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

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Abstract

Vibrio cholerae is a motile, gram-negative, rod-shaped bacterium and the etiologic agent responsible for the diarrheal disease cholera. Research into the pathogenesis of *V. cholerae*, which afflicts thousands of people worldwide each year, is critical for the development of more effective vaccines and therapeutics. ToxT is a key regulatory protein in *V. cholerae*, which 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. We are interested in determining which amino acids of ToxT are necessary for dimerization. Scanning alanine mutagenesis studies have shown that there are twenty-three N-terminal ToxT alanine substitution mutants defective for transcriptional activation. In the present study, we tested the effects of the individual N-terminal alanine substitutions on dimerization. Alanine mutations were introduced into a plasmid that expressed a ToxT^N-LexA^N protein fusion. These plasmids were transformed into a *sulAp-lacZ* reporter strain (JL1436) and then assayed for β -galactosidase activity. We expected that alanine substitutions that cause defects in dimerization will exhibit greater than 50% β -galactosidase activity in comparison to wild-type (ToxT^N-LexA^N) due to their inability to dimerize and repress *sulA* transcription. Data suggests that of twenty-three N-terminal alanine substitution mutations known to affect ToxT transcriptional activation, two mutations, which lie within a putative dimerization alpha helix, prevented ToxT dimerization. Future studies of the amino acids that are necessary for ToxT dimerization will help to further characterize the virulence

regulator ToxT. This research was supported in part by NIGMS MARC-U*STAR
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Introduction

V. cholerae is a motile, gram-negative, rod-shaped bacterium that belongs to the bacterial family *Vibrionaceae* (Reidl and Klose, 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 and Klose, 2002; Sack *et al.*, 2004). The first six pandemics occurred during the 19th century, affecting the Indian continent, continents in the southern hemisphere, North America, and Europe (Reidl and Klose, 2002; Sack *et al.*, 2004). The 7th pandemic, which occurred during the 20th century, began in Indonesia and eventually spread to South America, the Indian subcontinent, the Middle East, and Africa (Reidl and Klose, 2002; Sack *et al.*, 2004). In the past decade, there have been numerous 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 of cholera 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 reach up to 50-1000mL/h (Sack *et al.*, 2004). Severe dehydration typically follows if left untreated. Severe dehydration is characterized by undetectable blood pressure, poor skin turgor, sunken eyes, and wrinkled hands and feet (Sack *et al.*, 2004). Without proper medical attention

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

During its life cycle, *V. cholerae* persists in aquatic environments, and in the human host (Reidl and Klose, 2002). While inhabiting aquatic environments, *V. cholerae* can be found as biofilms or free swimming vibrios (Sack *et al.*, 2004). Biofilms are three-dimensional, multi-cellular micro-communities that help shield *V. cholerae* from the harsh aquatic environments (Reidl and Klose, 2002). Since *V. cholerae* persists in aquatic environments, the vehicle for infection is contaminated drinking water, or improperly prepared seafood, such as shellfish (Reidl and Klose, 2002; Sack *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 and Klose, 2002; Sack *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 *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 ϕ) (Waldor and Mekalanos, 1996). This bacteriophage transduces the genes that encode cholera toxin, the

other key virulence factor in *V. cholerae*, into the bacteria (Waldor and Mekalanos, 1996). In the small intestine *V. cholerae* secretes CT, which binds to the GM₁ ganglioside receptor of epithelial cells, and the toxin is then translocated into host cells where it catalyzes ADP-ribosylation of the G α . The end result is ionic imbalance that leads to fluid loss responsible for the voluminous watery diarrhea and if left untreated, death (King and van Heyningen, 1973; Pierce, 1973).

ToxT is 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 in 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 a DNA binding domain in the C-terminus. Studies in the Klose laboratory (Prouty *et al.*, 2005) have shown that ToxT contains dimerization determinants in the N-terminus and a DNA binding domain in the C-terminus, similar to AraC. It also has 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. We hypothesize that by altering the residues involved in ToxT dimerization by Ala substitution, we will negatively affect its ability to dimerize, and the resulting mutant protein will be defective for transcriptional activation.

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, 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 (twenty-three 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 was the focus of my project.

The N-terminal Ala substitutions were 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^N) 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^N fusion proteins in JL1436 will lead to repression of LacZ activity only if the domain fused to LexA^N contains dimerization determinants. The Klose laboratory has previously demonstrated that the ToxT amino terminus (ToxT^N) fused to LexA^N represses *sulAp-lacZ* transcription, providing evidence that dimerization determinants are located in ToxT^N.

In order to test the effects the twenty-three N-terminal alanine substitutions may have on the dimerization ability of ToxT, the ToxT^N-LexA^N fusion protein was utilized.

Each of the 23 Ala substitution mutations were introduced into the plasmid expressing ToxT^N-LexA^N by site-directed mutagenesis, as detailed below. Each mutation was confirmed by sequencing, and then each mutant plasmid was transformed into the reporter strain JL1436. Fusion protein expression was induced by the addition of isopropyl- β -D-thiogalactopyranoside (IPTG), and *sulAp-lacZ* transcription was measured by β -galactosidase activity. Controls included the (unmutated) ToxT^N-LexA^N, 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 had an effect on the dimerization ability of ToxT, the ToxT^N-LexA^N protein will be defective for binding *sulAp*, and will fail to repress the expression of β -galactosidase, resulting in high β -galactosidase activity. If the mutations do not affect dimerization, then the ToxT^N-LexA^N protein will bind the *sulA* promoter and repress β -galactosidase expression, resulting in low levels of β -galactosidase activity, similar to that caused by the (unmutated) ToxT^N-LexA^N.

Materials and Methods

Bacterial Strains - *Escherichia coli* strains DH5 α (Hanahan, 1983) and Top10 cells (Invitrogen) were utilized for cloning. The *E. coli* reporter strain JL1436 (Lin and Little, 1989) was used in the LexA-based dimerization assay.

Site-Directed Mutagenesis – The QuickChange Site-Directed Mutagenesis Kit (Stratagene) was used to perform all site-directed mutagenesis. Plasmid pKEK 522 contains the coding sequence for the ToxT^N-LexA^N 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 and Klose, 1999) and facilitates the detection of ToxT (see below).

Polymerase Chain Reaction (PCR): pKEK522 was used as the template plasmid for all mutagenesis reactions. Complimentary oligonucleotides were designed to change the codons of specific amino acids to encode alanine (GCX). Each PCR mutagenesis reaction contained two complimentary oligonucleotides that encoded the alanine mutation of interest and pKEK522 template plasmid. In addition, each PCR reaction also contained 1.25 μ l of complimentary primers, 1 μ l of DNTPs, 2 μ l of DMSO, 5 μ l of Pfu Turbo buffer, and 37.5 μ l of water. A negative control which consisted of the PCR mix mentioned above, excluding primers, was also included in each batch of PCRs. PCR reactions were screened using a 1% agarose gel. Positive PCR products were observed at 6,937 base pairs.

DNA Manipulations: 2 μ l DNA from PCR mutagenesis reactions were transformed into Top10 *E. coli* cells using a Bio Rad Gene Pulser II electroporator. Each reaction was added to 1 mL of Luria broth (LB) and incubated at 37°C for 1 hour. Cells were pelleted and re-suspended in 100 μ l of LB. Cultures were plated on LB agar containing 100 μ g/ml ampicillin, which selects for the cells containing the pKEK522 plasmid, and incubated overnight at 37°C. Four colonies were selected from each plate, and plasmids were isolated from each cell using a plasmid isolation kit (Qiagen). Each plasmid was sequenced, utilizing *toxT*-specific primers. One plasmid from each reaction which contained the correct alanine substitution mutation, confirmed by sequencing, was utilized in further assays, as described below.

β-Galactosidase Assays: Plasmids containing the amino terminal alanine substitution mutation in ToxT^N-LexA^N was transformed into the *sulAp-lacZ* reporter strain JL1436, as described above. Overnight cultures were added (1:50) to 5 mL of LB containing 100 μg/ml ampicillin and 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG); to induce expression of the ToxT^N-LexA^N fusion protein. Strains were grown to mid-log phase (OD₆₀₀ 0.3-0.6) at 37°C. Prior to each β-galactosidase assay, samples were normalized and stored in sample buffer to conduct Western immunoblots as described below. Cultures were assayed for β-galactosidase activity as described by Miller (1992). 100 μl of culture was added to 900 μl of Z buffer (10 ml Z-buffer + 27 ul of β-MET). 1 drop of 0.1% SDS and 2 drops of chloroform was added to each 1 mL sample and vortexed for 5 seconds. Tubes were incubated in a 28°C water bath for 5 minutes and then each reaction was started by adding 200 μl of o-nitrophenyl-β-D-galactopyranoside (ONPG) buffer (4 mg/ml ONPG in Z buffer) in 20 second intervals. Prior to beginning each reaction the start time was recorded. After the reactions produced a light yellow color, the reaction was stopped by adding 500 μl of 1M Na₂CO₃ and the stop time was recorded. After the assay was complete, samples were left undisturbed for 10 minutes to allow the cellular debris to sink to the bottom of the test tube. Afterwards, 200 μl from the top each sample was transferred to a 96 well plate and placed in an Eliza plate reader (BioRad). Readings for each sample were taken at OD₄₂₀. Miller units were then 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 were denatured and loaded into a 12% polyacrylamide gel and separated by electrophoresis. Proteins were transferred to a nitrocellulose membrane and ToxT^N-LexA^N fusion proteins were detected by Western immunoblot utilizing maltose binding protein antisera (New England Biolabs). Detection was expressed by using an ECL Western Blotting Analysis System kit (Amersham Biosciences).

Results

Site-Directed Mutagenesis

Twenty-three MBP-ToxT^N-LexA^N fusion-proteins containing N-terminal alanine mutations were constructed utilizing the QuickChange Site-Directed Mutagenesis Kit (Stratagene). 5 µl aliquots from each of the PCR reactions were mixed with 5X loading buffer and loaded into 1% agarose gel and subjected to electrophoresis to confirm constructs. Positive constructs displayed bands at ≈7 KB.

Structural Prediction by the Klose Laboratory

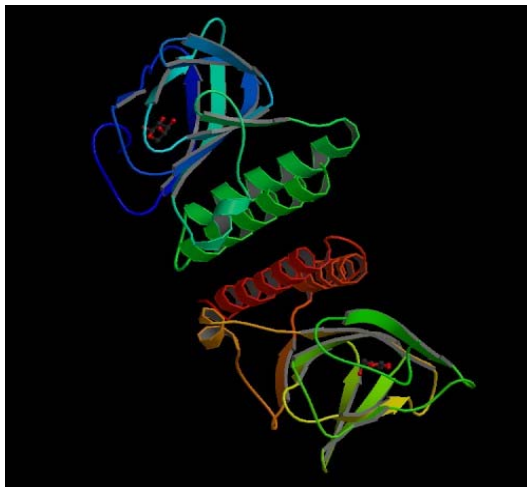


Figure 1. AraC N-terminus; possibly structurally related to ToxT N-terminus (as predicted by Soisson *et al.*, 1997). Schematic depicts two dimerized N-terminal AraC proteins. Each protein is made up of a β jelly roll and two alpha helices.

Structural prediction studies (THREADER) conducted by the Klose laboratory indicates that ToxT's amino terminus is structurally related to AraC's N-terminus and is composed of a beta jelly roll (aa 1-87), and two alpha helices (alpha helix 1: aa 88-98, alpha helix 2: aa 99-165). Amino

acids located in the second alpha helix are predicted to be involved in the dimerization interface, the region where two proteins interact. This assumption is further supported by the AraC structural model, which shows the second alpha helix being directly involved in dimerization (Figure 1).

β-galactosidase Assay

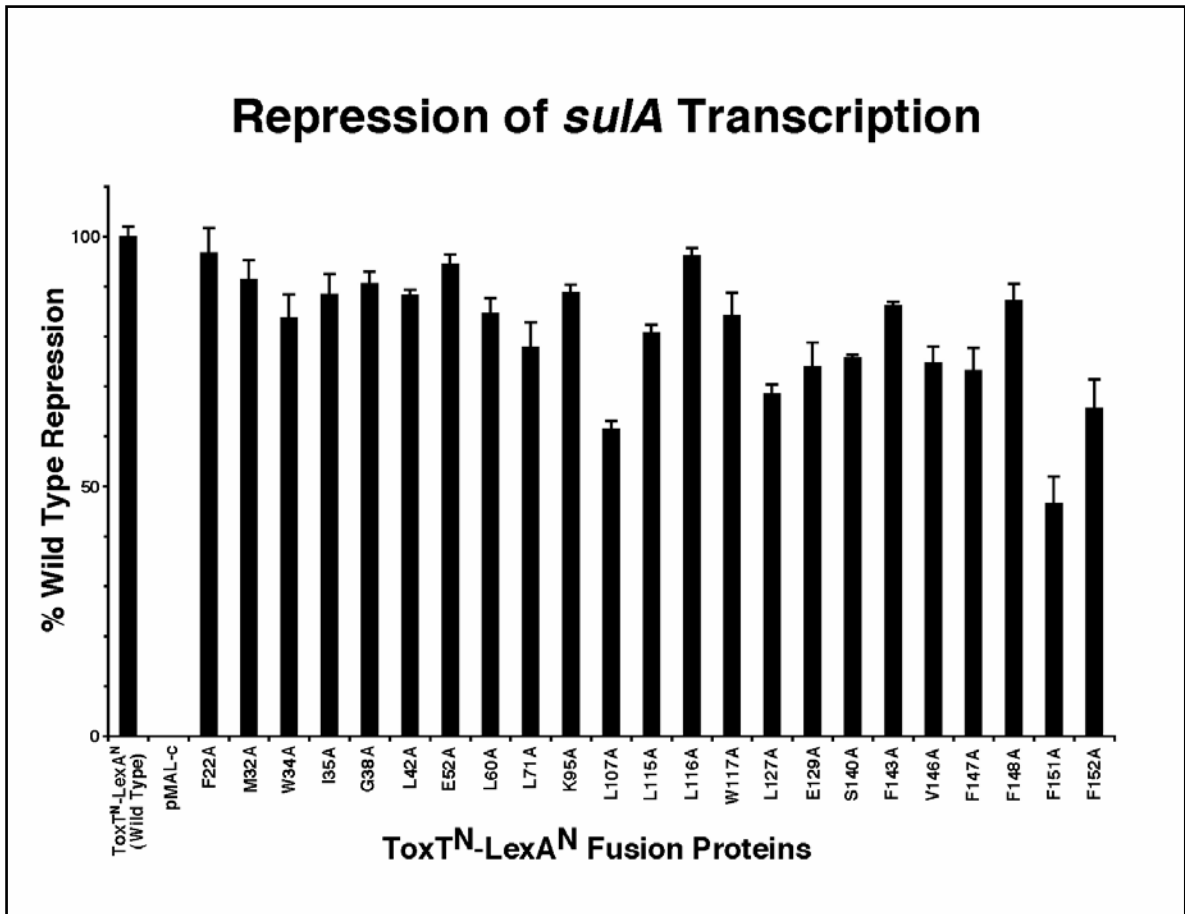


Figure 2. β-galactosidase Assay. Twenty-three N-terminal mutations assayed for β-galactosidase activity. Of the twenty-three, only two are statistically critical for dimerization: F151A ($p < 0.001$) and L107A ($p < 0.001$).

β-galactosidase assays indicated that amino acids located in the predicted beta jelly roll (aa 1-87) were found to exhibit wild type repression by 75% or more (Figure 2). Amino acids in the two alpha helical regions (alpha helix 1: aa 88-98, alpha helix 2: aa

99-165) displayed variable wild type repression ranging from less than 50% to nearly 100%. Student t-test indicates that F151A ($p < 0.001$) and L107A ($p < 0.001$) significantly affect ToxT dimerization (Figure 2). Interestingly, these amino acids lie within the predicted dimerization interface (alpha helix two) of ToxT, as illustrated by the structural model of AraC (Figure 1).

Western Immunoblot

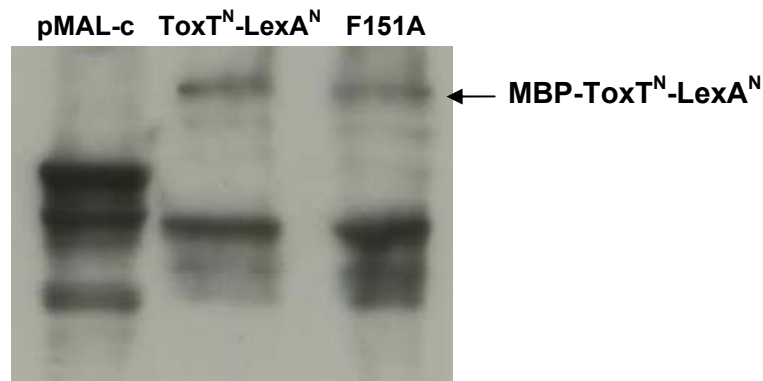


Figure 3. Western Immunoblot of MBP-ToxT^N-LexA^N F151A suggests protein stability. Expression of pMAL-c (negative control), ToxT^N-LexA^N (wild type), and F151A (mutant) was detected by Western immunoblot utilizing maltose binding protein antisera (New England Biolabs). Detection was visualized with an ECL Western Blotting Analysis System kit (Amersham Biosciences). Results indicate protein stability of F151A.

SDS-PAGE and Western Immunoblot was performed for normalized samples: pMAL-c (negative control), ToxT^N-LexA^N (wild type), and F151A (mutant). Detection was visualized with an ECL Western Blotting Analysis System kit (Amersham Biosciences). Expression of F151A was observed to be similar to ToxT^N-LexA^N (Figure 3), suggesting that the mutation of F151A does not result in protein degradation.

Conclusion

β-galactosidase Assay: ToxT's N-terminus contains dimerization determinants. Two amino acids, F151A ($p < 0.001$) and L107A ($p < 0.001$) were found to significantly affect dimerization. Interestingly, these amino acids are involved in a possible

dimerization contact region which would directly regulate dimerization. It appears that hydrophobic amino acids such as leucine, valine and phenylalanine located in the second alpha helix generally displayed a diminished ability to repress *sulAp* in comparison to hydrophobic amino acids in the beta jelly roll. Hydrophobic amino acids are typically found buried within a protein or can interact with other hydrophobic amino acids on a second protein (Lehninger *et al.*, 2005). This is consistent with the idea that the amino acids predicted to be located in the second alpha helix of ToxT's N-terminus may be involved in dimerization and represent the region that facilitates protein – protein interactions (Figure 1). These results are consistent with the speculation by Prouty *et al.* (2005) of the Klose laboratory that N-terminal coiled-coils are involved in dimerization. Lesk *et al.* (1980) found that hydrophobic and Van der Waals forces may provide alpha helices with the opportunity to explore each others' surfaces to find a good fit. This theory may explain the variability of wild-type repression observed for ToxT mutants in the residues 107 – 152 (Figure 2). The stability of the alpha helices, despite the Ala substitutions, still allows the monomers to interact and conform to a configuration which still allows them to dimerize, although perhaps not optimally.

Western Immunoblot: Evidence that the F151A mutation does not result in protein degradation indicates that the protein is indeed present. Thus, the depressed wild type activity of the F151A (Figure 2) is not the result protein instability, but rather of a decrease in dimerization.

Future Directions: Because the L107A mutant also displayed defects in dimerization in our LexA assay, although not to the same extent as F151A (Figure 2), a Western Immunoblot will be conducted for L107A in the near future to confirm that this

mutation affects dimerization rather than protein instability. Currently, the bacterial two-hybrid system, which is another assay that investigates protein-protein interactions, is being utilized to further study the N-terminal domain of ToxT. Specifically, F151A and L107A will be targeted for these studies. The bacterial two hybrid system will enable us to more fully characterize dimerization defects. Other have utilized alanine stretch mutagenesis to scan protein sequences in protein folding studies, investigate protein stability, and characterize the functional role of a stretch of residues (Lefèvre *et al.*, 1996). It would be interesting to introduce multiple alanine mutations in the beta-jelly roll, first alpha helix, and second alpha helix of ToxT separately in order characterize these three different N-terminal domains, and also to gain a deeper understanding of the structure – function of the N-terminus of ToxT.

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