

UNIVERSITY OF TEXAS AT SAN ANTONIO  
San Antonio, Texas

ELUCIDATION OF AMINO ACIDS NECESSARY FOR DIMERIZATION OF THE  
VIRULENCE REGULATORY PROTEIN TOXT OF *VIBRIO CHOLERAE*

A Proposal for a Thesis for the  
UTSA MARC U\*STAR Program

MARC Student's Name

Department of [Whatever is Appropriate]  
May 2006

PREPARED BY:

---

MARC U\*STAR Student Name

APPROVED BY:

---

Dr. Extraordinare, Thesis Advisor

---

Dr. M. I. Awesome, Thesis Reader

---

Dr. I. M. Smart , Thesis Reader

---

Dr. Andrew O. Martinez, Program Director MARC U\*STAR

RECEIVED BY THE MARC U\*STAR PROGRAM

May 2006

## Table of Contents

Abstract.....	1
Acknowledgements.....	3
Introduction.....	4
Background.....	5
Materials and Methods.....	9
Literature Cited.....	12

## Abstract

*Vibrio cholerae* is a motile, gram-negative, rod-shaped bacterium that causes the diarrheal disease cholera, which afflicts thousands of people worldwide each year. Research into the pathogenesis of *V. cholerae* is critical for the development of novel vaccines and therapeutics. ToxT is a key *V. cholerae* regulatory protein that directly activates the transcription of the two major virulence gene clusters, *ctx* and *tcp*, which encode cholera toxin (CT) and the toxin-co-regulated pilus (TCP), respectively. Previous data demonstrated that the N-terminus of ToxT (aa1-165) functions in dimerization of the protein, whereas the C-terminus (aa166-276) functions in DNA binding. Dimerization is necessary for DNA binding, which in turn is necessary for transcription activation, thus it is predicted that mutation of ToxT amino acids involved in dimerization should disrupt ToxT function. Scanning alanine mutagenesis studies have shown that there are approximately 24 N-terminal ToxT alanine substitution mutants defective for transcriptional activation. To test the effects of the individual N-terminal alanine substitutions on ToxT dimerization, we utilized a LexA based reporter assay. The N-terminus of ToxT (ToxT<sup>N</sup>) dimerizes the normally monomeric DNA binding domain of LexA (LexA<sup>N</sup>), which leads to repression of *sulA* transcription. The 24 N-terminal Ala substitution mutations were introduced into the ToxT<sup>N</sup>-LexA<sup>N</sup> fusion protein, and then each protein was assayed for its ability to repress a *sulAp-lacZ* reporter. To date, of the 17 N-terminal alanine substitution mutations known to decrease ToxT transcriptional activation, four caused significant increases in *sulA* transcription in the LexA-based reporter system, indicating these residues are involved in ToxT dimerization. Three of these residues are clustered in a predicted alpha helical region, thus identifying a putative

dimerization interface. Elucidation of the amino acids that are necessary for ToxT dimerization will help to further characterize the virulence regulator ToxT.

## **Acknowledgements**

I would like to express my gratitude to my thesis advisor, Dr. Karl E. Klose, for his guidance, patience, and support. I would also like to thank the following: my thesis readers, Dr. M. Neal Guentzel, and Dr. [Edwin J. Barea-Rodriguez](#) for their patience and understanding; the Klose laboratory for making me feel at home; Mike Prouty, Brandon Childers, and Jirong Liu for their guidance in the laboratory; Dr. Gail P. Taylor for her advice and support; the MARC-U\*STAR (Minority Access to Research Careers - Undergraduate Student Training for Academic Research) Program and the Honors College for providing such wonderful opportunities. This research was supported in part by NIGMS MARC-U\*STAR GM07717 and MH A151333.

## **Introduction**

*V. cholerae*, is a motile, gram-negative, rod-shaped bacterium that belongs to the bacterial family *Vibrionaceae* (Reidl, J., and Klose, K.E., 2002). In 1883 Robert Koch found this bacterium to be the etiologic agent responsible for the disease cholera. *V. cholerae* has caused seven documented pandemics (Reidl, J., and Klose, K.E., 2002; Sack, D.A. *et al.*, 2004). The first six pandemics occurred during the 19<sup>th</sup> century, affecting the Indian continent, continents in the southern hemisphere, North America, and Europe (Reidl, J., and Klose, K.E., 2002; Sack, D.A. *et al.*, 2004). The 7<sup>th</sup> pandemic, which occurred during the 20<sup>th</sup> century, began in Indonesia and eventually spread to South America, the Indian subcontinent, the Middle East, and Africa (Reidl, J., and Klose, K.E., 2002; Sack, D.A. *et al.*, 2004). In the past decade there have been high occurrences of cholera epidemics in Asia, Latin America, and Africa (United Nations Educational, Scientific and Cultural Organization (UNESCO) and the United Nations World Water Assessment Programme (WWAP), 2006). In fact, it is estimated that from the late 1990s and onward there have been between 100,000 and 200,000 cases officially reported in Africa each year, and in the year 2002 alone, there were 123, 986 cases and 3,763 deaths (UNESCO and WWAP, 2006).

Cholera has an incubation period of 18 hours to 5 days and is characterized by vomiting and profuse amounts of watery diarrhea, commonly referred to as “rice water” stool. In adults, fluid loss resulting from severe diarrhea can be 50-1000mL/h (Sack, D.A. *et al.*, 2004). If left untreated severe dehydration typically follows. Severe dehydration is characterized by undetectable blood pressure, poor skin turgor, sunken eyes, and wrinkled hands and feet (Sack, D.A. *et al.*, 2004). Without proper medical attention and

intervention, shock and death follow. In fact, when left untreated, severe cholera has a mortality rate of up to 50% (Sack, D.A. *et al.*, 2004).

During its life cycle, *V. cholerae* persists in aquatic environments and in the human host (Reidl, J., and Klose, K.E., 2002). While inhabiting aquatic environments, *V. cholerae* can be found as biofilms or as free swimming vibrios (Sack, D.A. *et al.*, 2004). Biofilms are three-dimensional, multi-cellular micro-communities that help shield *V. cholerae* from the harsh aquatic environments (Reidl, J., and Klose, K.E., 2002). Since *V. cholerae* persists in aquatic environments, the vehicle for infection is contaminated drinking water, or improperly prepared seafood, such as shellfish (Reidl, J., and Klose, K.E., 2002; Sack, D.A. *et al.*, 2004). Once ingested, *V. cholerae* must pass the gastric acid barrier of the stomach before it can reach the upper small intestine, where it typically colonizes and causes infection (Reidl, J., and Klose, K.E., 2002; Sack, D.A. *et al.*, 2004). To continue its life cycle, *V. cholerae* exits the host during excretion and then find its way back to an aquatic environment.

## **Background**

ToxT is the key regulatory protein in *V. cholerae* (DiRita *et al.*, 1991) because it is primarily responsible for the pathogenesis of *V. cholerae*. ToxT activates the transcription of *tcp* and *ctx* genes, which encode the two major virulence factors, the toxin co-regulated pilus (TCP) and the cholera toxin (CT), respectively (DiRita *et al.*, 1991). TCP is a Type IV pilus, a thin string-like protein structure, which is produced by *V. cholerae* when colonizing the intestine (Sack, D.A. *et al.*, 2004). TCP, aids the bacteria in adhering to neighboring vibrios, and is required for intestinal colonization. TCP also serves as a receptor for the cholera toxin-encoding phage (CTX $\phi$ ) (Waldor, M.K., and

Mekalanos, J. J., 1996). This bacteriophage transduces the genes that encode cholera toxin, the other key virulence factor in *V. cholerae*, into the bacteria (Waldor, M.K., and Mekalanos, J. J., 1996). In the small intestine *V. cholerae* secretes CT, which binds the GM<sub>1</sub> ganglioside receptor of epithelial cells, and the toxin is then translocated into host cells where it catalyzes ADP-ribosylation of the Gs $\alpha$ . The end result is ionic imbalance that leads to fluid loss responsible for the voluminous watery diarrhea and death if left untreated (King, C.A. and van Heyningen, W.A., 1973; Pierce, N.F., 1973).

ToxT is also an AraC family member. AraC family members are transcriptional activators that are classified based on a 99-conserved amino acid stretch, which is typically found within the C-terminus (Higgins *et al.*, 1992). AraC has been characterized by Bustos and Schleif (1993) to contain a dimerization domain in the N-terminus and DNA binding domain in the C-terminus. Studies in the Klose laboratory (Prouty, M.G. *et al.*, 2005) have shown that ToxT also contains dimerization determinants in the N-terminus and a DNA binding domain in the C-terminus, similar to AraC. It has also been concluded that in order for ToxT to bind DNA, it must first dimerize. Thus dimerization is an integral part of ToxT function, and the elucidation of the specific amino acids required for ToxT dimerization is important for a more thorough understanding of this critical virulence regulator.

### **Proposed Research**

I propose to examine dimerization characteristics of the *V. cholera* ToxT toxin. My *specific aim* is to determine which amino acids in the N-terminus are critical to ToxT dimerization. I *hypothesize* that by altering the residues involved in ToxT dimerization by

Ala substitution, I will negatively affect its ability to dimerize, and the resulting mutant protein will be defective for transcriptional activation.

To carry out this research, I will:

- 1) Conduct alanine substitution mutagenesis on selected amino acids in the ToxT N-terminus.
- 2) Introduce the Ala substitution ToxT mutants into a LexA-based dimerization assay to determine if the mutation affects ToxT dimerization.
- 3) Conduct SDS-PAGE and Western immunoblot to confirm the presence of the mutant ToxT proteins.
- 4) If time allows, confirm the results obtained with a LexA system utilizing a bacterial two hybrid system.

**Rationale:**

The Klose laboratory has already conducted scanning alanine mutagenesis on ToxT. Scanning alanine mutagenesis is site-directed mutagenesis that systematically replaces each individual non-alanine amino acid with alanine. Alanine is a useful amino acid to substitute for other amino acids because it is small, uncharged, and typically found both on the surface and buried within proteins, so its presence typically eliminates charged or hydrophobic interactions with minimal influence on protein structure (Chothia, C. 1976). This type of analysis by others in the Klose laboratory has identified a number of Ala substitution mutations within the N-terminus of ToxT (24 total) that prevent ToxT from activating two different ToxT-dependent promoters. Because these mutations lie within the N-terminus, it is possible that they disrupt ToxT dimerization.

Determining whether these specific mutations affect dimerization will be the focus of my proposal.

The N-terminal Ala substitutions will be tested for their effects on dimerization in a LexA-based reporter system. LexA is a DNA binding protein which is only able to bind DNA and repress transcription at the *sulA* promoter when dimerized (Lin and Little, 1989). Because the DNA binding domain of LexA is monomeric, the dimerization capabilities of any protein domain can be tested by fusing the domain of interest to the DNA binding domain of LexA (LexA<sup>N</sup>) and then testing the ability of this protein fusion to repress *sulA* transcription. An *E.coli* reporter strain (JL1436) has been constructed to contain a chromosomal *sulAp-lacZ* transcriptional fusion (Lin and Little, 1989). Expression of LexA<sup>N</sup> fusion proteins in JL1436 will lead to repression of LacZ activity only if the domain fused to LexA<sup>N</sup> contains dimerization determinants. The Klose laboratory has previously demonstrated that the ToxT amino terminus (ToxT<sup>N</sup>) fused to LexA<sup>N</sup> represses *sulAp-lacZ* transcription, providing evidence that dimerization determinants are located in ToxT<sup>N</sup>.

To test the effects the 24 N-terminal alanine substitutions may have on the dimerization ability of ToxT, the ToxT<sup>N</sup>-LexA<sup>N</sup> fusion protein will be utilized. Each of the 24 Ala substitution mutations will be introduced into the plasmid expressing ToxT<sup>N</sup>-LexA<sup>N</sup> by site-directed mutagenesis, as detailed below. Each mutation will be confirmed by sequencing, and then each mutant plasmid will be transformed into the reporter strain JL1436. Fusion protein expression is induced by the addition of arabinose, and *sulAp-lacZ* transcription is measured by  $\beta$ -galactosidase activity. Controls include the (unmutated) ToxT<sup>N</sup>-LexA<sup>N</sup>, as well as the plasmid containing no protein, expressed in

this reporter strain, as positive and negative controls, respectively. Thus, if any of the alanine substitutions have an effect on the dimerization ability of ToxT, the ToxT<sup>N</sup>-LexA<sup>N</sup> protein will be defective for binding *sulAp*, and will fail to repress the expression of  $\beta$ -galactosidase, resulting in high  $\beta$ -galactosidase activity. If the mutations do not affect dimerization, then the ToxT<sup>N</sup>-LexA<sup>N</sup> protein will bind the *sulA* promoter and repress  $\beta$ -galactosidase expression, resulting in low levels of  $\beta$ -galactosidase activity, similar to that caused by the (unmutated) ToxT<sup>N</sup>-LexA<sup>N</sup>.

## **Materials and Methods**

*Bacterial Strains* - *Escherichia coli* strains DH5 $\alpha$  (Hanahan, 1983) and TOP10 cells (Invitrogen) will be used for cloning. The *E. coli* reporter strain JL1436 (Lin and Little, 1989) will be used for the LexA-based dimerization assay.

*Site-directed Mutagenesis* - The QuickChange Site-Directed Mutagenesis Kit (Stratagene) will be used to carry out all site-directed mutagenesis. Plasmid pKEK 522 contains the coding sequence for the ToxT<sup>N</sup>-LexA<sup>N</sup> fusion protein, which is expressed from an IPTG-inducible promoter. The fusion protein also contains maltose binding protein (MBP) at its amino terminus; MBP has been shown to have no effect on ToxT activity (Schuhmacher, D.A. and Klose, K.E., 1999) and facilitates the detection of ToxT (see below).

*Polymerase Chain Reaction (PCR)*: pKEK522 will be used as the template plasmid for all mutagenesis reactions. Complimentary oligonucleotides have been designed to change the codons of specific amino acids to encode alanine (GCX). Each PCR mutagenesis reaction will contain two complimentary oligonucleotides that encode the Alanine

mutation of interest and pKEK522 template plasmid. In addition, each PCR reaction will also contain 1.25  $\mu$ l of complimentary primers, 1  $\mu$ l of dNTPs, 2  $\mu$ l of DMSO, 5  $\mu$ l of Pfu Turbo buffer, and 37.5  $\mu$ l of water. A negative control which will consist of the PCR mix mentioned above, excluding primers, will also be included in each batch of PCRs. PCR reactions will be screened using a 1% agarose gel. Positive PCR products will be observed at 6,937 base pairs.

*DNA Manipulations:* 1  $\mu$ l DNA from PCR mutagenesis reaction will be transformed into Top10 *E.coli* cells using a Bio Rad Gene Pulser II electroporator. Each reaction will then be added to 1 mL of Luria broth (LB) and incubated at 37°C for 1 hour. Cells will then be pelleted and re-suspended in 200  $\mu$ l of LB. Cultures will be plated on LB agar containing 100 $\mu$ g/ml ampicillin, which selects for the cells containing the pKEK522 plasmid, and incubated overnight at 37°C. Four colonies will be selected from each plate, and plasmids will be isolated from each cell using a plasmid isolation kit (Qiagen). Each plasmid will then be sequenced, utilizing *toxT*-specific primers. One plasmid from each reaction which contains the correct Alanine substitution mutation, to be confirmed by sequencing, will be utilized in further assays, as described below.

*$\beta$ -galactosidase assays:* Plasmids containing the amino terminal Alanine substitution mutation in ToxT<sup>N</sup>-LexA<sup>N</sup> will be transformed into the *sulAp-lacZ* reporter strain JL1436 as described above. Overnight cultures will be added (1:50) to 5 mL of LB containing 100  $\mu$ g/ml ampicillin and 0.3 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG); this induces expression of the ToxT<sup>N</sup>-LexA<sup>N</sup> fusion protein. Strains will be grown to mid-log phase (OD<sub>600</sub> 0.3-0.6) at 37°C. Prior to each  $\beta$ -galactosidase assay samples will be normalized and stored in sample buffer to conduct Western immunoblots

as described below. Cultures will be assayed for  $\beta$ -galactosidase activity as described by Miller (1992). 100  $\mu$ l of culture will be added to 900  $\mu$ l of Z buffer (10 ml Z-buffer + 27  $\mu$ l of  $\beta$ -MET). 1 drop of 0.1% SDS and 2 drops of chloroform will be added to each 1 mL sample and vortexed for 5 seconds. Tubes will be incubated in a 28°C waterbath for 5 minutes and then each reaction will be started by adding 200  $\mu$ l of ONPG buffer (4 mg/ml ONPG in Z buffer) in 30 second intervals. Prior to beginning each reaction the start time will be recorded. After the reactions produce a light yellow color, the reaction will be stopped by adding 500  $\mu$ l of 1M Na<sub>2</sub>CO<sub>3</sub> and the stop time will be recorded. After the assay is complete, samples will be left undisturbed for 10 minutes to allow the cellular debris to sink to the bottom of the test tube. Afterwards, 200  $\mu$ l from the top each sample will be used for spectrophotometer readings. Readings for each sample will be taken at OD<sub>420</sub> utilizing an Eliza plate reader (BioRad). Miller units will then be computed using the following equation:

$$\frac{OD_{420}}{OD_{600} * t * v} * 10^3 = \text{Miller Units}$$

where  $t$  = time and  $v$  = volume of culture (ml)

*SDS-PAGE and Western Blot Analysis* – Samples will be denatured and loaded into a 12% polyacrylamide gel and separated by electrophoresis. Proteins will then be transferred to a nitrocellulose membrane and ToxT<sup>N</sup>-LexA<sup>N</sup> fusion proteins will be detected by Western immunoblot utilizing maltose binding protein antisera (New England Biolabs). Detection will be expressed by using an ECL Western Blotting Analysis System kit (Amersham Biosciences).

## Literature Cited

- Bustos, S.A. and Schleif R.F. (1993) Functional domains of the AraC protein. *Proc Natl Acad Sci U S A.* **90**(12): 5638–5642.
- Chothia, C. (1976) The nature of the accessible and buried surfaces in proteins. *J. Mol. Biol.*, **105**: 1-14.
- Cunningham, B.C. and Wells, J.A. (1989) High-resolution epitope mapping of hGH receptor interactions by alanine-scanning mutagenesis. *Science*, **244**: 1081-1085.
- DiRita, V.J., Parsot, C., Jander, G., and Mekalanos, J.J. (1991) Regulatory cascade controls virulence in *Vibrio cholerae*. *Proc Natl Acad Sci USA* **88**: 5403-5407.
- Hanahan, D. (1983) Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166**: 557–580.
- Higgins, D.E., Nazareno, E., and DiRita, V.J. (1992) The virulence gene activator ToxT from *Vibrio cholerae* is a member of the AraC family of transcriptional activators. *J Bacteriol* **174**: 6974-6980.
- King, C.A. and van Heyningen, W.A. (1973) Deactivation of cholera toxin by a sialidase-resistant monosialosylgangliosides. *J. Infect. Dis.* 127, 639-647.
- Koch, R. (1884) An address on cholera and its bacillus. *Br. Med. J.* 2, 403-407.
- Lin, L.L. and Little, J.W. (1989) Autodigestion and RecA-dependent cleavage of Ind-mutant LexA proteins. *J Mol Biol.* **210**(3):439-52.
- Miller, J. H. 1972. Experiments in Molecular Genetics. In: *Assay of B-galactosidase*. Cold Spring Harbor Laboratory. Cold Spring Harbor, NY 352-359.
- Pierce, N.F. (1973) Differential inhibitory effects of cholera toxoids and ganglioside on the enterotoxin of *Vibrio cholerae* and *Escherichia coli*. *J. Exp. Med.* 137, 1009-1023.
- Prouty, M.G., Osario, C.R., and Klose, K.E (2005) Characterization of functional domains of the *Vibrio cholerae* virulence regulator ToxT. *Mol Microbiol.* **58**(4):1143-56.
- Reidl, J., and Klose, K.E. (2002) *Vibrio cholerae* and cholera: out of the water and into the host. *FEMS Microbiol Rev.* **26**(2):125-39. Review.

- Sack, D.A., Sack, B.R., Nair, B. G., and Siddique, A.K. (2004) Cholera. *Lancet*. **363**(9404):223-33. Review.
- Schuhmacher, D.A. and Klose, K.E. (1999) Environmental signals modulate ToxT-dependent virulence factor expression in *Vibrio cholerae*. *J Bacteriol*. **181**(5):1508-14.
- United Nations Educational, Scientific and Cultural Organization (UNESCO) and the United Nations World Water Assessment Programme (WWAP). (2006) The United Nations World Water Development Report 2: 'Water, a shared responsibility'. Berghahn Books, NY, pp 227.  
<<http://unesdoc.unesco.org/images/0014/001454/145405E.pdf>>  
[accessed 2006 April 1]
- Waldor, M.K., and Mekalanos, J. J. (1996) Lysogenic conversion by a filamentous phage encoding cholera toxin. *Science* **272**: 1910-1914.