. Lane 1 contains the protein marker broad range 2-212 kDa (NEB). Lanes 2 and 3 contain the supernatant from the lysate after centrifugation. Lanes 4 and 5 contain the flowthrough from the Ni-NTA column. Lanes 6 and 7 contain the 50 mM imidazole wash. The band at approximately 25 kDa corresponds to the DtxR monomer. The band at approximately 50 kDa could correspond to a small population of dimeric DtxR. A number of other bands are observed along with DtxR in the 50 kDa wash.

**Fig 2.2 SDS-PAGE Analysis of Ni-NTA Purified DtxR Using a Gradient**

Fig 2.2 SDS-PAGE analysis of purified lysate from BL21(DE3) *E. coli* cells overexpressing DtxR. Lane 1 contains the protein marker broad range 2-212 kDa (NEB). Lanes 2 through 10 contain fractions from a 0 mM to 10 mM imidazole gradient elution. The band at 25 kDa corresponds to the DtxR monomer, and the band at 50 kDa may correspond to a population of dimeric DtxR. As compared to the purification using bulk washes, a gradient appears to achieve higher overall purity.
Fig 2.3 BCA Concentration Assay of Purified DtxR

Fig 2.3 BCA concentration assay comparing aliquots of DtxR to known concentrations of BSA. After incubating samples with BCA reagents, the absorbance was determined at 562 nm. A linear regression was used, and the resulting equation was used to determine the concentration of DtxR from the spectroscopic readings at 562 nm. DtxR was determined to be at a concentration of 3.12 mg/mL, indicating a total yield of 97 mg.
Fig 2.4 Effects of Reactive Oxidative Species on Transcription Levels

Fig 2.4 β-galactosidase assays were performed to determine transcription levels when treated with three oxidative species: H$_2$O$_2$, CHP, and PQ. Plasmids contain the toxPO DtxR binding site upstream of the lacZ gene. After growing, cells were lysed and ONPG was added. Cleavage of ONPG by β-galactosidase can be followed spectroscopically. Lower activity levels indicate higher repressive activity of DtxR. Due to intrinsic activity differences between wild-type DtxR and C102D, relative levels of transcription compared to cells without oxidative species are shown. β-galactosidase activity increases relative to the reference in cells containing wild-type DtxR as oxidative species concentration increases, while activity remains relatively constant in cells containing the mutant C102D. (A) Cells were treated with H$_2$O$_2$ once they had reached an OD$_{600}$ of 0.4. When a concentration of 25 mM H$_2$O$_2$ had been added, wild-type cells demonstrated a 2400% increase in β-galactosidase activity. (B) Cells were treated with H$_2$O$_2$ once they had reached an OD$_{600}$ of 0.7. When a concentration of 50 mM H$_2$O$_2$ had been added, wild-type cells showed a 1000% increase in β-galactosidase activity. (C) Cells were treated with CHP once they had reached an OD$_{600}$ of 0.7. CHP concentrations over 1 mM resulted in cell death. (D) Cells were treated with PQ once they had reached an OD$_{600}$ of 0.7.
Discussion

The expression and purification of wild-type DtxR is a well-established procedure that yields relatively pure protein using Ni-NTA resin. Unlike many proteins which use an artificial His-tag, this procedure utilizes DtxR’s ability to bind divalent cations to purify it. However, this procedure does not remove all impurities from solution. As seen in figure 3.1, a number of other bands were present in the 50 mM imidazole elution. By using a gradient from 0-10 mM imidazole, however, many of these impurities can be removed (Fig 2.2). Although samples were boiled and treated with SDS and β-mercaptoethanol, a band can still be observed at 50 kDa and may correspond to the DtxR dimer.

Previous studies had shown that exposure of DtxR to air results in a loss of activity. In fact, DtxR was originally purified as an inactive, oxidized dimer. Activity was restored by exposing samples to reducing agents such as β-mercaptoethanol or DTT. Here, oxidized DtxR was generated by removing reducing agents from the buffer before the addition of oxidative species such as hydrogen peroxide. This oxidation could be measured by using 5,5’-dithiobis-(2-nitrobenzoic acid), also known as DTNB or Ellman’s reagent, a compound that is able to react with free-thiol groups. In the case of DtxR, there is only a single thiol group, cysteine 102.

Reaction of DTNB with a free-thiol releases 2-nitro-5-thiobenzoate (NTB), a yellow compound that can be quantified in a spectrophotometer by measuring the absorbance at 412 nm. By incubating with an excess of oxidative species, the majority of the free thiol groups are oxidized and unable to react with Ellman’s reagent. While disulfide bridges can be formed by exposure to air or hydrogen peroxide through a cysteine sulfenic intermediate, cysteine sulfinate can also be formed.
In order to determine the effects of oxidation on transcription rates, plasmids containing both DtxR and DtxR(C102D) were transformed into *E. coli* DH5α::λRS45toxPO/lacZ, and these cells were incubated with three reactive oxidative species: hydrogen peroxide, cumene hydroperoxide, and paraquat. While C102D is an active mutant, its repressive ability still varies from the wild-type repressor, and therefore the background in the data was corrected by showing the effect compared to cells without oxidative reagents. These results show that the transcription levels increase for wild-type DtxR upon the addition of oxidative species, but the levels for cells containing C102D are not affected. In other words, DtxR loses repressive ability in the presence of oxidative species while C102D does not.

*E. coli* cells are differentially sensitive to oxidative reagents depending on growth phase. Therefore, two O.D.₆₀₀’s at which reagents were added were used, 0.4 and 0.7. Incubation during logarithmic growth phase leads to larger increments in the transcription levels of β-galactosidase while incubation at the stationary phase allows the effect to be seen at higher oxidative reagent concentrations. This is due to the fact that *E. coli* cells in stationary phase are highly resistant to oxidative species due to the expression of a number of factors that are induced by RpoS, a sigma factor that is upregulated during the transition into stationary phase [35, 36].

Oxidants could react with either the cysteine or ferrous iron. DtxR is activated by binding ferrous iron. Cysteine is able to react with hydrogen peroxide to form either disulfide bridges or cysteine sulfenic acid. Hydrogen peroxide can oxidize ferrous iron forming ferric iron in a reaction known as the Fenton reaction. Therefore, two other oxidants, paraquat and cumene hydroperoxide, were also tested. Paraquat produces superoxide which attacks iron-sulfur clusters, releasing ferrous iron [37]. As the addition of paraquat results in an elevated
concentration of ferrous iron, it is not likely that the increased transcriptional rates can be explained by a depletion of ferrous iron by Fenton chemistry. Cumene hydroperoxide (CHP), a mutagenic alkyl hydroperoxide, was also tested, but it is lethal to E. coli even at very low concentrations. Therefore, the effects of CHP were unable to be determined beyond a concentration of 1 mM. Due to these varying oxidative mechanisms, it is likely that the oxidation of C102, and not Fenton chemistry, is responsible for the increased transcription levels. In addition, if the primary mechanism were the oxidation of ferrous iron, transcription levels in cells containing C102D would be expected to increase similarly to cells containing wild-type DtxR, but this is not observed. Fenton chemistry, however, may be able to explain the lower increases in transcription observed in cells containing the mutant protein.

These results suggest that DtxR may be involved in oxidative stress response. If DtxR were responsible for the repression of enzymes involved in oxidative stress, deregulation of DtxR by means of oxidation would lead to increased transcription and expression levels which would lead to the mitigation of the effects of oxidation.

The role of oxidation in DtxR function is only beginning to be understood. Structural studies of oxidized DtxR could reveal how oxidation leads to derepression. Further studies of gene regulation by DtxR under conditions of oxidative stress would address what genes, if any, are upregulated in the presence of oxidants. Finally, in vivo assays could show whether DtxR is able to protect cells from harmful Fenton chemistry by sequestering iron ions. This research suggests that DtxR, and DtxR-like repressors containing a cysteine, may be involved in the defense against oxidative stress.
Chapter 3: Crystallization Screening of Propionate Kinase

Introduction

Propionate kinase is a key metabolic enzyme and a potential drug target from the highly infectious bacterium Francisella tularensis. Here, crystallization conditions are determined for the apo-protein, and could be used for further structural studies of the apo-enzyme and holo-enzyme to better understand the mechanism of this protein.

Francisella tularensis is a gram-negative, highly infectious intracellular bacterium and is the causative agent of tularemia, a disease also known by the colloquial names rabbit fever, hare fever, deerfly fever, and lemming fever [38]. While there are several documented subspecies, the strain Francisella tularensis subspecies tularensis is the most virulent with an infectious dose of fewer than 10 CFU. In addition, the mortality rate from infections with this subspecies is around 5-6% if left untreated [38]. Due to mortality rates, its high infectivity, and the ease of dissemination, Francisella tularensis is considered a Category A agent by the Center for Disease Control (CDC, www.cdc.org) along with anthrax, hemorrhagic fevers, smallpox, and the plague. In particular, there are concerns that this bacterium could be used as an infectious agent in biological warfare [39].

Tularemia occurs only in the northern hemisphere [38] and cases are usually sporadic. Infections are frequently documented in Scandinavia, northern America, Japan, and Russia. More recently, however, cases have also been reported in Turkey, Yugoslavia, Spain, Kosovo, and Switzerland. Infections can be acquired through exposure to infected arthropod vectors such as deer flies, horse flies, ticks, and mosquitoes and through handling, ingesting, or inhaling infectious material [38, 39].
Several different clinical manifestations are known, and these depend primarily on the route of entry. One form, ulceroglandular tularemia, is a consequence of transmission by arthropod vectors [39], although some trappers and hunters have contracted the pathogen by handling infected meat [38]. This form of the disease is rarely fatal; mortality rates are less than 3% [38]. However, an acute form of the disease caused by the subspecies *tularensis* known as typhoidal tularemia has a mortality rate between 30% and 60%. A second form, oropharyngeal tularemia, is caused by the ingestion of contaminated food and water. It is characterized by a primary ulcer in the mouth and enlarged lymph nodes. A third form is respiratory, or pneumonic, tularemia. It is caused by the inhalation of contaminated material, and infections are usually related to farming activities or landscaping. In all manifestations of the disease, bacteria multiply at the initial site of infection before spreading to regional lymph nodes, the liver, and the spleen. Patients experience nonspecific flu-like symptoms including headaches, fever, chills, nausea, diarrhea, and pneumonia. Individuals who contract typhoidal tularemia also suffer from delirium and shock [38, 39].

Kinases are a class of enzyme that transfer terminal phosphoryl groups, and this is a fundamental process in signal transduction, gene regulation, and energy transfer. The *tdc* operon in *E. coli* and *Salmonella typhimurium* encodes proteins involved in the transport and metabolism of L-serine and L-threonine. L-threonine is cleaved non-oxidatively to propionate through the intermediate 2-ketobutyrate by pyruvate formate lyase or 2-ketobutyrate formate lyase, phosphotransacetylase, and propionate kinase. The final reaction in this pathway is performed by propionate kinase which converts propionyl phosphate and ADP to propionate and ATP [40].
Here, I expressed and purified recombinant propionate kinase from *Francisella tularensis* using an *E. coli* expression system. I attempted to determine crystallization conditions for the apo and holo-enzyme. By screening for possible crystallization conditions, I obtained apo-enzyme crystals that diffracted to a resolution of 3.5 Å.
Materials and Methods

Overexpression and purification of propionate kinase

The gene coding for propionate kinase was previously cloned into the pProEX HT prokaryotic expression system vector containing a cleavable N-terminal His-tag. Rosetta cells were transformed with pProEXHT-PK and grown in LB containing 100 µg/mL ampicillin. Protein expression was induced by adding a final concentration of 1.0 mM isopropyl-β-D-thiogalactosidase (IPTG) when the cells had reached an OD$_{600}$ of 0.6. The cells were allowed to express for 4 hours before being centrifuged. The resulting pellet was resuspended in 50 mL Ni-NTA lysis buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 10 mM Imidazole, pH 8 using NaOH) and frozen at -80°C.

The thawed solution was sonicated using the Sonic Dismembrator Model 500 (Fisher Scientific) for a total of 3 minutes using intervals of 15 seconds on and 30 seconds off. The resulting solution was centrifuged for 30 minutes at 12,000 rpm. The lysate was applied to 10 mL Ni-NTA Superflow resin (Qiagen) pre-equilibrated with 50 mL of Ni-NTA lysis buffer and the flowthrough was collected. The column was washed twice with Ni-NTA wash buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 20 mM Imidazole, pH 8 using NaOH). The protein was eluted 4 times with Ni-NTA elution buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 250 mM Imidazole, pH 8 using NaOH). Samples were analyzed using SDS-PAGE.

Pooled fractions were concentrated to a volume of 2 mL using Amicon® Ultra-15 Centrifugal Units with Ultracel-10 membranes (Millipore) before being loaded onto a Hiprep™ 16/60 Sephacryl™ S-200 High Resolution size exclusion column (GE Healthcare). The column was run using 50 mM NaH$_2$PO$_4$, 300 mM NaCl, pH 8. Elution was monitored by measuring the
absorbance at 280 nm. Peaks were analyzed using SDS-PAGE, fractions were collected, and the concentration was determined using the Bradford protein assay kit (Bio-Rad). Samples were concentrated to a concentration of 30 mg/mL with 15% glycerol and stored in aliquots at -80°C after flash freezing with liquid nitrogen.

**Crystallization**

Initial crystallization conditions were set up based upon previous results using .1 M Hepes pH 7, 5% Tacsimate (Hampton Research), and 10% PEG 5000 MME. Initial trials used a protein concentration of 20 mg/mL in the storage buffer with a drop to mother liquor ratio of 1:1, 1:2, and 2:2. Screens were then set up varying Tacsimate and PEG 5000 MME using protein in storage buffer with and without glycerol.

Multiple screens were then utilized to find and optimize crystal conditions for propionate kinase including the Crystal screen, Index screen, SaltRX screen, Natrix screen (Hampton Research), Wizard I screen, Wizard II screen, and Wizard III screen (Emerald Biosystems). Protein sample storage buffer was exchanged for 10 mM Hepes, pH 7. Screens were set up using the Phoenix™ liquid handling robot (Rigaku), setting up 0.3 µL drops with a sample to mother liquor ratio of 1:1 and a mother liquor volume of 50 µL.
**Results**

*Overexpression and purification of propionate kinase*

The molecular weight of the protein product was calculated to be approximately 42 kDa. Overexpressed propionate kinase was first purified using a Ni-NTA resin. After extensive washing with 20 mM imidazole buffer, the protein was eluted in four fractions using 250 mM imidazole buffer. Results were analyzed using SDS-PAGE. Several bands are seen in both washes, and a large, overexpressed band corresponding to PK is seen in all four elutions. Several additional faint bands are seen in the elution bands (Fig 3.1).

Elutions 1 and 2 were pooled, and the concentration was determined using the Bradford protein assay kit using known bovine serum albumin (BSA) samples as a standard. The concentration was determined to be approximately 2 mg/mL. The sample was concentrated down to 2 mL and injected onto a size exclusion column for further purification. Two peaks were primarily observed on the chromatogram from the size exclusion column: one after a run volume of 37 mL with a peak height of 430 mAU and another after a run volume of 55 mL with a peak height of 1100 mAU (Fig 3.2).

Fractions from both peaks were collected and analyzed using SDS-PAGE. Several faint bands of varying molecular weights were seen through fraction B9 corresponding to lane 6 in figure 3.3. Fractions B11 through C5 were collected and the concentration was determined using the Bradford protein assay kit. These results indicated that the concentration of the pooled PK samples were at an approximate concentration of 1.87 mg/mL (Fig 3.4). The protein solution was concentrated to a final concentration of 30 mg/mL and stored at -80°C with 15% glycerol.
Crystallization and co-crystallization of propionate kinase

Trays were set up based on previously determined crystallization conditions containing 0.1 M Hepes pH 7, 5% Tacsimate, and 10% PEG 5000 MME. A protein concentration of 20 mg/mL in the storage buffer was used while varying the protein to mother liquor ratio. Although heavy precipitate formed overnight, some small crystals formed. These were difficult to loop out of the drop and separate from the precipitate, and they differed morphologically from previously obtained crystals.

A second tray was set up using a protein to mother liquor ratio of 1:2 and a protein concentration of 15 mg/mL, and the concentration of Tacsimate was varied from 5% to 12%. Most drops remained clear, although light precipitate formed in some drops. Glycerol was removed from the protein storage buffer, and additional drops were set up. However, heavy precipitate formed overnight.

Attempting to stabilize the protein with substrate, drops were set up using the previously determined conditions with substrate and additional salts (4 mM ADP, 5 mM MgCl2, 20 mM Propionate, and 50 mM KNO3). However, results did not vary from the conditions without substrate. Heavy precipitate continued to form.

The storage buffer was then exchanged for 10 mM Hepes pH 7, and Hampton screens were utilized to obtain new crystallization conditions. Crystals formed from the Hampton SaltRX screen condition #94 containing 4.0 M Ammonium Acetate and 0.1 M Tris pH 8.5. A complete data set was obtained to a resolution of 3.5 Å, but the data could not be indexed to a space group as the crystal had fractured when originally looped.
Fig 3.1 SDS-PAGE of Ni-NTA Purified Propionate Kinase

Fig 3.1 SDS-PAGE analysis of purified lysate from rosetta *E. coli* cells overexpressing propionate kinase. Lane 1 contains the protein marker broad range 2-212 kDa (NEB). Lane 2 contains the flow through. Lanes 3 and 4 contain fractions washed with 20 mM imidazole. Lanes 5 through 8 contain fractions washed with 50 mM imidazole. An overexpressed band corresponding to 42 kDa PK is seen in lanes 4 through 8. Fractions 5 through 8 were pooled and further purified using size-exclusion chromatography.

Fig 3.2 Size Exclusion Chromatograph of Propionate Kinase

Fig 3.2 Size exclusion chromatograph of Ni-NTA purified propionate kinase. Sample was injected using a 2 mL loop and the column was washed with 50 mM NaH$_2$PO$_4$, 300 mM NaCl, pH 8. One peak is observed at 37 mL and a second at 55 mL. Fractions from each peak were further analyzed by SDS-PAGE.
Fig 3.3 SDS-PAGE of SEC Purified Propionate Kinase

Fig 3.3 SDS-PAGE analysis of PK purified from size-exclusion chromatography after purifying with Ni-NTA resin. Lane 1 contains the protein marker broad range 2-212 kDa (NEB). Lanes 2 through 9 contain fractions from the peak observed on the size exclusion chromatograph, and show a band corresponding to PK. Pure fractions were pooled, concentrated, and stored at -80°C.

Fig 3.4 Bradford Assay of Purified Propionate Kinase

Fig 3.4 Bradford Protein concentration assay comparing aliquots of PK to known concentrations of BSA. After reacting with the Bradford reagent, the absorbance at 595 nm was determined. The concentration of PK was determined to be 1.87 mg/mL.
Fig 3.5 Crystal Structure of Propionate Kinase

Fig 3.5 Crystal structure of propionate kinase solved by Dr. Dali Liu and Noah Wolfson '08. Structure was solved to a resolution of 2.2 Å using molecular replacement. Crystal belonged to space group P1 with unit cell parameters of $a=90.547\ \text{Å}$, $b=92.924\ \text{Å}$, $c=132.711\ \text{Å}$, $\alpha=107.21^\circ$, $\beta=103.71^\circ$, $\gamma=90.17^\circ$. 
Discussion

Following previously established expression and purification procedures, relatively large quantities of PK were obtained from *E. coli* rosetta cells. After elution from Ni-NTA resin, impurities were observed in addition to the 42 kDa band corresponding to PK. PK was able to be further purified by separating on a size exclusion column, resulting in relatively pure protein samples (Fig 3.3). In total, this procedure yielded 27 mg from 2 liters of culture.

Crystallization screens were then established using the highly purified protein. Previously, a structure of the apo-enzyme had been solved to 2.2 Å in this laboratory by Dr. Dali Liu and undergraduate Noah Wolfson ’08 (Fig 3.5). These results were unable to be reproduced. This same condition resulted in the formation of heavy precipitate and small crystals that different significantly in morphology from the aforementioned crystals. Protein working buffer, drop ratio, and temperature were varied in an attempt to replicate these crystallization conditions, but crystals could not be obtained. Additional screening used substrate, both ADP and propionate, in an attempt to stabilize the protein and obtain crystals of the holo-enzyme. Heavy precipitate was observed in these conditions as well. It was unclear what working buffer was originally used, and it is possible that this unknown variable impeded crystal growth using the same condition. Another crystallization condition was obtained by screening with Hampton high-throughput kits. Crystals were obtained that diffracted to approximately 3.5 Å. However, the crystal cracked when looped, resulting in data that has been difficult to index to a space group.

Further screening of this condition could result in a high resolution structure of PK. Additionally, cocrystallization with substrate and substrate analogues using this condition could
be performed to obtain a structure of the holo-enzyme. Structural information and comparisons between the apo- and holo-enzyme would reveal how, mechanistically, this enzyme transfers inorganic phosphate to ADP generating ATP. Ultimately, a better understanding of the structure and mechanism of this key metabolic enzyme could allow for rational drug design to develop specific small molecule inhibitors that could be used to treat tularemia.
References