1-1-2014

Mechanisms For Bicarbonate-Mediated Virulence In vibrio Cholerae

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MECHANISMS FOR BICARBONATE-MEDIATED VIRULENCE IN *VIBRIO CHOLERAE*

by

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DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

2014

MAJOR: IMMUNOLOGY &
MICROBIOLOGY

Approved by:

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Advisor Date

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ACKNOWLEDGEMENTS

While pursuing my Ph.D. at Wayne State University, I have had interactions with many people who deserve a lot of appreciation. My dissertation advisor, Dr. Jeffrey Withey, took me on with very little experience in lab settings. He taught me how to be a successful scientist, and his guidance will not be forgotten. Additional scientific assistance was provided to me by my committee members: Dr. Melody Neely, Dr. Timothy Stemmler, Dr. Philip Pellett, and for a time Dr. Judith Whittum-Hudson. I would like to thank them all for the support they have provided.

Additional support came from the members, past and present, of the Withey lab. All members were important in scientific discussion and made the lab enjoyable. Basel Abuaita and Michelle Bellair trained me in the lab and without them I would not have been as successful. I will carry their work ethic with me throughout my career. Additionally, I would like to thank Sarah Plecha, Jennifer Stone, Kristie Mitchell, and Paula Dietz. Without all of these personalities, the lab would not have been the same. All of the graduate students in the department deserve my appreciation for always being involved and providing support. I would also like to thank Hannah Rowe for being a great study partner during classes and always helping with technical problems.

Finally, I would like to thank the most important people in my life for all their help and support. My family has provided me with endless encouragement and cannot be thanked enough for all they have done for me. My parents, Nancy and Larry Thomson, my in-laws, Don and Sandy Fowler, and my brother, Paul, have all played roles in the completion of my graduate degree. Most of all, I would like to thank my amazing wife, Stacie. She has been extremely patient throughout the entire process and I am lucky to have her. Lastly, I would like to thank my beautiful daughter, Lena, who always gave a smile or laugh when I needed it the most.

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GENERAL INTRODUCTION

Cholera is a severe diarrheal illness, which is caused by the gram negative bacterium, *Vibrio cholerae*. The disease is initiated by ingestion of bacteria from contaminated food or water. Disease symptoms are characterized by voluminous watery diarrhea, referred to as "rice-water stool", because of its characteristic appearance. Cholera patients can lose up to 1 L of water every hour, leading to severe dehydration and hypotensive shock, termed "cholera gravis", within a few hours after initial symptoms. Most cholera-associated deaths occur within the first day due to the rapid dehydration. Additional symptoms include vomiting early in disease, cramping, clammy skin, and decreased skin turgor. If left untreated, there is a 50% lethality rate. However, with effective therapy, this rate can be reduced to less than 0.2% (205). Current therapy for cholera disease consists of rehydration through intravenous fluids and oral rehydration solution (ORS). ORS contains concentrations of sodium and glucose to maximize uptake of sodium into the small intestine (84, 184). Antibiotics can also aid in limiting the duration of the disease, but rehydration therapy is needed in combination. Another clinical manifestation of the disease is called "cholera sicca", in which fluid accumulates in the lumen of the intestine and leads to circulatory collapse before the first diarrheal symptoms occur (84).

In 2012, the World Health Organization (WHO) reported approximately 245,000 documented cases of cholera, resulting in 3034 deaths (252). However, this number is greatly under reported, due to problems with disease identification in developing countries. Additionally, countries may underreport due to fear of effects on tourism, travel, and trade. Historically, cholera affects approximately 3-5 million people annually, with an estimated 120,000 deaths (269). In regions where cholera is endemic, severe cases usually occur in children and adults that have not been previously exposed (77). When a cholera epidemic occurs, age does not play a role

as the population has not yet been exposed and thus has no developed immunity (37). Cholera is endemic in more than 50 countries, mainly in Asia and Africa, but has the capability to cause widespread epidemics. A recent example of an epidemic occurred after an earthquake hit Haiti in 2010. Infrastructure breakdown and lack of sanitation led to a cholera outbreak which spread throughout the entire country within a month. As a result of this outbreak, there have been over 500,000 cases and 7000 deaths (27). The disease has since extended from Haiti to the Dominican Republic and Cuba, showing its propensity for epidemic spread (252).

Cholera has been described dating back to the 5th Century, first depicted in Sanksrit. The disease has been found in the Indian subcontinent for centuries, but has since spread elsewhere. There were six pandemics between the years 1817 and 1923. The 7th and current pandemic began in 1961, and has involved almost the entire world. The main thought in the 19th century was that cholera disease was caused by "bad" air that would carry the disease and infect populations. Later in 1854, during the third pandemic which reached London, physician John Snow determined that the source of the disease was linked to a town water pump, and ordered that it be decommissioned (224). This action led to the cessation of the epidemic in London. With further epidemiological studies Snow discovered a water company was delivering sewage contaminated water to homes. Independently in 1854, Italian anatomist Filippo Pacini performed autopsies of recently deceased cholera patients and analyzed their intestines. He described comma-like shaped bacteria within the intestine, and believed that they were contagious and the cause of the disease and termed them *Vibrios* (14). However, his work was ignored by the scientific community until Robert Koch rediscovered the organism in 1884, linking the same comma-shaped bacilli to cholera patients (111). Pacini, several years later was recognized for his discovery and the bacterium was named *Vibrio cholerae* (127).

V. cholerae is a gram-negative bacterium that is classified based on the O antigen of its lipopolysaccharide. There are more than 200 serogroup classifications (57, 92, 205). Each of the seven pandemics has been attributed to the O1 serogroup of *V. cholerae*. Beginning in 1992, the O139 serogroup emerged with the ability to cause outbreaks, and is sometimes referred to as the eighth pandemic (16, 51, 102, 182). The O1 serogroup can be further divided into two biotypes: classical and El Tor (200). The classical biotype was responsible for the first six pandemics, while El Tor is responsible for the seventh and has currently replaced the classical biotype in the environment (57, 205). Classical biotype is known to initiate a more severe disease in humans, while El Tor is thought to survive better in the environment (57, 127).

Each *V. cholerae* biotype can be further subdivided into two major serotypes: Ogawa and Inaba. Ogawa strains produce the A and B antigens, while Inaba produces only the A and C antigen. Hikojima, a third serotype, albeit rare and unstable, produces all three antigens (205, 208). Classical and El Tor biotypes can be distinguished phenotypically using assays for hemolysis, hemagglutination, phage lysis or polymyxin B resistance (127). Additionally, biotype-specific genes can be genotypic distinguishing characteristics. Of late, new variants of El Tor that have some phenotypic similarity to classical biotype have been isolated from hospitalized patients (183).

Much work has been done to create a cholera vaccine, yet cholera vaccination is not yet a major part of worldwide control programs. Initially, a killed injectable vaccine was produced and required for international travel in the 1880s. Currently there are two oral killed vaccines that contain multiple biotypes of *V. cholerae* O1. One contains recombinant cholera toxin B subunit while the other contains *V. cholerae* O139 in addition to the O1 serogroup (105, 251). The vaccines can provide protection for adults for up to three years; however, the duration of

protection for children is much lower (38, 92). Live attenuated oral cholera vaccines have been used and efficacy results have been mixed (92). It has been suggested that the efficacy of vaccines can be substantiated by attenuated motile *V. cholerae*, which will penetrate the mucus layer and increase mucosal immunity (41, 130, 163). However, live attenuated cholera vaccines are not recommended by the WHO. Currently, cholera vaccines are being suggested for use during outbreaks and in areas at risk of outbreaks (251).

Studies of aquatic environments have shown that *V. cholerae* persist as normal inhabitants of freshwater, coastal, and brackish waters where they can also be transmitted to humans. The bacteria are known to associate with marine organisms, such as shellfish, copepods, Chironomid egg masses, and vertebrate fish (87, 109, 198, 204, 213, 234). Disease in developed countries usually stems from the consumption of contaminated food, while contaminated water is most often the problem in underdeveloped countries (78, 113, 217, 227). In areas where cholera is endemic, seasonal outbreaks occur in correlation with increases in surface water temperature, as well as phytoplankton blooms (124, 125, 234). Even with the association with marine life, cholera disease in nature is generally only seen in a human host (57, 205). The infectious dose of *V. cholerae* is approximately 10^8-10^{11} CFU for healthy North American volunteers (127). The bacteria must be at a concentration high enough to overcome innate immune defenses as well as the low pH of the stomach. Bacteria ingested with food that act as acid buffers, or ingested with the addition of bicarbonate, have a greatly decreased infectious dose and elevated infection rate (110, 206). Bacteria shed from an infected individual are in a hyperinfectious state that decreases the infectious dose to as low as 10-100 cells (25, 164, 186, 264), giving explanation for the rapid rate of spread during an epidemic.

Disease-causing *V. cholerae* exists in a biphasic life cycle: in the marine environment and in the human host. In aquatic environments outside of the human host, *V. cholerae* persists between epidemic seasons as free swimming planktonic cells, attached to biotic or abiotic surfaces, or in a viable but non-culturable (VBNC) state (1, 5, 87, 118, 218, 234). *V. cholerae*, *w*hen attached to chitonous surfaces such as crustacean shells, produce a chitinase which aids in using the surfaces as carbon and nitrogen sources (9, 161, 185). Additionally, *V. cholerae* can persist in the environment in a VBNC state, which makes them undetectable by normal culturing techniques (254). The signals for the bacteria to enter this state are not well defined; however, it is thought that it is in response to nutrient deprivation. *V. cholerae* in this state can regain the ability to multiply when grown in the presence of eukaryotic cells or in the human intestine (39, 214). The bacteria can also associate with marine organisms and form biofilms, which play an important role in survival in the environment.

It is thought that *V. cholerae* survives in the environment mostly in biofilms, and this is the form most likely consumed by humans (4). Biofilms are surface-attached micro-communities of bacteria that are usually coated in a matrix of polysaccharides or proteins (42). Increased biofilm formation by *V. cholerae* can be seen in a "rugose" colony phenotype due to increased secretion of VPS (vibrio polysaccharide), which results in resistance to chlorine (180, 201, 245). The role of the rugose colony morphology compared to that of the smooth colony phenotype in pathogenesis is not fully understood. However, biofilm-associated *V. cholerae* displays hyperinfectivity compared to the free swimming planktonic form (232, 268). Similarly, bacteria from cholera stool can be in planktonic form or biofilm-associated, the latter of which has greatly increased infectivity (58). This could be due to decreased acid susceptibility that the bacterium has when associated with a biofilm, increasing its odds of survival through the low pH of the

stomach (165). Additionally, the infectivity of water samples is greatly increased after removal of particles greater than 20uM in size, showing the importance in biofilms and disease (40). The existence of biofilms in the marine environment and increased infectivity of biofilm-associated *V. cholerae* illustrates the importance of biofilms in each phase of the bacterium's life cycle.

In the other phase of the life cycle, the human host, the bacteria are ingested and eventually colonize the intestine. Once the bacteria pass through the low pH of the stomach, they enter the duodenum and colonize the intestinal crypts and villi surfaces (83, 211). Motility and chemotaxis genes are expressed in the environment and are thought to be needed for the initiation of diseases. Upon entry into the small intestine, the bacteria undergo a change in gene expression, where genes involved in regulating motility are down-regulated and genes involved in pathogenesis and disease are up-regulated (24, 143). Colonization of the intestine occurs as well as progression of the disease. Towards the end of disease, the bacteria initiate the "mucosal escape response", where another inversion of gene expression occurs and the bacteria are shed back into the environment in the hyperinfectious state (188). The response additionally requires up-regulation of other genes, such as proteases to allow detachment from the epithelium, and motility genes for exit from the intestine (188).

Two traits are required of strains with pandemic potential. These are obtained through the acquisitions of the cholera toxin bacteriophage (CTXΦ) and vibrio pathogenicity island (VPI) (128, 246). These are horizontally acquired mobile genetic elements that provide the organism with genes essential for causing cholera. CTXΦ is a 6.9kb lysogenic filamentous bacteriophage that encodes the genes for the cholera toxin (CT), which is directly responsible for the voluminous watery diarrhea seen during disease (246). Additionally, CTXΦ carries the genetic information encoding the zona occludens toxin (Zot) and accessory enterotoxin (Ace), which

also have enterotoxic activity (60, 123, 243). The 40kb VPI contains the genes encoding the other major virulence factor, toxin co-regulated pilus (TCP), as well as other accessory virulence factors and genes with unknown function (128). Non-toxigenic strains of *V. cholerae*, i.e. strains that do not cause cholera, can be converted into toxigenic strains capable of causing cholera (246). This involves the production of extracellular CTXΦ particles by toxigenic strains. These particles are then taken up by non-toxigenic strains and integrate into the chromosome and act as stably expressed lysogenic phages (246). However, TCP must first be expressed by the non-toxigenic strains, as this is the receptor for the CTXΦ (246). This transduction event could happen in the intestine, effectively creating new toxigenic strains.

CT, which is responsible for the watery diarrhea, was first discovered and proved to act as an enterotoxin in 1959 by S.N. De (45). He showed that cell-free culture supernatants from classical biotype, when injected into rabbit ligated ileal loops, could cause fluid accumulation similar to what is produced when cultures of *V. cholerae* were injected. The toxin became further defined by the work of Finkelstein and LoSpalluto, in 1969 (63, 64). They purified the toxin and determined that it was made of two moieties, one of which could elicit vascular permeability. Later it was shown that cholera toxin is an 84 kDa protein consisting of five "light" subunits that are responsible for binding of the toxin to its target, and one "heavy" subunit, responsible for the action of the toxin (151). The "light" subunits refer to what is now called the B subunits, for binding, while the "heavy" subunit is the active A subunit. Together, these subunits make up the AB_5 holotoxin. Around the same time, the receptor for cholera toxin was identified as ganglioside GM_1 (106, 107, 131), found mainly organized into lipid rafts on the surface of intestinal epithelial cells (179). The active A subunit is translated as a single polypeptide chain, but it then post-translationally modified by a *V. cholerae* protease into two fragments: A1 and

A2. The two fragments remain attached by a disulfide bond with the active domain residing in the A1 fragment. The A2 fragment functions to insert into the B subunit pentameric ring (75).

Once the holotoxin is assembled in the periplasm, it is secreted extracellularly via a type II secretion system (26). Upon reaching the lumen of the intestine, the toxin binds to the GM_1 ganglioside by way of the five pentamerically arranged B subunits (99, 106, 107, 131). Once bound, CT is endocytosed and undergoes retrograde transport. After reaching the endoplasmic reticulum, the A subunit dissociates from the B subunits (61, 153). The A subunit translocates to the cytosol of the epithelial cell, where the A1 fragment ADP-ribosylates the $G_{s\alpha}$ subunit of adenylate cyclase (76). This leaves adenylate cyclase locked in its GTP-bound active state, thus increasing the concentration of cellular cAMP and causing an electrolyte transport imbalance. The imbalance consists of decreased sodium uptake with an increase in chloride and bicarbonate efflux (62). Decreased sodium uptake is followed by decreased water absorption, while chloride and bicarbonate efflux increases water secretion into the lumen of the intestine (62, 178). The combined effects of the electrolyte imbalance lead to fluid accumulation in the intestine and the severe watery diarrhea seen in cholera patients.

The second major virulence factor essential for *V. cholerae* strains to cause cholera disease is TCP (98, 128). The toxin co-regulated pilus is a type IV bundle-forming pilus and is termed “toxin co-regulated” due to its dependence on the same culture conditions as CT for expression (132, 237, 238, 240). TCP is responsible for mediating microcolony formation at the epithelial surface by bacterium-bacterium interactions (132), secretion of the colonization factor TcpF (140), and providing defense against antimicrobials and bile (141). Genes involved in TCP production and assembly are encoded by the 12 gene *tcp* operon, located on the VPI (57). The first gene in the operon, *tcpA*, encodes the major pilin subunit (132). Once assembled, TCP is

composed of 5-6nm filaments made up of many repeating subunits (238). A *tcpA* mutant strain of *V. cholerae* is unable to colonize the laboratory infant mouse model or healthy human volunteers, demonstrating its importance in initiation of disease (98, 240). TCP has been shown to mediate attachment and formed pilin matrices that aided in protection in the infant mouse model of colonization using field-emission scanning electron microscopy (FESEM) (141). Nevertheless, it remains unclear if TCP mediates direct attachment to the epithelium and colonization. Most of the *tcp* operon is involved in the production and assembly of the pilus. However, *tcpF* encodes a soluble factor important for colonization of intestinal epithelial cells that is secreted by TCP (140). Additionally, *tcpJ* encodes a leader peptidase that is important for processing of TcpA during secretion (129).

Other genes located on the VPI are coordinately regulated along with the *tcp* operon. including *tagA, aldA, tcpI,* and the *acfABCD* genes (47, 55, 90, 91, 192, 194, 247, 249). The roles in colonization and disease are largely unknown in regard to these coordinately regulated genes. *tagA* encodes a metal-dependent mucinase that is involved in cleaving mucin proteins to potentially modify the cell substrate for enhanced *Vibrio* binding (231). The aldehyde dehydrogenase encoded by *aldA* caused no defect in colonization when mutated (192). The *acfABCD* genes make up the accessory colonization factor (ACF) and are required for efficient colonization of the infant mouse; however, their exact role in pathogenesis is unclear (194). *acfB* and *tcpI* encode methyl accepting chemoreceptors that when mutated on their own do not cause colonization defects. However, when mutated simultaneously, they cause a defect in colonization, suggesting redundancy in their chemotactic properties (30, 54, 91). Additionally, the small RNAs encoded by *tarA* and *tarB* are located on the VPI upstream of *tcpI* (21, 202). TarA is involved in regulating expression of *ptsG,* which encodes a glucose transporter, and *V.*

cholerae with mutation in *tarA* have a colonization defect (202). TarB is involved in negative regulation of the secreted colonization factor TcpF prior to *V. cholerae* penetration of the intestinal epithelial layer (21).

In addition to CT and TCP, other virulence factors are important for colonization and disease. In particular, the roles of motility and chemotaxis have been intensely studied for their function in pathogenesis. *V. cholerae* is a highly motile bacterium, establishing this motility through the action of a single, polar flagellum. There is much debate in regard to the requirement of motility for intestinal colonization, as results differ depending on strains, mutations, and animal models used (67-69, 74, 203, 239, 256, 257). Non-motile mutants of classical biotype strains did not have impaired colonization in the infant mouse model compared to WT (74). Conversely, non-motile mutants in El Tor were shown to be ineffective at colonization of the infant mouse (23, 145, 220). The polar flagellum is driven by sodium motive force (133) and disruption of the genes involved in sodium uptake for the flagella can lead to increased CT and TCP when cultured in unpermissive conditions (94). Current thought is that motility is needed in the intestinal environment for penetration of the mucus layer, allowing access of *V. cholerae* to the crypts of the villi (24).

In the aquatic environment, *V. cholerae* is free-swimming with motility genes expressed and virulence genes down-regulated (74, 93, 94). *V. cholerae* contains three chemotaxis operons, only one of which is essential for chemotaxis (80, 96). Chemotaxis provides a mechanism for the bacterium to move toward resources or away from damaging agents. The requirement of chemotaxis in pathogenesis is disputed similarly to the role of motility. Reports have shown that chemotaxis is dispensable for colonization, as non-chemotactic mutants colonize the infant mouse intestine 70-fold better than WT (145). However, current thought is that non-chemotactic

mutants colonize the entire mouse intestine aberrantly, rather than just the proximal intestine (24). Further, positive chemotaxis toward the intestinal crypts where *V. cholerae* colonizes could lead to interaction with antimicrobial peptides that kill the bacteria, which a non-chemotactic mutant would not encounter as regularly, which could be an explanation for higher colonization of the mouse intestine by a non-chemotactic mutant (68, 191). *V. cholerae* has genes encoding 43 potential methyl-accepting chemotaxis proteins (MCP), compared to *Escherichia coli* which encodes only 4 MCPs (19, 24). These proteins respond to environmental stimuli and initiate direction change of the flagellum. *V. cholerae* having such a large number of MCPs demonstrates the complexity of chemotactic signaling, possibly needed because resources in marine environments are sparse. However, MCPs may also be active in the human host. a*cfB* and t*cpI*, located on the VPI and co-regulated with *ctxAB* and *tcp*, encode proteins that share homology with MCPs (30, 54, 91). However, their roles in infection are not fully understood.

Strains that have acquired the CTXΦ and VPI regulate these elements with a complex network of transcription factors. Coordinate expression of CT and TCP genes is dependent on this network, termed the "ToxR regulon" (194). ToxR was the first regulator identified (194, 221); however, it is not the transcriptional regulator that directly activates transcription of the *tcp* operon and *ctxAB* genes. The transcription activator directly responsible for production of the major virulence factors, CT and TCP, is ToxT (29, 47, 115, 262). Once produced, ToxT binds to virulence promoters and activates transcription of these genes (248). *toxT* is under control of the aforementioned ToxR, as well as another regulator, TcpP (95, 100, 144). An overview of the regulatory cascade that results in production of ToxT, and subsequently CT and TCP, is shown in Figure 1.

ToxR is a 32 kDa integral membrane protein that is constitutively produced and is present in all strains of *V. cholerae*, as well as other *Vibrio* species (177). It is not linked to the VPI, unlike ToxT (175). The winged helix-turn-helix DNA-binding domain of ToxR is cytoplasmically localized. ToxR, along with another integral membrane protein, ToxS, acts at the promoter of *toxT* to initiate transcription (174). ToxS is the interaction partner for ToxR, and acts to increase dimerization of ToxR, which is needed for full ToxR activity (52, 190). ToxS increases ToxR stability in *E. coli*; however, this is not seen in *V. cholerae* (10). ToxR also has been shown to partially activate *ctxAB* directly in the presence of bile acids, however full activation does not occur without ToxT (116, 175).

In addition to the major virulence factors, ToxR regulates expression of outer membrane porins OmpU and OmpT, allowing the bacteria to change the composition of its cell membrane in new environments (43, 150, 176). ToxR increases expression of *ompU*, which encodes a porin that has been shown to protect the bacteria from the cytotoxic effects of bile and may also act as an adhesin (196, 197, 226). This increase in transcription of *ompU* by ToxR is also enhanced in the presence of bile (170). Conversely, ToxR decreases expression of *ompT*, which is the dominant porin expressed in nutrient limiting conditions, such as the aquatic environment (150). Addition of asparagine, arginine, glutamic acid, and serine (NRES) to minimal media or growth in rich media increases expression of *ompU* while repressing *ompT* (170).

Another pair of integral membrane proteins, TcpP and TcpH, works in conjunction with ToxR to activate transcription of *toxT* (95). Similar to ToxR, TcpP shares homology with members of the PhoB/OmpR family of transcriptional regulators and contains an amino-terminal, cytoplasmically localized winged helix-turn-helix DNA-binding domain (156). TcpP, through its periplasmic domain, interacts with TcpH. In cells that lack TcpH, TcpP is rapidly degraded (10).

Additionally, even when TcpH is present, if conditions are unfavorable for virulence, TcpP will be degraded (158) . The protease responsible for the degradation of TcpP is the metalloprotease YaeL, which is also present in conditions not optimal for virulence (158). The intestinal bile salt, taurocholate, induces dimerization of TcpP through residues in the periplasmic domain, increasing its activation potential (261). TcpP/H binds the promoter of *toxT* between -51 and -32, while ToxR/S binds between -100 and -69 as shown by DNase I footprinting of the region (144). There is enhanced interaction between ToxR/S and TcpP/H under oxygen limiting conditions that resemble the environment of the small intestine (56). TcpP overexpression can overcome the need for ToxR for full activation of *toxT* (95, 144). Current thought is that when ToxR is present it recruits and guides TcpP to the *toxT* promoter. When ToxR is not present, TcpP relies on DNA-binding specificity to locate the *toxT* promoter (81).

ToxR is constitutively produced, while TcpP/H is under control of various transcription factors. In particular, *tcpPH* is activated by AphA and AphB (139, 222). AphA shares homology with the PadR repressor, which is known for its role in controlling gene expression that is required for the detoxification of phenolic acids (8). AphB, which is encoded by a gene that is not linked to *aphA*, shares homology with LysR regulators (139). DNase I footprinting has shown that AphA binds upstream of *tcpPH* between -101 and -71, while AphB binds at a position between -78 and -43 (137). These two transcription regulators directly interact with each other at the promoter for optimal activation (136). AphB changes conformation in response to potential host stimuli such as changes in pH and anaerobiosis and allows it to better bind the *tcpPH* promoter (135, 236). AphA and AphB are not the only transcription factors that act at the promoter of *tcpPH*. cAMP receptor protein (CRP), which normally plays a role in regulating carbon metabolism, binds to and represses the promoter of *tcpPH* (137, 221, 223). Footprinting

with purified CRP shows protection of a region that is overlapped completely by the binding sites of AphA and AphB (137). Another protein, PepA, responds to variations in pH and temperature, both environmental cues *V. cholerae* encounters while being passaged to the intestine. At non-permissive pH, PepA acts to repress transcription at the *tcpPH* promoter. The mechanism for this repression is still unclear, but PepA is thought to bind directly to the promoter (11).

Other signaling systems enable *V. cholerae* to identify and respond to environmental stimuli and transition to the human host. These include quorum sensing and the secondary messenger 3', 5'-cyclic diguanylate (c-di-GMP). Quorum sensing is a system that allows cell-to-cell communication between bacteria through the constitutive production of small peptides called autoinducers (AIs), leading to changes in gene expression depending on population density (172). The secondary messenger system also is involved in changes in gene expression in *V. cholerae*. Intracellular levels of c-di-GMP dictate gene expression of virulence genes as well as genes involved in biofilm formation (242). There is significant overlap between these two systems and the gene expression they influence.

V. cholerae has three quorum sensing systems, all of which lead to control of HapR, which then represses transcription of *aphA* (138, 173). One system involves the use of autoinducer CAI-1 and the sensor kinase CqsS, while the other system employs the autoinducer AI-2 and the sensor kinase LuxQ and its periplasmic partner LuxP (33). *V. cholerae* constitutively produces the autoinducers and when the population is at low cell density, quorum sensing is essentially turned off due to the lack of a significant concentration of proximal autoinducer. In this scenario, CqsS and LuxQ act as kinases and transfer phosphates to LuxU, which then transfers the phosphate group to LuxO. Phosphorylated LuxO initiates transcription

of four small RNAs termed *qrr1-4*, which stands for Quorum Regulatory RNA (65, 66). These RNAs, together with Hfq, bind to the 5'UTR of HapR mRNA and initiate is degradation (147, 148). HapR does not get produced and thus does not repress *aphA*, which allows activation of the virulence cascade. At high cell density, the autoinducers bind their respective sensor kinases, which alternatively act as phosphatases, removing phosphates from LuxU and LuxO. Now inactivated, LuxO no longer activates transcription of the *qrr1-4* and HapR is produced. HapR represses *aphA*, and the virulence cascade is disrupted (138). A third quorum sensing system in *V. cholerae* uses the VarS/VarA system to independently activate LuxO. Three small RNAs CsrB, CsrC, and CsrD are activated by the VarS/VarA two-component signaling system and deactivate CsrA. Inactivation of CsrA relieves inhibition of LuxO, and converges with the other systems to regulate expression of Qrr1-4 (147).

The correlation between genes involved in quorum sensing and their relation to virulence is unmistakable. However, in some epidemic El Tor strains, there is a frameshift mutation in *hapR*, rendering it non-functional (268), and raising the question of biological significance in the host. In addition to the virulence activator AphA, HapR controls production of other genes involved in pathogenesis. HapR increases expression of HapA, the hemagglutin/protease of El Tor biotype strains that is involved in mucosal escape (ref). Additionally, HapR also represses the formation of biofilms by repressing the VPS (vibrio polysaccharide) operon. In addition to virulence gene activation, biofilms are activated at low cell density due to the lack of the repressor HapR (88).

Further regulation of gene expression is provided by signaling pathways involving the secondary messenger, c-di-GMP. Secondary messengers play a large role in altering gene expression in response to external stimuli. The secondary messenger, c-di-GMP, has been

associated with controlling properties of the cell surface (44). Two-component systems recognize a signal as the first messenger and control production of enzymes that modulate intracellular levels of c-di-GMP, the second messenger. Levels of the second messenger are able to modulate the transcriptome. Synthesis of c-di-GMP is controlled by diguanylate cyclases, while degradation is controlled by phosphodiesterases. Diguanylate cyclases contain the conserved motif GGDEF and are important for synthesis of c-di-GMP, while phosphodiesterases contain the EAL motif and are important for its degradation (7, 193, 210). Analysis of the *V. cholerae* genome has revealed many genes encoding proteins that contain one or both of these motifs needed for regulation of c-di-GMP levels (73). Increased levels of c-di-GMP correspond to increases in the vibrio polysaccharide (vps) genes required for biofilm formation, extracellular protein secretion system (EPS), and mannose sensitive hemagglutinin (MSHA) type IV pilus biogenesis genes. Conversely, high levels of c-di-GMP decrease the level of transcript of *fla* genes that are required for flagellar biosynthesis (15, 241). The VieSAB system is an important three-component system that is involved in down-regulation of biofilm (157). VieA is a phosphodieterase that decreases the amount of intracellular c-di-GMP (233). The VieSAB system is also important in CT production, relating secondary messengers with virulence (145, 146). For maximal CT production, a functional VieA must be present to keep low levels of c-di-GMP (242).

The 276 amino acid major virulence regulator, ToxT, is the primary transactivator of *ctxAB* and *tcp* transcription (29, 47, 115, 262). ToxT is a 32 kDa member of the AraC/XylS family of transcription regulators (101). The amino-terminal domain (NTD) of ToxT spans amino acids 1-160. A small linker region between amino acids 160-169 separates the NTD from the carboxy-terminal domain (CTD), which spans amino acids 170-276 (152). Within the CTD

resides the region that shares homology with other AraC/XylS family members. This region is approximately 100 amino acids and contains two helix-turn-helix DNA binding motifs (72, 155). The NTD of ToxT does not share significant sequence homology with any other AraC/XylS family members. However, the solved ToxT crystal structure revealed some structural similarity with AraC, despite the absence of significant sequence similarity (152).

ToxT activates virulence genes by binding to 13 base pair degenerate sequences called toxboxes (248). In addition to the major virulence factors, CT and TCP, ToxT activates transcription of other genes involved in pathogenesis, including *acfA, acfD, tagA, aldA, tcpI,* and the small regulatory RNAs *tarA* and *tarB* (21, 202, 247-249). Toxboxes exist in different orientations among ToxT-dependent genes. The promoters of *tcpA, ctxAB,* and *tarA* contain two toxboxes arranged as a direct repeat (202, 248). In contrast, two toxboxes are arranged as an inverted repeat at the promoters of *acfA, acfD, tagA,* and *tcpI* (202, 247-249). Finally, there is only a single toxbox at the promoter of *aldA (249).* In each of these promoters, the ToxT binding sites lie upstream from the -35 and -10 RNA polymerase (RNAP) binding sites. These types of promoters are termed class I promoters and suggest that ToxT interacts directly with the RNAP α subunits in order to induce transcription (22).

ToxT activates transcription from the promoter of *tcpA.* As a result, the operon containing all of the *tcp* genes is activated (115, 262). Additionally, the polycistronic RNA transcript containing all of these genes reads through *toxT* (262). Thus, once ToxT is produced via the activity of TcpP/H and ToxR/S, ToxT activates its own production by initiating transcription through *tcpA.* During infection, virulence must eventually be shut off for mucosal escape (188) and this would be impossible with ToxT continually activating its own production. Abuaita and Withey discovered that ToxT is cleaved by at least one ATP-dependent protease as

a mechanism to turn off virulence. This cleavage results in the production of an intermediate degradation product of ToxT. The production of the ToxT protease occurs when *V. cholerae* is grown under virulence repressing growth conditions. The protease/s that cleaves ToxT was also found to be present in *E. coli*. The cleavage site for the ToxT protease is located between amino acids 100-110 of ToxT, in a region that was unstructured in the solved toxT crystal structure (3).

The hypothesis that ToxT acts as a homodimer to activate transcription has been thoroughly investigated due to the existence of multiple toxboxes present in the promoters of most of the ToxT-dependent promoters. However, the discussion about the multimerization of ToxT is incomplete. The AraC/XylS family includes members that act as dimers, including AraC, RhaS, and RegA, while other members act as monomers, such as MarA, SoxS, and Rob (53, 97, 154, 250). Studies using LexA dimerization assays, as well as bacterial two-hybrid systems, have suggested that the NTD of ToxT is involved in dimerization (34, 35, 152, 195, 216). Dimerization has also been shown to be required for full virulence gene activation by ToxT. The NTD alone, when induced *in trans* has the ability to act as a dominant negative, reducing the overall activity of ToxT and further suggesting the requirement for dimerization (195). Amino acids throughout the NTD have been identified as essential for the dimerization process (34, 35). Additionally, a small molecule inhibitor of virulence gene activation, virstatin, was discovered and found to decrease dimerization of ToxT as its mechanism of repression (117, 216). Conversely, using DNA footprinting, ToxT has been shown to bind to the *tcpA* promoter as a monomer . Altering the spacing between toxboxes, eliminating the capability of ToxT bind as a homodimer, revealed that ToxT was still able to protect DNA in the experiments (247, 248). However, ToxT was unable to activate transcription from constructs having significantly altered spacing between toxboxes, illustrating the importance of proximity between binding sites for full

activation. Also, the promoter of *aldA* contains only one toxbox, suggesting that ToxT is not required to act as a dimer (249). Additionally, ToxT can bind and inhibit expression of genes required for biosynthesis of the anti-colonization factor, MshA. ToxT is able to bind and repress independently of the N-terminal dimerization domains proposed (112). Further work to determine the mechanism of dimerization for ToxT binding and virulence gene activation is still needed.

The ability of ToxT to activate transcription from certain promoters is altered by the presence of the global repressor of transcription, H-NS. The histone-like protein H-NS is found in many gram-negative organisms and plays a role in modulating activity of genes located on acquired mobile genetic elements (228). In *V. cholerae,* H-NS has been shown to repress activation of *ctxAB, toxT,* and *tcpA* (189, 263). In an *hns* deletion strain, expression of *ctxAB* no longer requires ToxR or ToxT, suggesting that the activators displace H-NS and allow transcription (189). This work was furthered by showing that H-NS binds to the *ctxAB* promoter and ToxT competes for binding (229).

Laboratory culture conditions that result in the production of virulence factors differs between the biotypes. For the El Tor biotype, a biphasic culture condition called AKI induces production of CT. AKI conditions consist of growth in AKI medium (1.5% peptone, 0.3% yeast extract, 0.5% NaCl, 0.3% sodium bicarbonate) statically at 37º C for a few hours. Then, a fraction of the phase one culture is shifted to shaking for another two hours (120-122). For classical biotype, CT and TCP are produced when grown in Luria-broth (LB) pH 6.5 at 30ºC while shaking, known as virulence inducing or ToxR-inducing growth conditions due to role ToxR plays in these conditions (176). These *in vitro* virulence inducing conditions result in the production of ToxT, and therefore the production of genes essential in pathogenesis. ToxT, and

consequently TCP and CT, are not produced under virulence repressing conditions: LB pH 8.5, 37º C, and shaking (176).

The *in vitro* virulence inducing conditions mentioned before have enabled the determination of physical and chemical signals that modulate production of virulence factors. However, the *in vivo* signals that *V. cholerae* encounters as it transitions from the environment to colonizing the human intestine differ from some of the *in vitro* growth conditions. For instance, the upper small intestine has a higher pH with a temperature of 37ºC. This is unlike the *in vitro* virulence inducing conditions of pH 6.5 and 30ºC. Therefore, the bacteria must encounter different signals in the host that result in the production of virulence factors.

AphA, AphB, ToxR, TcpP, as well as other virulence proteins are able to respond to signals found throughout the host to modulate their activity (56, 116, 135, 138, 261). Additionally, ToxT can modulate its activity in response to signals found in the host. Signals within the intestine include temperature, pH, the molecular constituents of the intestine, as well as components of the consumed food. Host signals that negatively regulate ToxT activity include bile, unsaturated fatty acids, and the natural compound capsaicin (31, 32, 86, 212). Alternatively, bicarbonate positively effects ToxT activity and is also abundant in the small intestine where *V. cholerae* colonizes (2). The current model for the modulation of ToxT activity within the upper small intestine is shown in Figure 2.

ToxT-dependent transcription of virulence genes can be significantly reduced with the addition of bile or with alterations in temperature (212). Bile, secreted from the gall bladder into the lumen of the intestine to emulsify fats, is a heterogenous mixture of saturated and unsaturated fatty acids, bilirubin, cholesterol, inorganic salts, and phospholipids. Fractionation of bile components revealed that unsaturated fatty acids (UFA) were directly responsible for the

reduction in ToxT activity (31). Additionally, the ToxT crystal structure revealed a buried 16-carbon fatty acid, cis-palmitoleate, bound between the NTD and CTD (152). Further analysis proved a variety of different chain lengths of unsaturated fatty acids also reduced ToxT-dependent activation of CT (34). Other negative effectors include natural compounds from foods. In particular the red chili component, capsaicin, has been shown to decrease CT expression (32). It was originally thought to affect ToxT activity, but has also recently been shown to increase the transcription of *hns*, resulting in decreased CT expression (32, 255). All the negative effectors mentioned have been shown to decrease ToxT NTD dimerization in bacterial two-hybrid assays, similar to the effect of virstatin (34).

On the other hand, ToxT is positively modulated by bicarbonate. Bicarbonate (HCO_3^-), is secreted by intestinal epithelial cells to buffer stomach acid in the small intestine and protect the epithelial border (104). Bicarbonate has been shown to increase *tcp* expression and CT production in a ToxT-dependent manner. Bicarbonate does not increase the levels of ToxT itself but enhances its ability to activate virulence gene transcription. The carbonic anhydrase inhibitor, ethoxzolamide (EZA), has been shown to decrease the ability of bicarbonate to activate ToxT, showing the importance of the conversion of CO_2 to HCO_3^- (2). The mechanism for action of bicarbonate on ToxT is currently unresolved.

Understanding the signals that *V. cholerae* senses during the transition from the environment to the human host are essential for the development of new therapies. Substantial work has been done to elucidate the mechanisms for negative modulation of ToxT activity within the intestine. The work in this dissertation is aimed at identifying the mechanisms for bicarbonate-mediated positive modulation of ToxT activity, leading to activation of *V. cholerae* virulence.

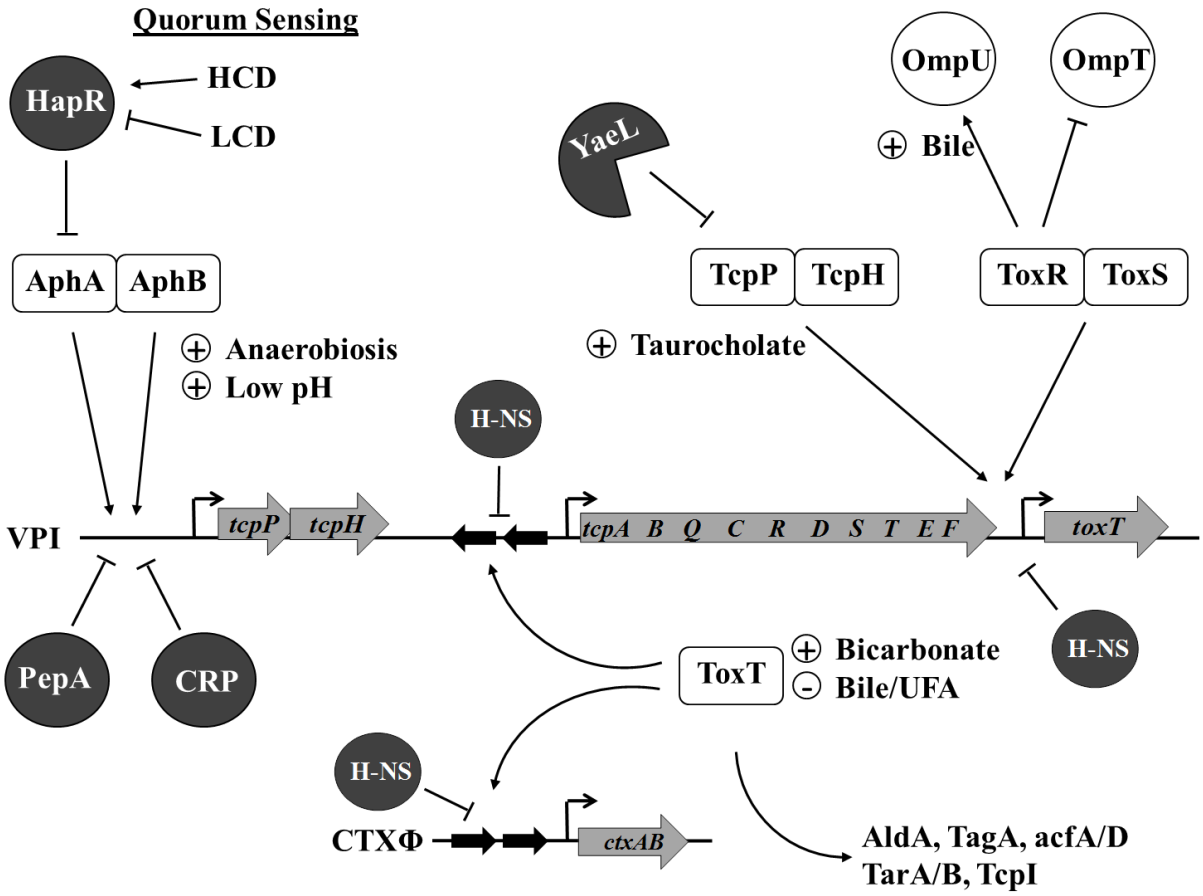

Figure 1. Virulence regulatory cascade in *V. cholerae* that results in production of CT and TCP. Light gray arrows represent genes located of the VPI or CTXΦ that are controlled by the cascade. Thick black arrows upstream of genes refer to ToxT binding sites. Rectangles signify transcriptional activators in the cascade, while circles are transcription repressors. YaeL is a protease that cleaves TcpP. Arrows extending from transcription regulators signify positive regulation, and lines with flat end represent inhibition. Plus signs symbolize positive effectors of the adjacent transcription regulator. Minus signs are negative effectors.

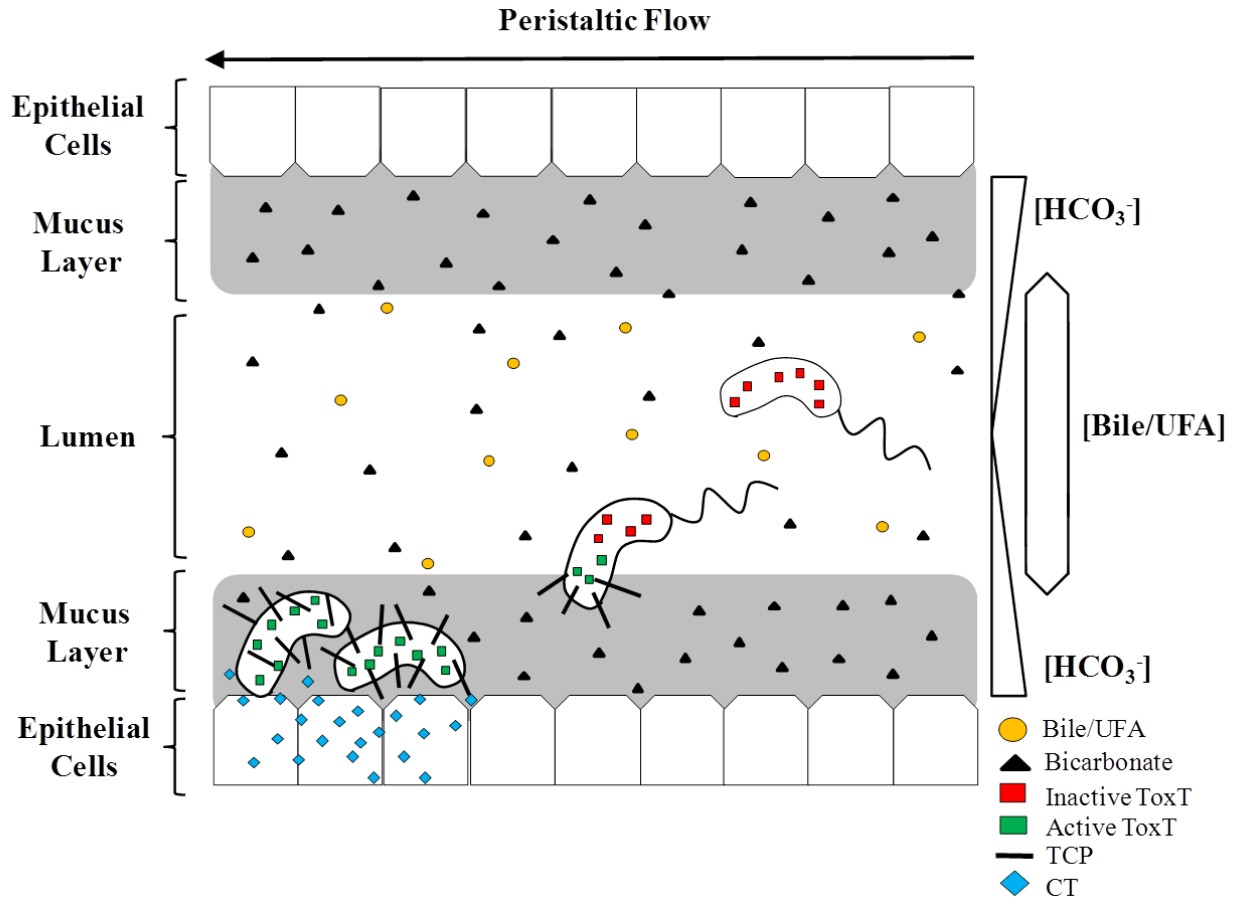

Figure 2. Model for induction of virulence gene expression by ToxT effector modulation in the upper small intestine. On the right, motile bacteria in the lumen of the intestine encounter high concentrations of bile/unsaturated fatty acids (UFA) which inactivate ToxT and virulence genes are not expressed. Bicarbonate exists in the lumen but the concentration of bile/UFA is larger. As the bacterium swims into the mucus layer, bile/UFA concentration decreases as it cannot enter the layer. The concentration of bicarbonate increases as the bacterium gets closer to the epithelium. Bicarbonate activates ToxT. Toxin co-regulated pilus (TCP) is expressed first by *V. cholerae* mediating microcolony formation. Closer to the epithelium cholera toxin (CT) is produced causing the characteristic watery diarrhea.

CHAPTER ONE

Bicarbonate increases binding affinity of *Vibrio cholerae* ToxT to virulence gene promoters

ABSTRACT

The major *Vibrio cholerae* virulence gene transcription activator, ToxT, is responsible for production of the diarrhea-inducing cholera toxin (CT), and the major colonization factor, toxin co-regulated pilus (TCP). In addition to the two primary virulence factors mentioned, ToxT is responsible for activation of accessory virulence genes such as *aldA, tagA, acfA, acfD, tcpI,* and *tarAB.* ToxT activity is negatively modulated by bile and unsaturated fatty acids found in the upper small intestine. Conversely, previous work identified another intestinal signal, bicarbonate, which enhances the ability of ToxT to activate production of CT and TCP. The work presented here further elucidates the mechanism for enhancement of ToxT activity by bicarbonate. Bicarbonate was found to increase activation of ToxT-dependent accessory virulence promoters in addition to those that produce CT and TCP. Bicarbonate is taken up into the *V. cholerae* cell, where it positively affects ToxT activity by increasing DNA binding affinity for the virulence gene promoters that ToxT activates, regardless of toxbox configuration. The increase in ToxT binding affinity in the presence of bicarbonate explains the elevated level of virulence gene transcription.

INTRODUCTION

Vibrio cholerae has two distinct phases in its life cycle. In the planktonic state in the aquatic environment, *V. cholerae* represses expression of pathogenesis genes, while increasing transcription of genes involved in environmental survival, such as those required for motility (24,

143). Upon entry into a human host, via consumption of contaminated water or food, the bacteria encounter signals resulting in an inversion of their transcriptome profile. In this new, virulent state, expression of genes involved in motility and environmental survival is repressed, while expression of genes involved in host survival and pathogenesis is activated, resulting in the diarrheal disease cholera (24, 143). Virulence is controlled by a cascade of positive transcription regulators known as the ToxR regulon (194). Many of the signals required to initiate change in the transcriptional profile have been identified in recent years (24, 143, 159).

The main signals that induce transcription of *V. cholerae* pathogenesis genes in the host act on different steps in the virulence regulatory cascade. At the uppermost level of the cascade, AphA and AphB activate transcription of *tcpPH* (136, 137, 139). AphB activity is enhanced by both low pH and anaerobic conditions (11, 135), such as would be found as *V. cholerae* transitions through the stomach and into the upper small intestine. The next level of the cascade includes the aforementioned TcpP, along with the constitutively produced ToxR, both of which are integral membrane proteins that work in tandem to activate *toxT* transcription (144). TcpP activity has recently been shown to be enhanced by taurocholate (261), a bile salt, and interaction between TcpP and ToxR is enhanced under oxygen-limiting conditions (56). The final level of the cascade consists of ToxT, which directly activates production of the major virulence factors cholera toxin (CT) and toxin co-regulated pilus (TCP) (29, 47, 115, 262). ToxT also directly controls transcription of other accessory virulence genes including *aldA, tagA, acfA, acfD, tcpI, tarA*, and *tarB* (21, 202, 247-249). ToxT activity is reduced in the presence of the unsaturated fatty acid components of bile (31, 212) and enhanced in the presence of bicarbonate (2), both of which are present in the upper small intestine where *V. cholerae* preferentially colonizes.

ToxT is a 276 amino acid member of the AraC/XylS family of transcriptional regulators. The ToxT C-terminal domain contains two helix-turn-helix motifs responsible for DNA binding (101). ToxT activates virulence genes by binding to a 13bp degenerate DNA sequence called a toxbox (248). All ToxT-dependent virulence genes have two toxboxes upstream of their transcriptional start site in either direct or inverted repeat configurations, with the exception of *aldA*, which has only a single toxbox (248). ToxT can bind toxboxes as a monomer (12, 247, 248), but is presumed to dimerize to fully activate at least some virulence genes (34, 35, 152, 195, 216).

Activation of the ToxT-dependent virulence genes can be altered in the presence of the positive and negative ToxT effectors. After egress from the stomach into the intestine, the bacteria encounter high concentrations of both bile and bicarbonate. As mentioned above, the unsaturated fatty acid components of bile are negative effectors of ToxT activity (31, 34, 152, 195). The N-terminus of ToxT is involved in the response to these effectors, presumably by decreasing ToxT dimerization (34). Bicarbonate is present in the upper small intestine to buffer stomach acid and to also protect the epithelial layer (104). Bicarbonate enhances the ability of ToxT to increase CT and TCP production (2). How bicarbonate enhances ToxT activity is not well understood.

Here, we report that bicarbonate increases transcription activation of other ToxT-dependent genes in addition to the genes encoding the two major virulence factors. We show that bicarbonate enters the bacterium, where it could interact with ToxT in the cytoplasm. Furthermore, we have determined that the mechanism for bicarbonate-mediated enhancement of ToxT activity is due to increased binding affinity of ToxT for the promoters it activates. This increase in binding occurs at promoters of each ToxT-dependent gene, regardless of toxbox

configuration. This work establishes a direct mechanistic link between a signal from the host and increased transcription of pathogenesis genes that occurs in the host.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains and plasmids used in this work are listed in Table 1. All strains were maintained at -70°C in LB containing 20% glycerol. Wild-type *V. cholerae* classical biotype strain O395 and an isogenic Δ*toxT* (29) with corresponding plasmids were used for β-galactosidase assays. The *tcpA::lacZ* and *ctxAB::lacZ* promoter fusions were constructed using pTL61t (48, 248) and contained 138bp and 76bp upstream of the transcriptional start site, respectively. The pTL61t fusions *aldA::lacZ* and *tagA::lacZ*, contained 158bp and 92bp, respectively, upstream of their transcriptional start sites (249). Fusions *acfA::lacZ* and *acfD::lacZ* were constructed in (247) and pTL61t contained 104bp and 99bp upstream of the transcriptional start site, respectively. The *tcpI::lacZ* fusion contained 76bp (unpublished work). Strains and plasmids used in this study are listed in Table 1. The antibiotic concentration for strains with the pTL61t plasmid was 100ug/mL ampicillin and without plasmid were 100ug/mL streptomycin.

β-galactosidase assay. Bicarbonate virulence inducing conditions were as previously described (2). Briefly, *V. cholerae* classical biotype strain O395 was grown overnight in LB at 37°C and subcultured 1:100 into AKI medium in the presence or absence of freshly prepared 0.3% sodium bicarbonate (36mM) for 4 hours. Cultures were grown statically for 4 hours at 37°C and analyzed. The β-galactosidase assay was performed as previously described (171).

$H^{14}CO_3$ uptake assay. *V. cholerae* classical biotype strain O395 was grown overnight in LB at 37°C and subcultured 1:100 into AKI medium in the absence of bicarbonate for 3 hours.

At 3 hours, 1μCi $NaH^{14}CO_3$ (54mCi/mmol) (Perkin-Elmer) was added for each milliliter of *V. cholerae* subculture. Immediately, 1mL of culture was centrifuged and supernatant was discarded. The cell pellet was washed 3 times with 1mL of AKI medium and re-centrifuged. Cell pellets were resuspended in 100uL AKI medium and added to 5mL Scintillation cocktail (Fisher Scientific). The same procedure was followed in 15 minute intervals for two hours. After uptake, cpm was measured for each time point using an LS6000IC liquid scintillation counting system (Beckman).

Protein Purification. Maltose binding protein (MBP)-ToxT and MBP-AraC purification was performed as previously described (48). Briefly, fusion proteins were purified from *Escherichia coli* strain BL21(DE3) with plasmid pMAL-c2e containing MBP-ToxT or MBP-AraC. *E. coli* cells were subcultured until the optical density at 600nm reached 0.5. Then, the fusion protein was induced with the addition of 0.25mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were lysed by French press and lysate was run over an amylose column (New England Biolabs). Fractions containing MBP-ToxT were dialyzed against buffer containing 50 mM Na_2HPO_4 (pH 8.0), 10 mM Tris-HCl (pH 8.0), and 100 mM NaCl and then dialyzed again against the same solution with 20% glycerol. The protein concentration was determined using a Qubit 2.0 Fluorometer (Invitrogen).

Electrophoretic mobility shift assays (EMSA). EMSA was performed as previously described (48). DNA probes were produced by PCR of pTL61T containing appropriate promoter sequence with one unlabeled primer and one primer end labeled with γ-^{32}P(Perkin-Elmer) by T4 polynucleotide kinase (New England BioLabs). The binding reactions were prepared to a final volume of 30 μL containing: 10 μg/mL salmon sperm DNA, 10 mM Tris-acetate (pH 7.4), 1 mM Potassium EDTA (pH 7.0), 100 mM KCl, 1 mM dithiothreitol (DTT), 0.3 mg/mL bovine serum

albumin (BSA) and 10% glycerol. Binding reactions contained various concentrations of purified MBP-ToxT and all reactions had a constant concentration of labeled DNA probe. For assays using MBP-AraC, binding reactions had a final concentration of 50 mM KCl, 5% glycerol, and 50 mM L-arabinose. Sodium bicarbonate, sodium biselenite, and sodium acetate were added to a final concentration of 36 mM in binding reactions containing the respective molecules. The binding reactions were incubated at 30°C for 30 minutes and immediately loaded into 6% polyacrylamide gel and run at 4°C. Gels were dried and analyzed by autoradiography.

Binding curve analysis. Autoradiographs were analyzed using ImageJ software (NIH). Percent of labeled DNA bound by protein was determined for each lane. Graphpad Prism 5 software was used for curve fitting to the equation %Bound=B_{max}*[Protein]^h/(K_d^h+ [Protein]^h) with B_{max} constraint set to 100. The K_d for each condition was calculated and significance was determined. Nonspecific binding was omitted due to the excess of nonspecific salmon sperm DNA added to binding reactions.

RESULTS

Bicarbonate increases activation of ToxT-dependent promoters. Bicarbonate has previously been shown to increase production of the major ToxT-dependent virulence factors, TCP and CT (2); however, the effect of bicarbonate on other ToxT-activated promoters had not been assessed. Earlier work had demonstrated that activation of genes *aldA, tagA, acfA, acfD,* and *tcpI* are dependent on production of ToxT (247-249). We assessed transcriptional activity of these ToxT-dependent promoters using plasmid-borne β-galactosidase promoter fusions in the classical biotype *V. cholerae* strain O395 and an isogenic *toxT* deletion (29). The location and orientation of the ToxT binding sites, or toxboxes, within each ToxT-dependent promoter is shown (Fig. 3A,B). Fusion construct activity from *ctxAB::lacZ, aldA::lacZ, tagA::lacZ,*

acfA::lacZ, acfD::lacZ, and *tcpI::lacZ* was measured in the presence and absence of 36 mM sodium bicarbonate to determine if bicarbonate enhanced the ability of ToxT to activate transcription of these genes as it does with the major virulence genes *tcpA* and *ctxAB.* Culture conditions used to assess activity were adopted from previous work with bicarbonate (2). Our results confirm that bicarbonate activates each of these ToxT-dependent promoters in wild-type O395, while having no effect on a Δ*toxT* strain (Fig. 3C). Therefore, bicarbonate has a global effect by enhancing ToxT activity at all ToxT-dependent promoters.

Radiolabeled bicarbonate is taken up over time by *V. cholerae.* Previous work (2) and our findings (Fig. 3C) strongly suggested that the positive effect of bicarbonate on ToxT activity was the result of a direct interaction, but this had not been confirmed. ToxT protein levels are roughly equivalent in the presence or absence of bicarbonate (2), and therefore ToxT is thought to be in a less active state in the absence of bicarbonate. The simplest mechanism for bicarbonate activation of ToxT would be a direct interaction between bicarbonate and ToxT within the cytoplasm of the bacterial cell. To determine whether such a direct interaction between bicarbonate and ToxT is possible, we performed a $NaHC^{14}O_3$ uptake assay to measure bicarbonate import into the *V. cholerae* cell. First, classical biotype *V. cholerae* was subcultured statically in AKI medium for two hours in the absence of bicarbonate. Then, C^{14}-labeled bicarbonate was added to the culture. Aliquots were centrifuged at 15 minute intervals. Cell pellets were washed with AKI medium three times and ^{14}C-radiolabel in the cell pellet was quantified using a scintillation counter.

Results of the bicarbonate uptake assay displayed a linear increase of radiolabel within the cell pellet of the culture (Fig. 4). Linear regression of the dataset revealed an equation of the best-fit line of $y=27.46x+525$, with an R^2-value of 0.984. The results from the time course uptake

show that bicarbonate is entering the bacteria at a linear rate. This finding suggests that bicarbonate can interact directly with ToxT in the bacterial cytosol.

Bicarbonate increases ToxT equilibrium binding affinity to P_{tcpA}. The simplest mechanism for a direct interaction between bicarbonate and ToxT that results in increased transcription activation by ToxT would be increased occupancy of toxboxes by ToxT, leading to enhanced interactions between ToxT and RNA polymerase. In the murine pathogen *Citrobacter rodentium*, an interaction between bicarbonate and another AraC/XylS protein family member, RegA, leads to stabilization of RegA binding to the *grlA* promoter (235, 259). To determine whether bicarbonate directly affects ToxT binding to its specific DNA sites, we characterized the binding pattern of ToxT to virulence gene promoters using EMSA. The assays were designed to establish an estimation of the equilibrium dissociation constant, K_d, thus quantifying the interaction of ToxT with the promoter of ToxT-dependent virulence genes in the absence and presence of bicarbonate. In regard to this work, K_d represents the concentration of ToxT needed to bind 50% of the promoter DNA at equilibrium in the EMSA. Mathematically speaking, there is an inverse relationship between K_d and binding affinity; thus meaning the lower the K_d, the higher the binding affinity of ToxT to the DNA.

To determine the K_d of the interaction between ToxT and P_{tcpA}, we titrated purified MBP-ToxT, in the presence or absence of 36 mM bicarbonate, with a constant concentration of ^{32}P labeled P_{tcpA} PCR product below the empirically estimated K_d. MBP-ToxT was used because the independently folded MBP helps to solubilize ToxT and prevent aggregation. The binding reactions for each of the MBP-ToxT concentrations were run on a 6% polyacrylamide gel and subjected to autoradiography. The percentage of bound DNA at each concentration of MBP-ToxT was calculated using densitometry with ImageJ software. Curve fitting was performed with

Graphpad software using the equation, %Bound=B_{max}*[Protein]^h/(K_d^h+ [Protein]^h). B_{max} is the amount of bound DNA where the curve plateaus, which we set to a constraint of 100%.This constraint represents all free DNA being bound by ToxT. When the Hill slope, h, is greater than one, the curve adopts a sigmoidal shape representing multiple binding sites and cooperativity, in this case between MBP-ToxT and P_{tcpA}. These data are shown in Figure 5A. Binding reactions that correspond to lanes 1-7 were allowed to equilibrate in the absence of bicarbonate, while binding reactions in lanes 8-14 included bicarbonate. The percentage of bound DNA was calculated and curves were fit for both sets of reactions (Fig. 5B). With the addition of bicarbonate, the concentration of MBP-ToxT to bind 50% of P_{tcpA} was 145.3 nM, significantly lower than the 287.9 nM K_d without bicarbonate. The lower K_d in the presence of bicarbonate indicates a higher binding affinity for the *tcpA* promoter. Importantly, the Hill slope of the binding curves is greater than one and the curves take on a sigmoidal shape. This indicates that there are multiple binding sites for ToxT binding and that there is a degree of cooperativity in binding both sites. The increase in binding affinity in the presence of bicarbonate in an *in vitro* EMSA indicates a proximal relationship between the bicarbonate ion and ToxT, and likely a direct interaction.

The addition of bicarbonate to the binding reaction increases the pH from 7.4 to 8.6. To confirm that the increase in binding affinity is due to the bicarbonate ion itself and not an increase in pH, we performed an EMSA in which binding reactions at pH 7.4 (Fig. 5C, lanes 1-7) and 8.6 (Fig. 5C, lanes 8-14) were compared in the absence of bicarbonate. Densitometric analysis and nonlinear regression provided the binding curves and K_d values for the EMSA (Fig. 3D). The binding curves at the two pH values overlapped and the K_d values for each curve were not statistically different. The K_d of these two conditions was lower than in Figure 3A because

the concentration of active ToxT protein varies among batches and decreases over time. However, the overall response to the different binding conditions remains similar in different purified protein batches.

Both ToxT and RegA, transcriptional regulators of the AraC/XylS family, exhibit increased binding in the presence of bicarbonate (235, 259). We next analyzed whether another family member, AraC, showed signs of stabilized promoter architecture in the presence of bicarbonate to assess whether this was a common feature shared among the entire AraC family. Our results indicate that the addition of bicarbonate inhibited binding of AraC to the *araBAD* promoter (Fig. 5E,F). Therefore, not all AraC/XylS family members are positively modulated by bicarbonate.

Other small effector molecules do not increase ToxT binding to P_{tcpA}. The results described above suggest that addition of bicarbonate to binding reactions increases the binding affinity of ToxT to P_{tcpA}. However, it is possible that this phenomenon is not limited to bicarbonate specifically and could also be mediated by other small molecules similar to bicarbonate. We next tested the binding response of ToxT to molecules with similar structure or molecular weight to bicarbonate. Sodium biselenite has a similar structure to bicarbonate with a different central atom. ToxT had a greatly reduced binding affinity in the presence of biselenite (Fig. 6A,B). We also tested sodium acetate, which has similar molecular weight to sodium bicarbonate. ToxT also showed decreased binding to P_{tcpA} with the addition of this small molecule (Fig. 6C,D). The increase in binding affinity of ToxT to P_{tcpA} appears to be bicarbonate-specific as other small molecules do not enhance ToxT binding or activity (data not shown).

Bicarbonate increases binding to ToxT-dependent promoters regardless of promoter orientation. EMSA equilibrium binding experiments indicated that bicarbonate increases ToxT binding affinity for P_{tcpA}. However, there is significant diversity in the configuration and spacing of toxboxes at different ToxT-activated promoters (248). ToxT directly controls transcription of several other virulence genes besides *tcpA*, including *ctxAB, aldA, tagA, acfA, acfD,* and *tcpI* (247-249). The *tcpA* promoter contains two toxboxes in a direct repeat configuration. P_{ctxAB}, like P_{tcpA}, is also configured as two direct repeat toxboxes (Fig. 1A). However, the orientation of the toxboxes is in the opposite direction relative to the promoter at P_{ctxAB} and the spacing between toxboxes differs between P_{tcpA} and P_{ctxAB} (48, 248, 263). In contrast to P_{tcpA} and P_{ctxAB}, P_{tagA}, P_{acfA}, P_{acfD}, and P_{tcpI} each contain a pair of toxboxes in an inverted repeat configuration and with variations in spacing between toxboxes (Fig. 3B). Finally, P_{aldA} contains a single toxbox (Fig. 3A). As described earlier, addition of bicarbonate to *V. cholerae* cultures increases activity from all of these promoters (Fig. 3C).

To determine whether bicarbonate affects binding affinity of ToxT for each of these unique promoters, we performed EMSAs and calculated K_d as previously described for P_{tcpA}. First, we tested the ability of bicarbonate to increase the binding affinity of ToxT to P_{ctxAB}, which controls production of CT and has two direct repeat toxboxes. Figure 5A shows the autoradiograph of ToxT binding to P_{ctxAB} in the absence and presence of bicarbonate. Densitometry and linear regression revealed that the K_d is reduced from 328.7 nM MBP-ToxT to 231.4 nM with bicarbonate, indicating an increase in binding affinity. Next, we investigated if bicarbonate increased ToxT binding at the inverted repeat promoter of *tagA.* We used P_{tagA} as an example of inverted repeat toxbox configurations that also occur in the promoters of *acfA, acfD,* and *tcpI*. As with the direct repeat promoters, bicarbonate increased the binding affinity of ToxT

to P_{tagA} (Fig. 7C, D). Finally, we performed EMSAs to determine if bicarbonate increased binding to the single toxbox promoter of *aldA*. The EMSA with binding reactions of MBP-ToxT and P_{aldA} in the absence and presence of bicarbonate is shown in Fig. 7E. The binding curve was developed and revealed that bicarbonate decreased the K_d for ToxT binding to P_{aldA} from 173.7 nM to 113.9 nM. The increase in binding affinity to each of the promoters with bicarbonate agreed with the results of the analysis of each promoter in the β-galactosidase assays. Together, these data demonstrate that bicarbonate increases binding at each promoter regardless of toxbox orientation, including a single toxbox promoter, and reveals that the mechanism by which bicarbonate enhances ToxT activity is most likely a change in protein conformation, resulting in increased DNA binding affinity.

DISCUSSION

The regulatory network that controls production of the major virulence factors responsible for disease symptoms of cholera culminates with the production of ToxT. ToxT is the transcription regulator directly responsible for activation of *tcpA* and *ctxAB* transcription (29, 47, 115, 262). Previous studies have shown that bicarbonate, a signal located in the upper small intestine where *V. cholerae* colonizes, enhances the ability of ToxT to activate these major virulence genes (2). In addition to these genes, we have shown that bicarbonate increases transcription of other ToxT-dependent virulence genes, *aldA*, *tagA*, *acfA*, *acfD*, and *tcpI*.

ToxT activity is modulated by different effector molecules. It is negatively regulated by bile and its unsaturated fatty acid components (31, 212), as well as the small molecule virstatin (117, 216). Bile is located at high concentration in the lumen of the upper small intestine and the interaction between the unsaturated fatty acids of bile and ToxT are thought to decrease ToxT's

ability to dimerize effectively and bind to DNA (34, 152, 195). Our findings have shown that bicarbonate, which is also present at high concentration in the upper small intestine, has a mechanism converse to bile. Our results indicate that bicarbonate-mediated activation of virulence gene transcription occurs as a result of an increase in ToxT binding affinity to virulence gene promoters. The increase in binding affinity in response to bicarbonate can be seen at each ToxT-dependent promoter, regardless of toxbox configuration and positioning.

The binding curves generated from EMSAs performed on different ToxT-dependent promoters were sigmoidal. Sigmoidal binding curves generally relate to cooperative binding to multiple binding sites of a protein, similar to O_2 binding by hemoglobin (82). Cooperativity occurs when one bound molecule increases the binding affinity of subsequent molecules. Positive cooperativity can also be seen in terms of transcription factors binding to promoters with multiple binding sites. One example of positive cooperativity of transcription factors is seen with the TtgR operator of *Pseudomonas putida* strain DOT-T1E (142). TtgR, which can form dimers in solution, exhibits biphasic cooperative binding at this promoter (142). ToxT is thought to have optimal activity when acting as a dimer (34, 35, 152, 195, 216). In contrast, it has also been shown that ToxT can bind DNA as a monomer (12, 247, 248). The sigmoidal ToxT binding curve at two toxbox promoters (Fig. 5B, 7B, 7D) suggests that a single ToxT monomer binds the DNA first, coinciding with previous findings. Additionally, positive cooperativity derived from the sigmoidal binding curve implies that the binding of the first monomer increases the binding affinity of the second monomer. This can be explained by an increase in the incidence of dimerization between ToxT monomers after one has already bound the promoter, increasing the binding affinity of the second ToxT monomer. Interestingly, the ToxT binding curve of the single toxbox promoter of *aldA* also exhibited a sigmoidal shape. Previous studies have shown

that this promoter contained a single toxbox; however, cooperative ToxT binding at this promoter suggests that another ToxT binding site may be present in the vicinity of the promoter.

Potential mechanisms for the increased ToxT DNA binding affinity in response to bicarbonate include a change in conformation of ToxT or increased frequency of ToxT dimerization. Indirect mechanisms that increase ToxT binding can be ruled out as the increase in binding was seen in binding experiments using purified components. Work on an AraC/XylS family member of *C. rodentium*, RegA, revealed that bicarbonate increases RegA binding to promoter regions (235, 259). RegA can act as a monomer or dimer, similar to ToxT, and it was shown that bicarbonate did not increase the incidence of RegA dimerization (258). As a primary assessment of ToxT dimerization in the presence of bicarbonate we analyzed the ability of bicarbonate to increase ToxT binding to the single toxbox of P_{aldA}, in which dimerization should not be evident. However, as stated before, our findings revealed the possibility of more than one toxbox in or near the promoter of *aldA*. Consequently, further investigation into the precise mechanism of increased ToxT binding affinity in the presence of bicarbonate is needed.

In order for bicarbonate to interact with ToxT, it must first enter the bacterium. We report here that bicarbonate does get taken up by the cell. However, further investigation into the mechanism of uptake is needed. Previous work has shown that addition of the carbonic anhydrase inhibitor, ethoxzolamide (EZA), decreases activation of P_{tcpA} in the presence of bicarbonate (2). *V. cholerae* contains genes encoding each of the three classes of bacterial carbonic anhydrase. A triple carbonic anhydrase deletion did not reveal a decrease in $H^{14}CO_3$ uptake compared to WT O395 (data not shown). This suggests that the mechanism for uptake is not completely reliant on carbonic anhydrase-dependent conversion of HCO_3^- to CO_2.

Signals that *V. cholerae* encounters during passage to the intestine result in the production of ToxT (159), but due to the presence of a high concentration of bile and unsaturated fatty acids in the lumen of the intestine, ToxT is initially in an inactive form (31, 34, 195, 212). Bicarbonate is also present in the lumen where bile is present and could potentially be activating some ToxT protein. The bacteria in the lumen still express flagella and undergo positive chemotaxis towards mucin proteins (6, 24). As the bacteria swim closer to the intestinal epithelium, the concentration of bile decreases as the large molecules that comprise bile cannot enter the mucus layer. The concentration of bicarbonate increases as the bacteria get closer to the epithelium due to direct secretion of bicarbonate from these cells (104), but more importantly, the ratio of bicarbonate to UFAs increases. ToxT protein becomes active as it reaches the high bicarbonate/low UFA breakpoint and active ToxT increases transcription of genes involved in virulence, such as CT and TCP (2). In addition to these major virulence factors, bicarbonate activates accessory virulence factors important in disease progression. Our findings here reveal that the mechanism for bicarbonate-mediated enhancement of ToxT activity is due to an increase in ToxT binding affinity to virulence gene promoters. This provides a direct relationship between an intestinal signal and increased expression of genes required for pathogenesis.

Table 1. Strains used in this study

Strain or Plasmid	Description	Source
Strains		
JW 467	*Escherichia coli* BL21(DE3)	Lab Collection
JW 1560	JW 467 + pJW 407 (MBP-ToxT)	Lab Collection
JW 9	*Vibrio cholerae* classical strain O395	Lab Collection
JW 150	O395 Δ*toxt*	Lab Collection
JW 18	pJW 54 + JW 9	19
JW 441	pJW 211 + JW 9	31
JW 87	pJW 82 + JW 9	17
JW 97	pJW 89 + JW 9	17
JW 86	pJW 81 + JW 9	18
JW 89	pJW 84 + JW 9	18
JW 99	pJW 91 + JW 9	Lab Collection
JW 515	pJW 54 + JW 150	Lab Collection
JW 1809	pJW 211 + JW 150	31
JW 167	pJW 82 + JW 150	Lab Collection
JW 168	pJW 89 + JW 150	Lab Collection
JW 165	pJW 81 + JW 150	Lab Collection
JW 166	pJW 84 + JW 150	Lab Collection
JW 169	pJW 91 + JW 150	Lab Collection
Plasmids		
pJW 407	pMAL-c2e-ToxT	31
pJW 54	pTL61t containing 138bp upstream of *tcpA*	19
pJW 211	pTL61t containing 76bp upstream of *ctxAB*	31
pJW 82	pTL61t containing 158bp upstream of *aldA*	17
pJW 89	pTL61t containing 92bp upstream of *tagA*	17
pJW 81	pTL61t containing 104bp upstream of *acfA*	18
pJW 84	pTL61t containing 99bp upstream of *acfD*	18
pJW 91	pTL61t containing 76bp upstream of *tcpI*	Lab Collection

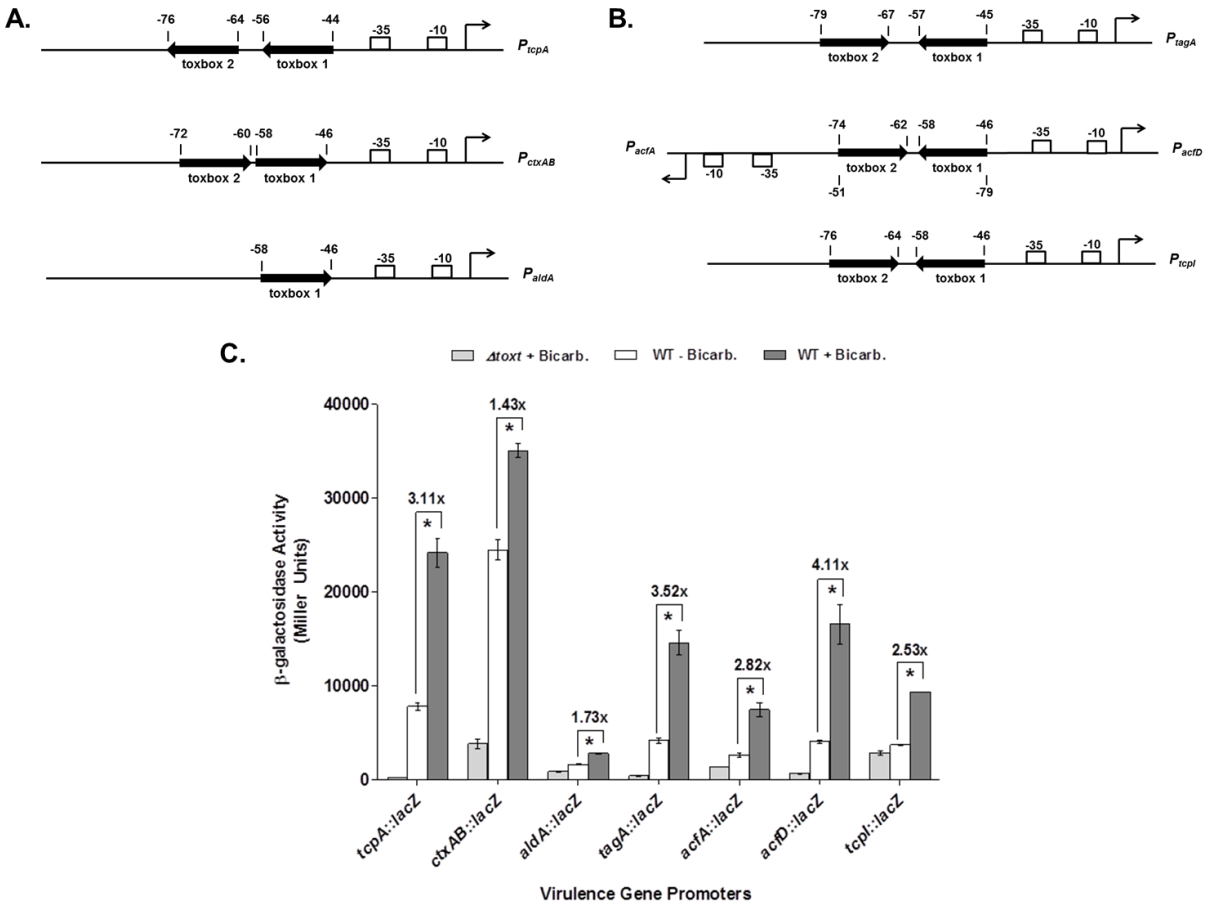

FIGURE 3. Transcriptional effects of exogenous bicarbonate on ToxT-dependent virulence gene promoters. (A) Orientation and positioning of direct repeat toxbox virulence gene promoters P_{tcpA} and P_{ctxAB}, and single toxbox promoter, P_{aldA}. (B) Orientation and positioning of inverted repeat toxbox virulence gene promoters, P_{tagA}, P_{acfA}, P_{acfD}, and P_{tcpI}. Toxbox represents known ToxT binding sites. (C) β-galactosidase activity produced from plasmid-borne virulence gene promoter fusion constructs in classical strain O395 and isogenic Δ*toxT* strain. Cultures were grown in AKI media in the absence or presence of 36 mM $NaHCO_3$. Statistical significance determined using Student's t-test (*, $P < 0.00025$). Error bars represent +/- standard deviation.

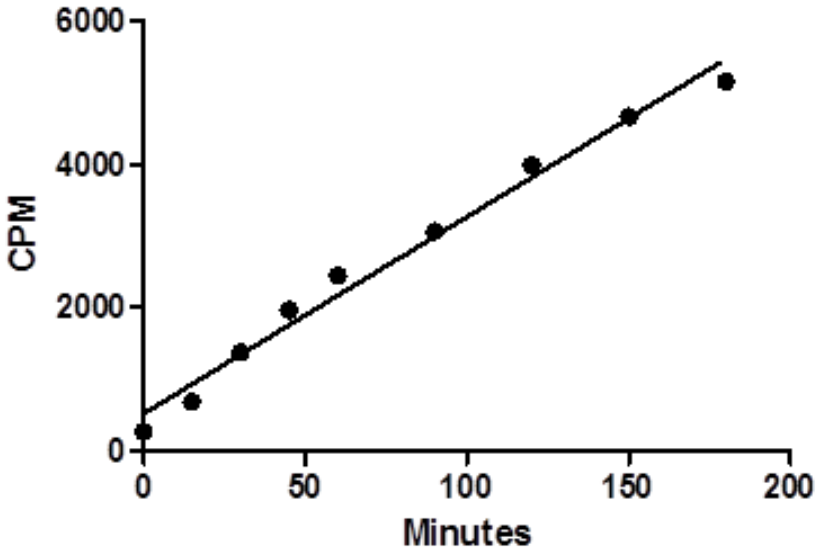

FIGURE 4. Radiolabeled bicarbonate is taken up by *V. cholerae*. Classical strain O395 cells were grown in AKI media in the absence of bicarbonate for 2 hours. $NaH^{14}CO_3$ was added at time 0 and uptake was analyzed every 15 minutes by liquid scintillation counting. $NaH^{14}CO_3$ uptake was measured as CPM, reflecting the amount of radioactive ^{14}C found within *V. cholerae* culture. Error bars represent +/- standard deviation of three separate experiments.

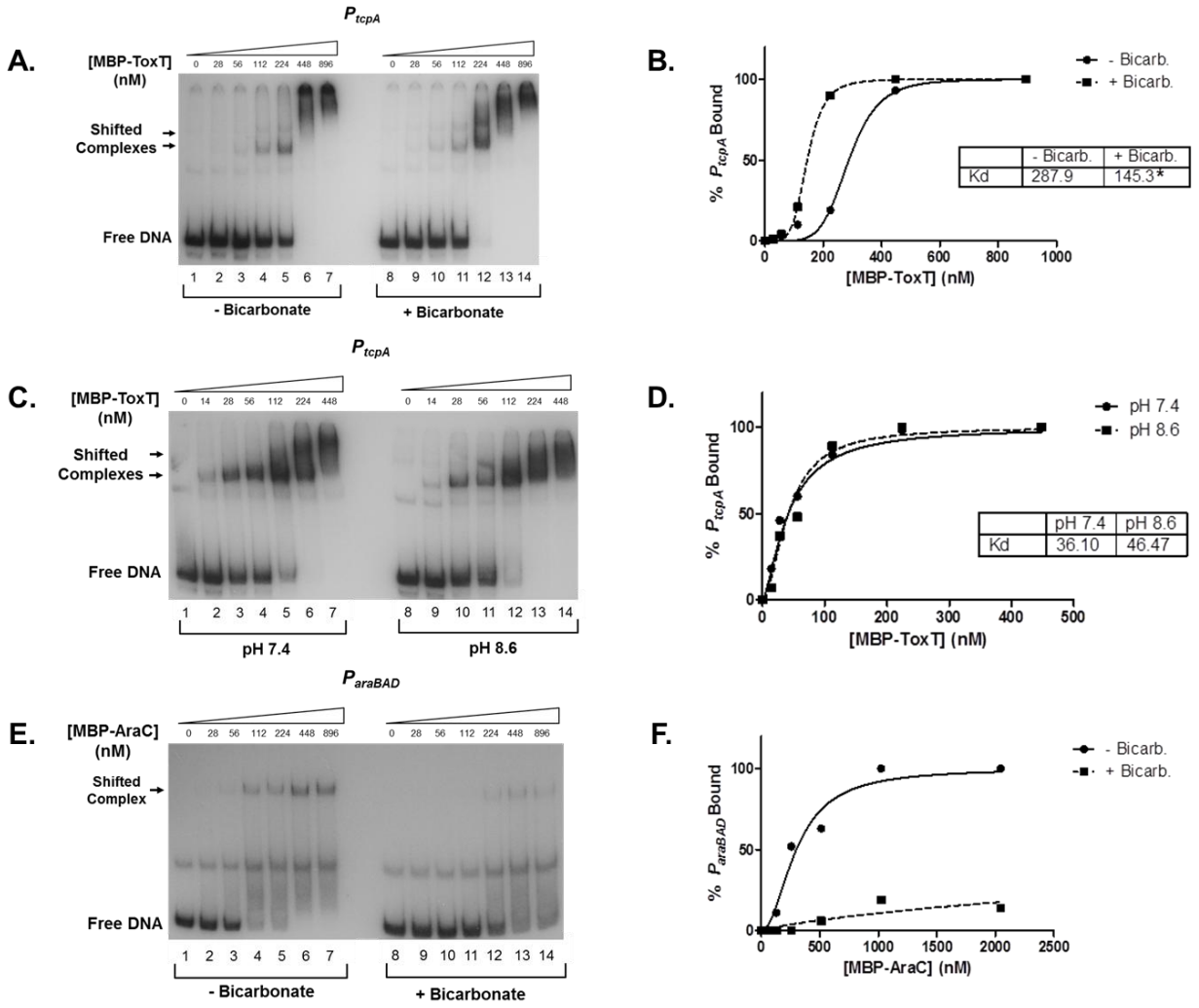

FIGURE 5. Bicarbonate increases binding affinity of MBP-ToxT to P_{tcpA}. MBP-ToxT binding to P_{tcpA} was analyzed using EMSA. Autoradiographs of EMSAs presented are representative of three or more independent experiments. (A) Binding reactions between MBP-ToxT and P_{tcpA} in lanes 1-7 were conducted in the absence of $NaHCO_3$. Lanes 8-14 were incubated in the presence of 36 mM $NaHCO_3$. Lanes 1 and 8 contained P_{tcpA} DNA in the absence of MBP-ToxT. Subsequent lanes contained a titration of MBP-ToxT with concentrations labeled in the figure. (B) Binding curve for the autoradiograph shown in (A). Densitometry of autoradiograph was performed with ImageJ software. Circles represent percent P_{tcpA} bound by MBP-ToxT in the absence of bicarbonate. Solid line corresponds to the binding curve for MBP-ToxT to P_{tcpA} determined by the equation %Bound=B_{max}*[Protein]^h/(K_d^h+ [Protein]^h) with B_{max} constraint set to 100 using Graphpad Prism 5 software. Squares and dashed line represent percent bound and binding curve, respectively, in the presence of bicarbonate. K_d for each condition is inset and significant difference between the best-fit values of each data set is denoted by * ($P < 0.00025$). (C) Autoradiograph of EMSA showing titration of MBP-ToxT bound to P_{tcpA} at pH 7.4 (Lanes 1-7) and pH 8.6 (Lanes 8-14). (D) Binding curves of each pH condition with K_d. (E) Autoradiograph of EMSA showing titration of MBP-AraC bound to P_{BAD} in the absence of $NaHCO_3$ (Lanes 1-7) and presence of 36 mM $NaHCO_3$ (Lanes 8-14). (F) Binding curves of MBP-AraC in the absence and presence of 36 mM $NaHCO_3$.

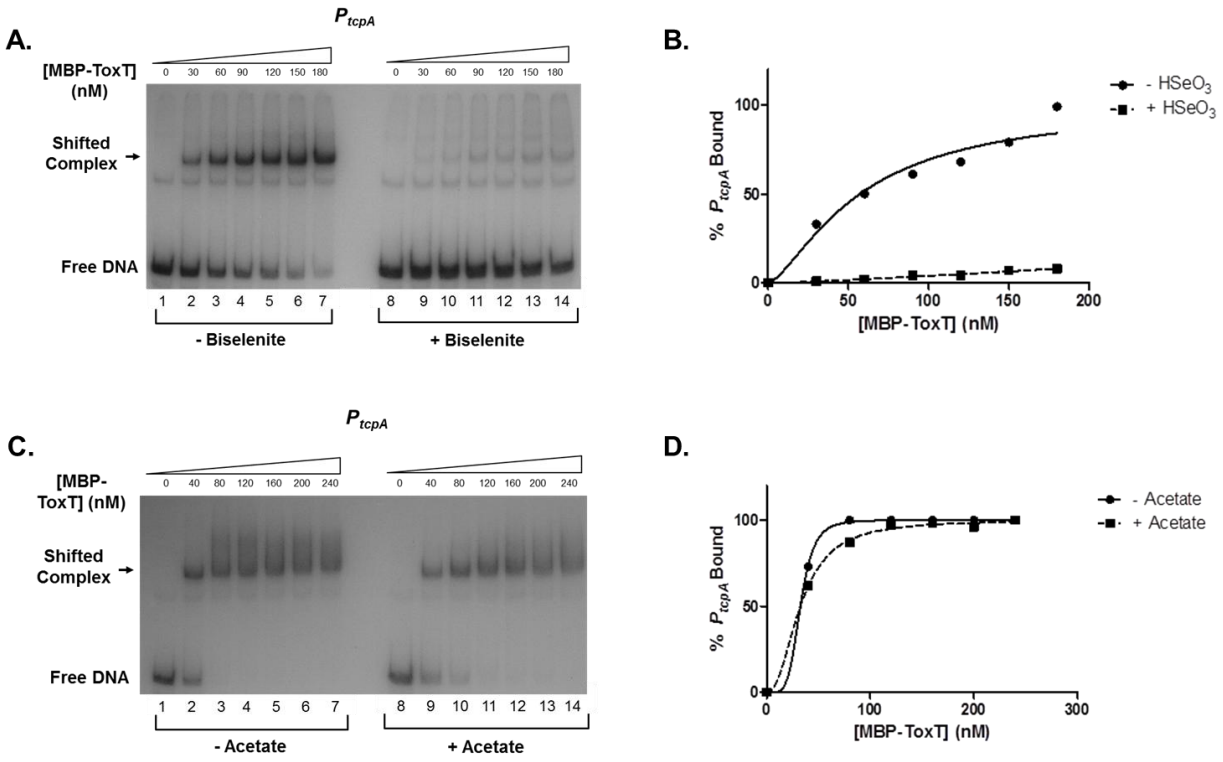

FIGURE 6. Small molecules, biselenite and acetate, do not enhance MBP-ToxT binding to P_{tcpA}. Autoradiograph of EMSA showing binding reactions of MBP-ToxT to P_{tcpA} with different small molecules similar to $NaHCO_3$. (A) Binding reactions in the absence and presence of 36 mM $NaHSeO_3$ with corresponding binding curves (B). (C) Binding reactions in the absence and presence of 36 mM $NaC_2H_3O_2$ along with corresponding binding curves (D).

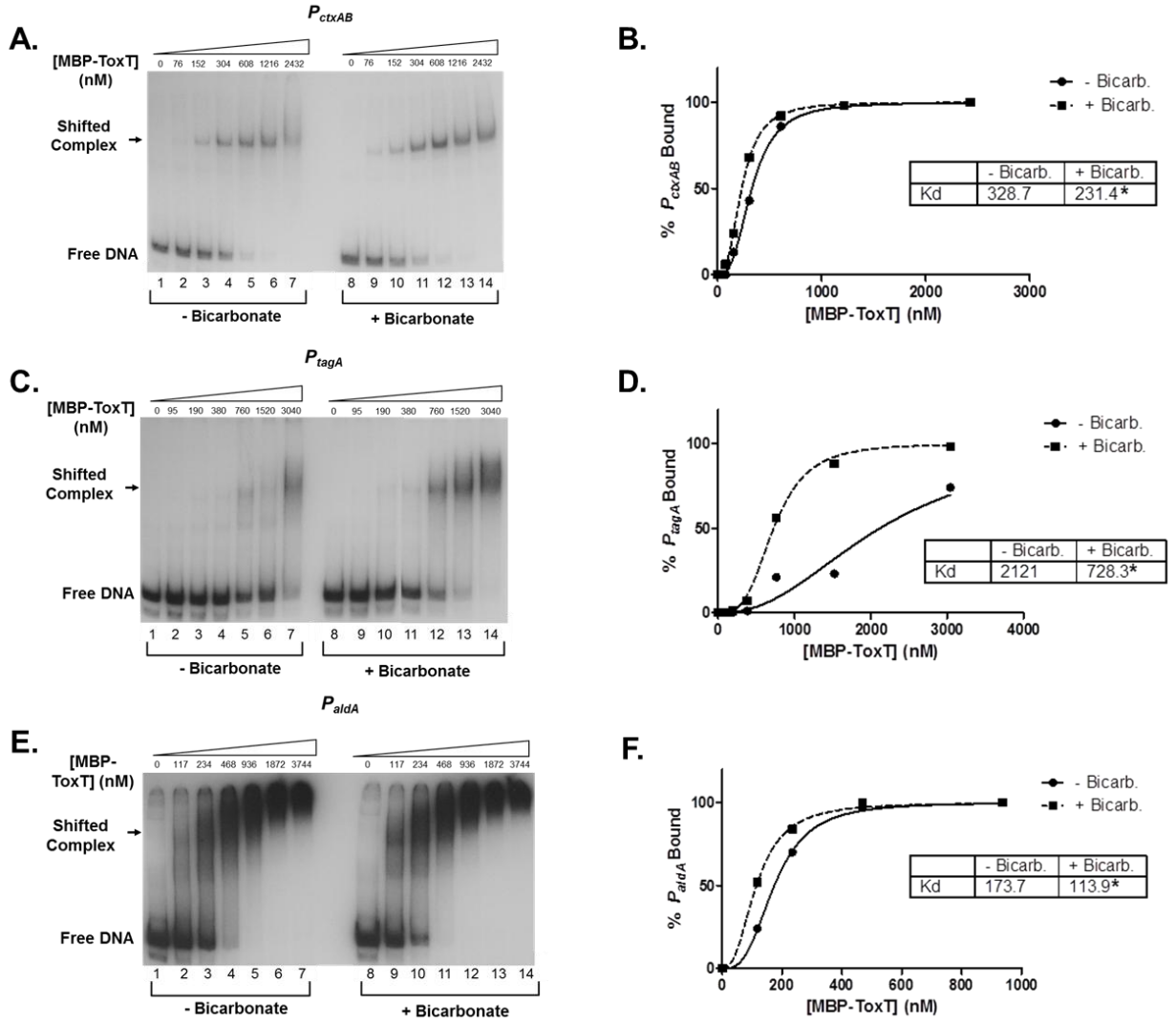

FIGURE 7. Bicarbonate increases ToxT binding affinity to promoters having all known toxbox configurations. Each autoradiograph is a representative or three or more independent experiments. Binding reactions in lanes 1-7 in all autoradiographs were conducted in the absence of $NaHCO_3$. Binding reactions in lanes 8-14 in all autoradiographs were conducted in the presence of 36 mM $NaHCO_3$. All binding curves were generated using Graphpad Prism 5 software using the equation %Bound=B_{max}*[Protein]^h/(K_d^h+ [Protein]^h) with B_{max} constraint set to 100. (A) Autoradiograph of EMSA showing MBP-ToxT binding to P_{ctxAB}, in which two toxboxes are oriented as direct repeats. Representative binding curves shown in (B). (C) Autoradiograph of EMSA showing MBP-ToxT binding to P_{tagA}, in which two toxboxes are oriented as inverted repeat. (D) Binding curves corresponding to MBP-ToxT binding to P_{tagA}. (E) Autoradiograph of EMSA showing MBP-ToxT binding to single toxbox promoter, P_{aldA}. (F) Binding curves of P_{aldA} EMSA.

CHAPTER TWO

A small unstructured region in *Vibrio cholerae* ToxT mediates the response to positive and negative effectors and ToxT proteolysis

*The work presented in this chapter was completed in part by Sarah C. Plecha. She performed β-galactosidase assays involving the negative effectors, bile and linoleic acid

ABSTRACT

Vibrio cholerae is the causative agent of the severe diarrheal disease cholera. Production of the virulence factors that are required for human disease is controlled by a complex network of transcriptional and post-transcriptional regulators. ToxT is the transcription regulator that directly controls the production of the two major virulence factors: toxin co-regulated pilus (TCP) and cholera toxin (CT). The solved crystal structure of ToxT revealed an unstructured region in the N-terminal domain between residues 100 and 110. This region and the surrounding amino acids have been previously implicated in ToxT proteolysis, resistance to inhibition of virulence induction by negative effectors, and ToxT dimerization. To better characterize this region, site-directed mutagenesis was performed to assess the effects on ToxT proteolysis and bile sensitivity. This analysis identified specific mutations within this unstructured region that prevent ToxT proteolysis and other mutations that limit the inhibition of virulence gene activation by bile and unsaturated fatty acids. In addition, we found that mutations that affect the sensitivity of ToxT to bile also affect the sensitivity of ToxT to its positive effector, bicarbonate. These results suggest that a small unstructured region in the ToxT N-terminal domain is involved in multiple aspects of virulence gene regulation and response to human host signals.

INTRODUCTION

Vibrio cholerae is the etiological agent of the severe diarrheal disease, cholera. Cholera disease is characterized by extreme water loss and dehydration due to diarrhea, and if left untreated can result in death. The bacteria are usually ingested through contaminated food or water and colonize the upper small intestine (108). When the *V. cholerae* bacterium is in the optimal environment within the intestine, it begins producing the major virulence factors responsible for causing disease: cholera toxin (CT) and toxin co-regulated pilus (TCP) (176, 237, 238). CT is an ADP-ribosylating toxin composed of five binding B subunits and one enzymatic A subunit (49). After binding the GM_1 ganglioside via the B subunits, the A subunit is translocated into the intestinal epithelial cell, where it modifies $G_s\alpha_1$ leading to aberrant secretion of chloride, water, and other electrolytes (205). TCP is a type IV bundle-forming pilus responsible for bacteria-bacteria interactions that result in microcolony formation during intestinal colonization (132, 238, 240).

TCP and CT are produced via a virulence regulatory cascade known as the ToxR regulon. Expression of CT and TCP is directly activated by the major virulence transcription regulator, ToxT (47, 115). ToxT binds "toxbox" motifs in the promoters of *ctxAB* and *tcpA*, as well as in the promoters of other accessory virulence factors, such as *acfA*, *acfD*, *tagA*, *aldA* ,and *tcpI*, and small regulatory RNAs *tarA* and *tarB*, resulting in expression of these genes under appropriate conditions (21, 115, 202, 247-249, 262, 263). ToxT is a 276 amino acid protein that is part of the AraC/XylS family of transcription regulators (101). ToxT consists of two domains, the N-terminal domain (1-160) (NTD) and the C-terminal domain (170-276) (CTD), separated by a short linker (161-169) (152). The CTD comprises the DNA-binding domain, consisting of two helix-turn-helix motifs, which shares homology with AraC (155). The NTD shares no significant

sequence similarity with any other protein, but shares secondary structural similarity with AraC (152) despite having only 14% identity at the amino acid level.

Transcription of *toxT* is initially activated by both TcpP/H and ToxR/S (47, 95, 100, 144). After ToxT protein is present, it can produce more of itself independently of TcpP/H and ToxR/S by binding to the promoter of *tcpA* and activating transcription of a long, polycistronic mRNA containing *toxT* (100, 262). Proteolysis of ToxT is required to break this auto-regulatory loop and completely shut off virulence gene expression prior to escape from the host (3). A region of the ToxT NTD between amino acids 100-109 was found to be required for proteolysis of ToxT (3). This region was not resolved in the ToxT crystal structure, indicating the absence of a fixed structure, at least in ToxT crystals (152).

Activation of ToxT-dependent promoters is further regulated by effector molecules that act on ToxT. ToxT activity is inhibited by bile and, to a greater extent, the unsaturated fatty acid (UFA) components of bile, including oleic, linoleic, and arachidonic acid (31, 212). The ToxT crystal structure contained a buried 16-carbon fatty acid, palmitoleic acid that was shown to decrease binding of ToxT to the *tcpA* promoter when added exogenously (152). Another inhibitor of ToxT, virstatin, decreases ToxT activation of *ctxAB* and *tcpA* by inhibiting ToxT dimerization (117, 216). On the other hand, ToxT activity is enhanced by bicarbonate, which is abundant within the upper small intestine where *V. cholerae* colonizes (2). Given their high concentrations in the upper small intestine, bile and bicarbonate are likely to be in vivo effectors used by *V. cholerae* to determine the optimal location for colonization.

In the solved ToxT crystal structure, a region between amino acids 100-109 was not visible, indicating that it lacked a consistent structure, at least in the contact of the crystal. This unstructured region was shown to contain the site critical for proteolysis (3), and, together with

the surrounding amino acids, has also been implicated in responding to bile, unsaturated fatty acids (UFAs), and virstatin (34, 35, 117). Due to its importance for ToxT proteolysis and sensing of ToxT inhibitory substances, we performed site-directed mutagenesis on this unstructured region and the surrounding amino acids to identify specific amino acid changes that alter ToxT function. Mutational analysis of amino acids 100-110 confirmed that this region is important for control of ToxT proteolysis. We have further identified specific mutations that prevent the ToxT effectors bile, UFAs, virstatin, and bicarbonate from affecting ToxT activity. Our results suggest that the unstructured region in the ToxT NTD plays a central role in control of *V. cholerae* virulence by impacting ToxT activity at multiple levels.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. *Escherichia coli* JM101 was used for cloning. All strains were maintained at -70°C in LB containing 20% glycerol. For determination of response to negative effectors, overnight cultures of classical O395 *V. cholerae* were diluted 1:40 into LB pH 6.5 in the presence or absence of 0.05% sodium choleate (Sigma-Aldrich) as a substitute for crude bile, 32μM linoleic acid (Acros Organics), or 50μM virstatin (Santa Cruz Biotechnology). Cultures were grown shaking for 3 hours at 30°C and analyzed. Bicarbonate virulence inducing conditions were as previously described (2). Briefly, classical O395 strains were grown overnight in LB and subcultured 1:100 into AKI medium (1.5% peptone, 0.3% yeast extract, 0.5% NaCl) in the presence or absence of freshly prepared 0.3% sodium bicarbonate (36mM). Cultures were grown statically for 4 hours at 37°C and analyzed. Induction of protein expression from pMAL-c2e derivatives in all of these conditions was done using a final concentration of 25μM isopropyl-β-D-thiogalactopyranoside (IPTG). Proteolysis conditions were

conducted as previously described (3). Overnight cultures were subcultured 1:40 into LB pH 8.5 and grown at 37°C for 4 hours. pBAD33 derivatives were induced using a final concentration of 0.2% arabinose. To assess proteolysis with additional effectors, pBAD33-ToxT was induced with or without the addition of the following final concentrations of effectors: linoleic acid (32μM, 160μM, 320μM), palmitic acid (380μM)(Sigma), virstatin (100μM, 500μM), sodium bicarbonate (36mM). To evaluate ToxT degradation after shifting to repressing conditions, bacteria were cultured in virulence inducing conditions (LB pH 6.5/30°C, shaking) for three hours with the addition of 0.2% arabinose. Then, cells were harvested by centrifugation and resuspended in virulence repressing conditions (LB pH 8.5/37°C, shaking). Linoleic acid was added to appropriate cultures after shift to a concentration of 320 μM.

Plasmid and strain construction. Construction of site-directed ToxT mutants was done using splicing by overlap extension PCR (103). For cloning C-terminal His-tagged *toxT* into pBAD33, outside primers BP22 and BP195 (3) were paired with inside primers containing desired mutations using *V. cholerae* O395 *toxT* as a template. PCR products were inserted into pBAD33 using restriction enzymes *XbaI* and *PstI*. For cloning MBP-ToxT, outside primers BP171 and BP172 (3) were paired with inside primers and PCR products were inserted into the pMAL-c2e vector using restriction enzymes *KpnI* and *PstI*. A list of primers used in this study is available in the supplementary material (Table 2). pBAD33 and pMAL-c2e derivatives were electroporated into a previously constructed *V. cholerae* classical biotype strain O395 Δ*toxt* mutant with a chromosomal *tcpA::lacZ* fusion (2).

Western blot analysis of ToxT. Western blot analysis was performed as previously described (2). Briefly, subcultures were normalized by optical density at 600nm, harvested by centrifugation and resuspended in 10ul water and 10ul 2x protein buffer (123 mM Tris-HCl, 4%

sodium dodecyl sulfate, 1.4 M 2-mercaptoethanol, 20% glycerol, 0.2% bromophenol blue). Samples were boiled for 5 min and subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was blotted on nitrocellulose and probed with 1:5000 dilution of mouse monoclonal anti-His tag antibody (Millipore). Secondary goat anti-mouse IgG conjugated to alkaline phosphatase (AP) was used at a dilution of 1:5000 (Southern Biotech). After blots were washed, they were developed using immuno-BCIP (5-bromo-4-chloro-3-indolylphosphate)-nitroblue tetrazolium liquid substrate (MP Biomedicals). Densitometry of western blots was performed using ImageJ software (NIH). Percent ToxT degradation was quantified by comparing degradation band to total ToxT.

β-galactosidase assays. Cells were grown under the appropriate conditions and β-galactosidase activity was measured and expressed in Miller units as previously described (2, 3, 12, 171).

Protein purification and electrophoretic mobility shift assays (EMSA). Protein purification and EMSAs were performed as previously described (48). Maltose-binding protein (MBP) fusions were purified from *E. coli* BL21(DE3) with plasmid pMAL-c2e containing either MBP-ToxT WT or MBP-ToxT N106F. DNA probes for EMSA were produced by PCR of plasmid pTL61t containing P_{tcpA}. Binding reactions were performed in 30uL total volume containing 10 μg/mL salmon sperm DNA, 10 mM Tris-acetate (pH 7.4), 1 mM Potassium EDTA (pH 7.0), 100 mM KCl, 1 mM dithiothreitol (DTT), 0.3 mg/mL bovine serum albumin (BSA) and 10% glycerol, along with titrations of MBP-ToxT WT or N106F. Binding reactions were performed in the presence or absence of 36 mM sodium bicarbonate or 32 μM linoleic acid for 30 minutes at 37°C, and run through a 6% polaycrylamide gel at 4°C. Binding reactions for comparison to linoleic acid reactions contained 3.33% DMSO.

Binding curve analysis. Autoradiographs were analyzed using ImageJ software (NIH) as previously described (data in submission). Briefly, the percent of labeled DNA bound by MBP-ToxT WT or N106F was determined for each lane and Graphpad Prism 5 software was used for curve fitting to the equation $\%Bound=Bmax*[Protein]^h/(K_d^h + [Protein]^h)$ with Bmax constraint set to 100. The K_d for each condition was calculated and significance of K_d between conditions was determined.

RESULTS

Identification of residues essential for ToxT proteolysis. The crystal structure of ToxT revealed a small, unstructured region in the NTD of the protein (152). This region, as well as the surrounding amino acids, has been implicated in ToxT proteolysis, resistance to negative effectors of ToxT activity, and dimerization (3, 34, 35, 117, 195, 216). To further characterize this region in regard to these roles, we performed site-directed alanine mutagenesis of ToxT amino acids 100-110, shown in Fig. 8. Mutations were made in *toxT*, incorporated into vector pBAD33, and electroporated into a Δ*toxT* derivative of classical biotype *V. cholerae* strain O395. To assess ToxT proteolysis, we grew *V. cholera*e expressing plasmid-borne WT or mutated ToxT proteins, carrying a polyhistidine tag at the C-terminus (ToxT-6His) for detection, under repressing conditions (LB pH 8.5, 37°C, shaking) for 4 hrs. Cells were harvested and subjected to western blot analysis using a monoclonal anti-His tag antibody. The previously described degradation intermediate of ToxT (24) was present in cell extracts from strains carrying each of the mutations in the unstructured region between amino acids 100-109, except for the G100A and M103A mutant strains (Figure 9A). Densitometry was performed using ImageJ software (Figure 9B) and revealed that the L107A and S109A mutants also had decreased levels of

degradation compared to WT. Mutations I104A and R105A resulted in increased degradation of ToxT, possibly due to decreased ToxT stability. The identification of mutations in this region affecting ToxT proteolysis both confirms previous work demonstrating the importance of amino acids 100-109 (3) and pinpoints amino acids 100 and 103 as crucial for ToxT proteolysis.

To further evaluate the absence of the intermediate degradation product seen in the G100A and M103A mutant strains, we performed culture condition shift experiments. Shift experiment methods from (3) were amended slightly for use with mutant ToxT strains. Briefly, *V. cholerae* strain O395 containing plasmid-borne, histidine tagged WT ToxT or mutant ToxT was grown in virulence inducing conditions (LB pH 6.5/30º C, shaking) for three hours with the addition of arabinose to induce ToxT protein production. Cells were centrifuged and resuspended in virulence repressing/proteolysis positive media (LB pH 8.5) and grown shaking at 37º C. Cultures were normalized by OD_{600} at T=0 and equivalent culture volume was taken at every time point to monitor the degradation of full length ToxT. The degradation of full length WT ToxT and ToxT M103A was analyzed by western blot analysis with anti-His tag antibody (Fig. 10A). Densitometry of the western blot was performed using ImageJ software (Figure 10B). WT ToxT was almost completely degraded 30 minutes after being shifted to virulence repressing conditions. ToxT M103A was also degraded; however 30% of the starting ToxT remained after 45 minutes. Degradation of ToxT G100A was also evaluated after a shift to repressing conditions and was degraded similarly to WT ToxT (data not shown). A likely explanation for the lack of intermediate degradation product seen with the G100A mutant (Figure 9A) is enhanced proteolytic degradation of the proteolysis intermediate. Together, these findings reveal amino acid 103 as an important recognition site by the protease and that the loss of the intermediate

degradation product under virulence repressing conditions is not only due to a decrease in stability of the intermediate.

Identification of ToxT residues involved in responding to natural negative effectors, bile and linoleic acid. The unstructured region of ToxT contained residues that, after mutagenesis, produced forms of ToxT that altered normal proteolysis of the protein. Previously published evidence has also shown that residues in this region are required for the normal response of ToxT to negative effectors, such as bile and UFAs (34, 195). Bile and UFAs decrease the ability of ToxT to activate transcription of virulence genes (31, 212). Two ToxT mutations, M103A and N106A, have previously been described as insensitive to bile and UFAs (34). We tested strains carrying these ToxT mutations, as well as strains carrying other alanine substitutions in the unstructured region, to determine whether their response to these negative ToxT effectors was altered. Overexpression of ToxT eliminates the effect of bile and linoleic acid on ToxT activity. Therefore, we chose to use the IPTG-inducible expression vector pMAL-c2e in place of arabinose-inducible pBAD33 to easily decrease the overall production of ToxT, and to facilitate protein purification for downstream applications. Previous work that used MBP-ToxT fusions showed no significant difference in activity as compared to untagged ToxT (212). As a further control for any effect the MBP tag may have had on ToxT activity, we also performed experiments with untagged ToxT mutants of interest and found that they respond to effector molecules similarly to MBP fusions using the pMAL-c2e vector (data not shown). The MBP-ToxT mutants were induced from pMAL-c2e in a Δ*toxT* derivative of classical biotype *V. cholerae* strain O395 containing a chromosomal *tcpA::lacZ* transcriptional fusion. Induction of *tcpA* transcription in the presence and absence of negative effectors was measured by β-galactosidase reporter assay and used as a metric for ToxT activity. The fold change upon

addition of effector molecules was calculated for each individual experiment. The mean fold change for each mutant was determined and will be referred to as ToxT's response to effectors. Significance of the fold change of each ToxT mutant compared to the fold change of WT ToxT in response to negative effectors was determined using a Student's t-test.

Fig. 11A shows the activity and response of the ToxT mutants to 0.05% sodium choleate, a crude derivative of bile. 0.05% sodium choleate was the lowest concentration that still decreased ToxT activity in titration experiments (data not shown). Alanine mutations to amino acids 100-104, 107, and 108 caused reductions in overall activity of ToxT in the presence and absence of bile as compared to WT MBP-ToxT. These results agree with previous reports that mutations at these residues reduce activation of *acfA::phoA* (35). The L107A mutation completely eliminated *tcpA::lacZ* expression. Prior reports have suggested that this mutation inhibits ToxT dimerization and therefore inhibits ToxT activity (34, 35). Additionally, ToxT R105A had higher overall activity compared to WT as has been previously reported (35). The fold decrease in activation of *tcpA::lacZ* upon addition of bile was calculated for each mutant (Fig. 11A). This analysis revealed ToxT mutations (G100A, D101A, I104A, Y108A, and E110A) that caused a greater sensitivity to the negative effect of bile. The M103A mutation did not affect the sensitivity of ToxT to bile as compared to WT, which was unexpected as this mutant reportedly had decreased sensitivity to bile when measuring CT and TCP production (34). However, a mutation at amino acid 106, which had been previously shown to decrease sensitivity to bile when measuring CT and TCP production (34) also decreased sensitivity to bile in our work.

The ability of these ToxT mutants to activate *tcpA::lacZ* in the presence or absence of 32 μM linoleic acid was also assessed (Fig. 11B). As was observed in the bile experiments,

mutation to residues 100-104, 107, and 108 caused decreased overall transcriptional activity with and without added effector. Also, mutants D101A, M103A, I104A, and Y108A had increased sensitivity to linoleic acid, as we had observed with bile. Previous work suggested that the M103A ToxT mutant has diminished sensitivity to another UFA, palmitoleic acid (34). However, we observed increased sensitivity of M103A to the negative effector linoleic acid. Mutant N106A, which was previously reported to cause a reduced response to palmitoleic acid in terms of CT and TCP production (34), also exhibited decreased activation of *tcpA::lacZ* in the presence of linoleic acid, verifying the importance of this residue for the response to negative effectors.

Identification of ToxT residues involved in responding to a positive effector, bicarbonate. We have previously shown that bicarbonate induces virulence gene expression by enhancing ToxT activity (2). It is possible that the response of ToxT to this positive effector could be mediated by the same region of the protein that mediates the response to negative effectors. To determine whether the unstructured region is involved in the response of ToxT to bicarbonate, as well as to bile/UFAs as shown above, we analyzed these ToxT mutants in the absence or presence of the positive effector.

V. cholerae was grown in the presence and absence of 36mM bicarbonate while expressing WT or mutant MBP-ToxT from a plasmid. β-galactosidase assays were used to determine the activation levels of chromosomal *tcpA::lacZ* (Figure 12). ToxT alanine substitutions at amino acids 100-104, 107, and 108 caused decreased overall ToxT activity, as we observed in the bile/UFA experiments described above. Additionally, mutation to S109 and E110 caused a slight decrease in overall activity compared to WT. The L107A mutant was completely inactive and bicarbonate had no activating effect on this variant of ToxT. Bicarbonate

increased the activity of WT MBP-ToxT 2.6-fold in these experiments (Fig. 12). ToxT mutants G100A, D101A, M103A, I104A, and Y108A all had increased response to the activating effect of bicarbonate. Similarly, ToxT mutants G100A, D101A, I104A, and Y108A had increased sensitivity to bile or linoleic acid. The R105A, N106A, and E110A ToxT mutants had a significantly reduced fold increase in *tcpA::lacZ* expression upon addition of bicarbonate. The N106A mutant is notable because it also showed decreased sensitivity to the negative effectors, bile and linoleic acid. These results suggest that the same ToxT residues within the unstructured 100-109 region may be involved in the response to both positive and negative effectors.

Alternate substitutions in the ToxT unstructured region affect response to positive and negative effectors. The results described above show that alanine mutagenesis within the unstructured region of ToxT alters the protein's sensitivity to bile, unsaturated fatty acids, and bicarbonate. Because amino acids in the unstructured region showed reduced sensitivity to both bile/UFA and bicarbonate when mutated to alanine, we generated alternate substitutions of amino acids 105 and 106 to further investigate the role of these residues in ToxT function. We also made mutations to amino acid 107 because the L107A mutant was completely inactive and a previous report showed that mutation L107F had decreased response to bile (195). Finally, we made an alanine substitution of amino acid L114, which has been shown to cause insensitivity to the inhibitory effects of bile, UFAs, and virstatin and is located in close proximity to the 100-109 unstructured region (34, 117). ToxT mutants R105K, R105Q, R105F, N106S, N106F, L107F, L107S, L114A, and a double mutant R105A/N106A were tested using the β-galactosidase assay described above in bile/linoleic acid inhibiting conditions (Figs. 13A,B), as well as bicarbonate inducing conditions (Fig.14). Fold change with addition of effectors was calculated for each mutant and compared to the fold change response of WT ToxT.

Alternate substitutions at amino acid R105 generally exhibited higher than normal ToxT activity in the absence of effector molecules, similar to what was observed after mutation to alanine. ToxT R105Q had increased response to the negative effector bile, while ToxT R105F exhibited a decreased response to bile (Fig. 13A). On the other hand, ToxT R105K exhibited increased sensitivity to linoleic acid (Fig. 13B). The increased sensitivity to negative effectors of the R105K and R105Q mutants may be due to a conformational change in the protein that increases accessibility of the negative effector binding sites. ToxT L107F, which has previously been reported as having decreased response to bile and UFAs (195), had increased response to both negative effectors compared to WT. ToxT L107S was similar to L107A in having inactivity. ToxT N106S had a response to bile similar to WT, while the double mutant R105A/N106A had decreased sensitivity to bile, presumably due to the N106A mutation (Fig. 13A). The N106S mutation caused an increase in response to linoleic acid, but the double R105A/N106A mutant displayed reduced sensitivity to linoleic acid (Fig. 13B). L114A was insensitive to bile and UFAs as previously described (34). Another mutation, N106F, revealed complete insensitivity to the negative effectors, bile and linoleic acid. This is a novel mutation that exhibits greatly decreased response to negative effectors.

In addition to showing reduced responsiveness to the negative effectors, ToxT mutants N106F, R105A/N106A, and L114A exhibited decreased sensitivity to the positive effector, bicarbonate (Fig. 14). All mutations to amino acid 105 resulted in decreased response of ToxT to bicarbonate. Of all the mutations in this region, the N106F mutation caused the greatest decrease in response to bicarbonate, followed by L114A and the double mutant R105A/N106A.

ToxT N106F has decreased sensitivity to virstatin. In addition to bile and UFAs, mutations to amino acid residue L114 have been found to cause resistance to the effects of a

synthetic inhibitory molecule, virstatin (34, 117, 216). We have shown above that the ToxT L114A mutant also has decreased sensitivity to bicarbonate (Fig. 14). The N106F mutant that we discovered to play a role in resistance to bile, UFAs, and bicarbonate behaves similarly to L114A under the same conditions. In order to determine if the N106F mutant is also insensitive to virstatin, we grew the bacteria containing either WT or the N106F mutant ToxT under virulence-inducing conditions in the presence and absence of 50 μM virstatin. In the presence of virstatin there is a 9.7-fold reduction in *tcpA::lacZ* when expression of WT MBP-ToxT is induced (Fig. 15). The effect of virstatin on ToxT activity was decreased in the resistant L114A mutant, consistent with previous work (34, 117, 216). The effect of virstatin was also greatly decreased in the N106F mutant, as mentioned above.

Change in binding affinity of ToxT N106F is reduced in presence of linoleic acid or bicarbonate. Previous work has suggested that palmitoleic acid decreases the ability of ToxT to bind to P_{tcpA} *(152)*. Additionally, we have shown that bicarbonate increases the binding affinity of ToxT to virulence gene promoters regardless of toxbox orientation (Chapter One). To assess the ability of linoleic acid and bicarbonate to alter the binding affinity of ToxT N106F to P_{tcpA}, we subjected WT MBP-ToxT and the N106F derivative to electrophoretic mobility shift assays (EMSA). Experiments were designed to determine the equilibrium binding affinity of ToxT for the major virulence gene promoter, P_{tcpA}, in the absence and presence of effector. DNA was added to binding reactions at a concentration below the estimated K_d along with a titration of MBP-ToxT.

A representative autoradiograph containing the titration of WT MBP-ToxT binding in the absence (Lanes 1-6) and presence of 32 μM linoleic acid (Lanes 7-12) if shown in Fig. 16. Binding reactions in the absence of linoleic acid contained a volume control of DMSO. The

binding curves for each condition were generated using Graphpad Prism 5 software and the equation $\%Bound=B_{max}*[MBP\text{-}ToxT]h/(K_dh + [MBP\text{-}ToxT]h)$. From this equation, the equilibrium binding affinity, K_d, was determined and significance was calculated (Fig. 16A). The K_d of WT ToxT for P_{tpA} was increased with linoleic acid, corresponding to a decrease in binding affinity. The MBP-ToxT N106F mutant was also assessed for changes in K_d in the presence of linoleic acid. The EMSA and resulting binding curve for reactions containing linoleic acid revealed no significant change in K_d when compared to the DMSO control (Fig. 16B).

Similarly, we examined the binding affinity of WT MBP-ToxT and N106F MBP-ToxT for P_{tcpA} in the absence and presence of 36 mM bicarbonate (Fig. 17). Binding reactions with no added bicarbonate are shown in lanes 1-7, while binding reactions with bicarbonate are shown in lanes 8-14. The binding curves were generated and revealed that bicarbonate decreased the K_d for WT MBP-ToxT (Fig. 17A), while there was no statistical difference in binding affinity of ToxT N106F to P_{tcpA} when bicarbonate was added (Fig. 17B). The lack of change in binding affinity of ToxT N106F with the addition of these ToxT effector molecules correlates with the transcriptional reporter assays described above (Fig. 13B,14), providing a direct link between changes in DNA binding and transcriptional activation.

Addition of unsaturated fatty acids prevents ToxT proteolysis. The work described above strongly suggests that the short unstructured region of ToxT between amino acids 100-110 is involved both in the response to positive and negative effectors and in ToxT proteolysis. Since such a small segment of ToxT was implicated in these different functions, we next investigated the possibility that addition of effector molecules could inhibit proteolysis by altering this region of ToxT and/or potentially blocking access to it by protease(s). We induced production of WT ToxT using the pBAD33 vector under virulence repressing conditions (LB pH 8.5/37° C,

shaking) that normally result in proteolysis of ToxT and allow for detection of proteolytic intermediates (24). We added various concentrations of effector molecules known to modulate ToxT activity positively or negatively and compared levels of ToxT proteolysis. We found that proteolysis of ToxT was eliminated in a dose-dependent manner by the UFA, linoleic acid (Figure 18). In contrast, a saturated fatty acid, palmitic acid, which does not inhibit ToxT activity (31), also does not inhibit ToxT proteolysis. Addition of effectors virstatin and bicarbonate did not significantly affect proteolysis.

Elimination of proteolysis by linoleic acid could be due to a conformational change in ToxT upon binding of the effector, a steric interruption due to the large size of the effector, or inhibition of the protease responsible for degradation of ToxT. An additional possibility is that linoleic acid increases the degradation rate of the ToxT intermediate, which would resemble a defect in proteolysis. To assess this possibility, we used the assay discussed above (Fig. 10A), in which ToxT is induced under virulence inducing conditions and shifted to virulence repressing conditions. The ToxT protease is produced or activated under virulence repressing conditions, and when the cultures are shifted, the ToxT protein present from virulence inducing conditions is degraded over time. Again, WT ToxT was almost completely degraded at 30 minutes in the absence of linoleic acid (Fig. 19A). However, when linoleic acid was present after the shift, full length ToxT persists to a higher extent over time as shown by densitometry (Fig. 19B). A portion of full length ToxT was degraded when linoleic acid was present, but that could be attributed to the on/off rate of linoleic acid allowing access by the protease.

To determine whether linoleic acid is sterically interfering with proteolysis in the unstructured region due to binding at this site, we analyzed proteolysis of effector-insensitive ToxT mutants (R105A, N106A, and N106F) in the absence and presence of linoleic acid (Fig.

20A). Proteolysis of WT ToxT was almost fully inhibited by the addition of 320 μM linoleic acid, as shown earlier in Fig. 11. ToxT R105A exhibited a lower degree of protection from proteolysis in the presence of linoleic acid, confirmed by densitometry (Fig. 20B). These data suggest that linoleic acid is not inhibiting activity of the protease itself, because there would be similar detection of the degradation intermediate of ToxT R105A as with WT ToxT with linoleic acid present if the protease were inactivated. The effector-insensitive mutants N106A and N106F showed complete inhibition of proteolysis similar to WT ToxT. Therefore, it is unlikely that linoleic acid is binding to these amino acids and sterically interfering with the protease. This is not surprising due to the previous finding that a palmitoleic acid molecule was found in the interface between the NTD and CTD in the crystal structure of ToxT (152). As a result of our findings, we hypothesize that the change in conformation of ToxT upon binding UFA, introduced in (152), precludes access of the ToxT protease to the unstructured region and thus inhibits degradation.

DISCUSSION

ToxT is the major transcription activator that induces CT and TCP production and thus is responsible for initiating cholera disease. It has previously been shown that amino acids in the region of 100-110 of ToxT are important for the response to negative effector molecules and dimerization (34, 35, 117, 195, 216). Also, this region was required for ToxT proteolysis (3). Interestingly, this region was not visible in the solved ToxT structure due to the absence of a fixed structure in ToxT crystals (152). We mutated each individual amino acid from positions 100-110 of ToxT and discovered that ToxT variants G100A and M103A abrogated normal proteolysis of ToxT, M103A being more important for protection from degradation. We also

discovered amino acids important for the response of ToxT to bile, linoleic acid, and bicarbonate. N106A, R105A/N106A, N106F, and L114A showed decreased response to bile, linoleic acid, and bicarbonate. N106F also showed reduced response to the negative effector virstatin, as did L114A, as previously reported (28). Most mutations to amino acid 105 had no effect on reducing the response to bile and linoleic acid, but caused reduced sensitivity to bicarbonate. Additionally, effector-insensitive ToxT N106F demonstrated no change in binding affinity to P_{tcpA} in response to UFA and bicarbonate.

ToxT proteolysis occurs as a mechanism to break the auto-regulatory loop that is initiated after ToxT protein production has begun. We showed that ToxT G100A and M103A had reduced ToxT proteolysis; however, upon further analysis discovered that G100A likely caused enhanced degradation of the proteolytic intermediate rather than reducing degradation of full length ToxT. Possible mechanisms for resistance of the M103A mutant to proteolysis are alteration of the cleavage site, inaccessibility of the protease to the cleavage site due to a structural change, or an increase in protein stability. Previous work indicated that the protease cleavage site is located between amino acids 100 and 109, so any of these mechanisms could be consistent with the known data (3). The M103A mutation, which renders ToxT resistant to proteolysis, was previously reported to activate 300% more than WT ToxT in *ctxA::lacZ* assays but 44% of WT in *acfA::phoA* fusion assays (35). In our study, M103A caused decreased activation of *tcpA::lacZ.* Due to the observed decrease in transcriptional activation by this mutant, it is probable that the M103A mutation either alters a protease cleavage site or makes the site inaccessible to a protease, rather than increasing protein stability. Similarly, the addition of linoleic acid inhibited proteolysis, presumably due to a conformational change upon binding, resulting in an inactive state of ToxT. The inactive state of ToxT with bound linoleic acid may

resemble the inactive conformation of ToxT M103A, preventing access to the unstructured region by the protease. However, mutations to amino acids 101, 102, 104, and 108 that also displayed decreased overall ToxT activity did not alter proteolysis of ToxT. Therefore, it is likely that M103 is part of a protease recognition site.

ToxT L107A and S109A also exhibited reduced degradation compared to WT. There was still a proteolytic intermediate present; however the substitution from leucine to alanine is very conservative and may not have changed the side chain enough to eliminate proteolysis completely. When tested, the L107F mutation caused no change in ToxT proteolysis, so residue L107 most likely is not directly involved in proteolysis (data not shown). Two other mutations in the unstructured region, I104A and R105A, increased ToxT degradation product formation. This could be due to a decrease in ToxT stability, an increase in access by the protease, or enhanced intermediate stability. We performed further analysis of these mutants using our culture shift assay to measure the loss of full length ToxT over time under repressing conditions. ToxT I104A exhibited a lower level of full length degradation than WT ToxT, implying that it has increased full length stability (data not shown). Therefore, the increased ToxT I104A degradation intermediate seen in Fig. 9A is most likely due to enhanced stability of the degradation intermediate itself. ToxT R105A had increased degradation compared to WT ToxT in the culture shift assay and we therefore conclude it is indeed degraded to a higher level than WT (data not shown). ToxT R105A has overall enhanced activity compared to WT (35). This form of ToxT, despite its higher activity, may have been evolutionarily selected against due to its increased proteolytic degradation.

The protease responsible for ToxT cleavage remains undiscovered, but locating a candidate cleavage site could aid in protease identification. Our work shows the involvement of

multiple amino acids, mainly M103, in proteolytic cleavage of ToxT and the current thought is that multiple ATP-dependent proteases may be involved (3). The discovery of multiple amino acid involvement in the proteolytic degradation of ToxT supports the possibility of cleavage by multiple proteases. ATP-dependent proteases such as Clp, Lon, and HsIV recognize stretches of hydrophobic amino acids that are normally buried within the protein structure (209). Mutation of methionine to alanine at amino acid 103 could change the degree of hydrophobicity of this region of the protein, resulting in altered proteolytic cleavage.

Our findings revealed that the addition of linoleic acid blocked proteolysis of ToxT. However, UFA-insensitive ToxT N106F did not reverse the effect of linoleic acid on ToxT proteolysis. Therefore, we conclude that amino acid N106 is not part of a binding site for UFAs, agreeing with results from (152). The protective effect of linoleic acid on proteolysis is likely due to a ToxT conformational change upon binding of the effector molecule, effectively blocking access to the unstructured region by the protease. Interestingly, degradation of ToxT was unchanged when bicarbonate was present in proteolysis culture conditions. These findings, together with results from our transcription reporter assays, led to the development of a model for modulation of ToxT activity under different culture conditions (Fig. 21). In this model, we propose that when ToxT is produced under the virulence inducing conditions it is in fluctuation between an active and inactive conformation in the absence of effector molecules. This fluctuation leads to mid-level virulence promoter activity. In the presence of negative effector molecules, ToxT becomes locked into an inactive conformation, resulting in low virulence gene promoter activity. In this conformation, it is proposed that the NTD and CTD are pulled together by the effector molecule (152), and we add that this conformation impedes access to the unstructured region by the ToxT protease. Alternatively, in the presence of the positive effector

molecule bicarbonate, ToxT is locked into an active conformation, causing the highest virulence gene promoter activity. We propose that the active conformation of ToxT has an exposed unstructured region leading to a high degree of proteolysis.

It has been proposed that the unstructured region of ToxT could be functionally related to the N-terminal arm of related family members, AraC and RegA (152). The N-terminal arms of these proteins are involved in effector control of transcription activity (225, 258). In particular, when the N-terminal arm of RegA is mutated, it leads to a constitutively active form of RegA, comparable to RegA in the presence of bicarbonate (258). It was proposed that the N-terminal arm normally blocks DNA binding by RegA, and mutation to the arm or the addition of bicarbonate relieves the inhibition and allows DNA binding (258). ToxT does not contain such an N-terminal arm. However, effector-insensitive ToxT mutants, N106F and L114A, exhibit a similar phenomenon, wherein virulence gene promoter activity is increased in the absence of effector compared to WT ToxT (Figs. 13,14). These mutants, regardless of effector presence, resemble the high-level activity of ToxT in the presence of bicarbonate. Therefore, we believe the unstructured region of ToxT may play a similar role as the N-terminal arm of RegA, where it normally functions to impair DNA binding in the absence of bicarbonate.

The negative effectors of ToxT activity, bile, UFAs, and virstatin, have been implicated in reducing dimerization of ToxT monomers, leading to reduced overall activity (34, 216). The inactive conformation that we propose could be due to decreased interaction between ToxT monomers through the unstructured region, which has been previously proposed (152). Bicarbonate has not been implicated in dimerization, although increasing interaction between ToxT monomers has been suggested as a possible mechanism for bicarbonate-dependent enhancement of ToxT activity (2).

The unstructured region of ToxT is a likely candidate for effector molecule binding due to its importance in the response to negative and positive effectors, solvent accessibility (152) for on/off binding, and its disorder in the crystal structure. Unstructured regions in crystal structures are often disordered due to the lack of a stabilizing molecule (50), which could also be the case with ToxT. It is unlikely that UFAs bind the unstructured region of ToxT, as mutations in the region do not reverse the proteolytic blockade by linoleic acid. Conversely, we hypothesize that the positive effector bicarbonate could bind in this region. First, it is possible that bicarbonate could bind to ToxT in the unstructured region, as a bulky phenylalanine substitution at N106 results in an active conformation of ToxT, similar to bicarbonate. Furthermore, the X-ray structure of *E. coli* aminopeptidase A (PepA) revealed a bicarbonate anion bound to an arginine side chain (230). Our work here shows the importance of an arginine (R105) in the ToxT response to bicarbonate, raising the possibility that bicarbonate may be binding to this residue.

We have introduced a model wherein ToxT becomes activated by bicarbonate as the concentration of bicarbonate increases when *V. cholerae* enters the mucus layer of the small intestine (2). This model illustrates that ToxT is in an inactive form in the lumen of the intestine due to a lower local concentration of bicarbonate. The model was adapted in (260), where bile/UFAs in the lumen of the intestine inactivate ToxT, and are replaced by bicarbonate as the bacterium moves closer to the mucus/epithelial layer. Using this model of ToxT-dependent virulence in the host, it is plausible that the inactive state of ToxT with UFA bound can be converted to the active state in the presence of enough bicarbonate, even though the effectors may not be bind in the same region.

The discovery that UFAs block ToxT proteolysis may support a mechanism for inhibiting premature degradation of ToxT. After ToxT is produced and *V. cholerae* is in the lumen of the

intestine, ToxT must remain intact but inactive so that CT and TCP are not produced prematurely in infection. UFAs in bile provide a mechanism for ToxT inactivity in the lumen and also protect ToxT from proteolysis. As the bacteria encounter higher concentrations of bicarbonate and lower concentrations of UFA closer to the epithelial surface, bicarbonate binds and converts ToxT to its active conformation. If bicarbonate also blocked proteolysis, *V. cholerae* could not undergo the mucosal escape response and be released from the intestine, as there would be no mechanism for breaking the ToxT positive auto-regulatory loop that causes continued production of TCP and CT.

In summary, we determined that the response of ToxT to both negative and positive effectors present in the intestine is mediated by an unstructured region in the NTD. Amino acids in this region were of high importance for proteolysis of ToxT and led to the finding that the negative effector linoleic acid blocks degradation of ToxT. This unstructured region plays a role in the response of ToxT to environmental signals and has implications for the temporal and spatial regulation of virulence factor production in the host.

Table 2. Primers used in this study

Primer Name	Description	Sequence 5' to 3' Direction
BP22	Outside Primer (Forward) for ToxT insertion in pBAD33 (*XbaI*)	GCTCTAGATTTAGGATACATTTTTATGATTGGGAAAAAATCTTTTCAAAC
BP195	Outside Primer (Reverse) for ToxT insertion in pBAD33 (*PstI*)	GATCCTGCAGTTAATGATGATGATGATGATGTTTTTCTGCAACTCCTGTC
BP171	Outside Primer (Forward) for ToxT insertion in pMAL-c2e (*KpnI*)	GACAAGGTACCGATGATTGGGAAAAAATCTTTTCAAAC
BP172	Outside Primer (Reverse) for ToxT insertion in pMAL-c2e (*PstI*)	GATCCTGCAGTTATTTTTCTGCAACTCCTGTCAAC
JT96	G100A Inside Primer (Reverse) Pair with BP22 or BP171	CCTTATCATGAGATCAGCAAGAATGTAAGATTTTAG
JT97	G100A Inside Primer (Forward) Pair with BP195 or BP172	CTAAAATCTTACATTCTTGCTGATCTCATGATAAGG
JT98	D101A Inside Primer (Reverse) Pair with BP22 or BP171	CTATATAAATTCCTTATCATGAGAGCACCAAGAATGTAAG
JT99	D101A Inside Primer (Forward) Pair with BP195 or BP172	CTTACATTCTTGGTGCTCTCATGATAAGGAATTTATATAG
JT100	L102A Inside Primer (Reverse) Pair with BP22 or BP171	CTATATAAATTCCTTATCATGGCATCACCAAGAATGTAAG
JT101	L102A Inside Primer (Forward) Pair with BP195 or BP172	CTTACATTCTTGGTGATGCCATGATAAGGAATTTATATAG
JT102	M103A Inside Primer (Reverse) Pair with BP22 or BP171	CACTATATAAATTCCTTATCGCGAGATCACCAAGAATG
JT103	M103A Inside Primer (Forward) Pair with BP195 or BP172	CATTCTTGGTGATCTCGCGATAAGGAATTTATATAGTG
JT104	I104A Inside Primer (Reverse) Pair with BP22 or BP171	CACTATATAAATTCCTTGCCATGAGATCACCAAGAATG
JT105	I104A Inside Primer (Forward) Pair with BP195 or BP172	CATTCTTGGTGATCTCATGGCAAGGAATTTATATAGTG
JT106	R105A Inside Primer (Reverse) Pair with BP22 or BP171	CTTTATTTTCACTATATAAATTCGCTATCATGAGATCACC
JT107	R105A Inside Primer (Forward) Pair with BP195 or BP172	GGTGATCTCATGATAGCGAATTTATATAGTGAAAATAAAG
JT71	N106A Inside Primer (Reverse) Pair with BP22 or BP171	CTTTATTTTCACTATATAAAGCCCTTATCATGAGATCACC
JT72	N106A Inside Primer (Forward) Pair with BP195 or BP172	GGTGATCTCATGATAAGGGCTTTATATAGTGAAAATAAAG
JT73	L107A Inside Primer (Reverse) Pair with BP22 or BP171	GATCTTTATTTTCACTATATGCATTCCTTATCATGAGATC
JT74	L107A Inside Primer (Forward) Pair with BP195 or BP172	GATCTCATGATAAGGAATGCATATAGTGAAAATAAAGATC
JT108	Y108A Inside Primer (Reverse) Pair with BP22 or BP171	GTAATAGATCTTTATTTTCACTAGCTAAATTCCTTATCATGAG
JT109	Y108A Inside Primer (Forward) Pair with BP195 or BP172	CTCATGATAAGGAATTTAGCTAGTGAAAATAAAGATCTATTAC
JT110	S109A Inside Primer (Reverse) Pair with BP22 or BP171	CAAAGTAATAGATCTTTATTTTCAGCATATAAATTCCTTATC
JT111	S109A Inside Primer (Forward) Pair with BP195 or BP172	GATAAGGAATTTATATGCTGAAAATAAAGATCTATTACTTTG
JT112	E110A Inside Primer (Reverse) Pair with BP171	CCAAAGTAATAGATCTTTATTTGCACTATATAAATTCCTTATC
JT113	E110A Inside Primer (Forward) Pair with BP172	GATAAGGAATTTATATAGTGCAAATAAAGATCTATTACTTTGG
JT75	L114A Inside Primer (Reverse) Pair with BP171	CATTATGTTCACAATTCCAAAGTAATGCATCTTTATTTTCAC
JT76	L114A Inside Primer (Forward) Pair with BP172	GTGAAAATAAAGATGCATTACTTTGGAATTGTGAACATAATG
JT144	R105K Inside Primer (Reverse) Pair with BP171	CTTTATTTTCACTATATAAATTCTTTATCATGAGATCACC
JT145	R105K Inside Primer (Forward) Pair with BP172	GGTGATCTCATGATAAAGAATTTATATAGTGAAAATAAAG
JT146	R105Q Inside Primer (Reverse) Pair with BP171	CTTTATTTTCACTATATAAATTCTGTATCATGAGATCACCAAG
JT147	R105Q Inside Primer (Forward) Pair with BP172	CTTGGTGATCTCATGATACAGAATTTATATAGTGAAAATAAAG
JT148	L107F Inside Primer (Reverse) Pair with BP171	GATCTTTATTTTCACTATAAAAATTCCTTATCATGAGATC
JT149	L107F Inside Primer (Forward) Pair with BP172	GATCTCATGATAAGGAATTTTTATAGTGAAAATAAAGATC
JT150	L107S Inside Primer (Reverse) Pair with BP171	GATCTTTATTTTCACTATAAGAATTCCTTATCATGAGATC
JT151	L107S Inside Primer (Forward) Pair with BP172	GATCTCATGATAAGGAATTCTTATAGTGAAAATAAAGATC
JT156	N106F Inside Primer (Reverse) Pair with BP171	GATCTTTATTTTCACTATATAAAAACCTTATCATGAGATC
JT157	N106F Inside Primer (Forward) Pair with BP172	GATCTCATGATAAGGTTTTTATATAGTGAAAATAAAGATC
JT180	N106S Inside Primer (Reverse) Pair with BP171	CTTTATTTTCACTATATAAAGACCTTATCATGAG
JT181	N106S Inside Primer (Forward) Pair with BP172	CTCATGATAAGGTCTTTATATAGTGAAAATAAAG
JT182	R105A, N106A Inside Primer (Reverse) Pair with BP171	CTTTATTTTCACTATATAAAGCCGCTATCATGAGATCACCAAG
JT183	R105A, N106A Inside Primer (Forward) Pair with BP172	CTTGGTGATCTCATGATAGCGGCTTTATATAGTGAAAATAAAG
JT184	R105F Inside Primer (Reverse) Pair with BP171	CTTTATTTTCACTATATAAATTAAATATCATGAGATCACCAAG
JT185	R105F Inside Primer (Forward) Pair with BP172	CTTGGTGATCTCATGATATTTAATTTATATAGTGAAAATAAAG

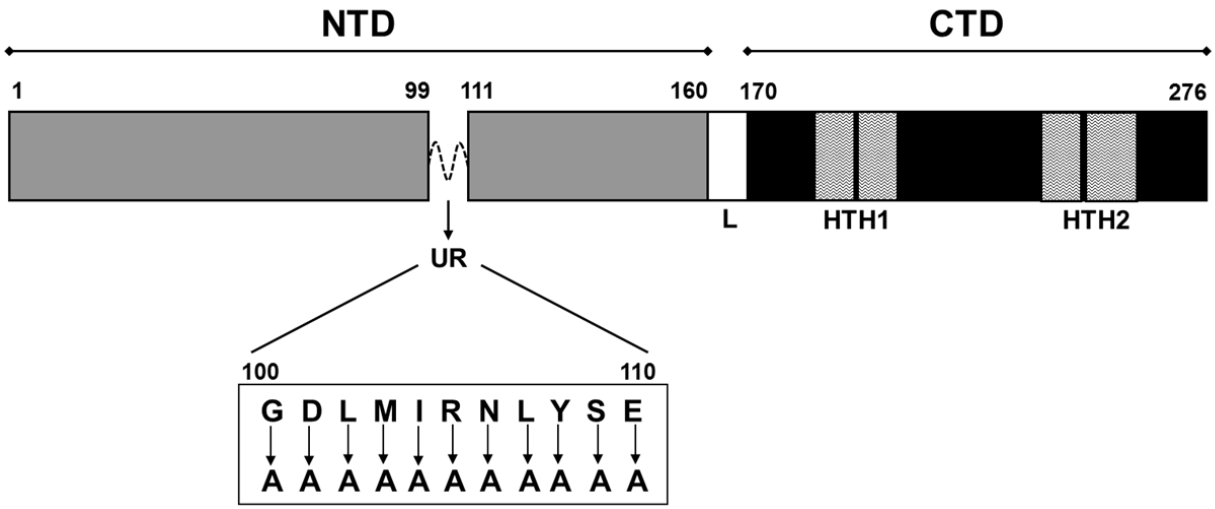

FIGURE 8. Linear representation of ToxT domain orientation and mutagenesis sites. Amino acids located in the unstructured region (UR) of ToxT between amino acids 100-110 were mutated to alanine for initial analysis of region. N-terminal domain (NTD) located between 100-160; Linker (L) located between 160-169; C-terminal domain (CTD) between 170-276. Helix-turn-helix domains 1 and 2 (HTH1,HTH2) located within the CTD.

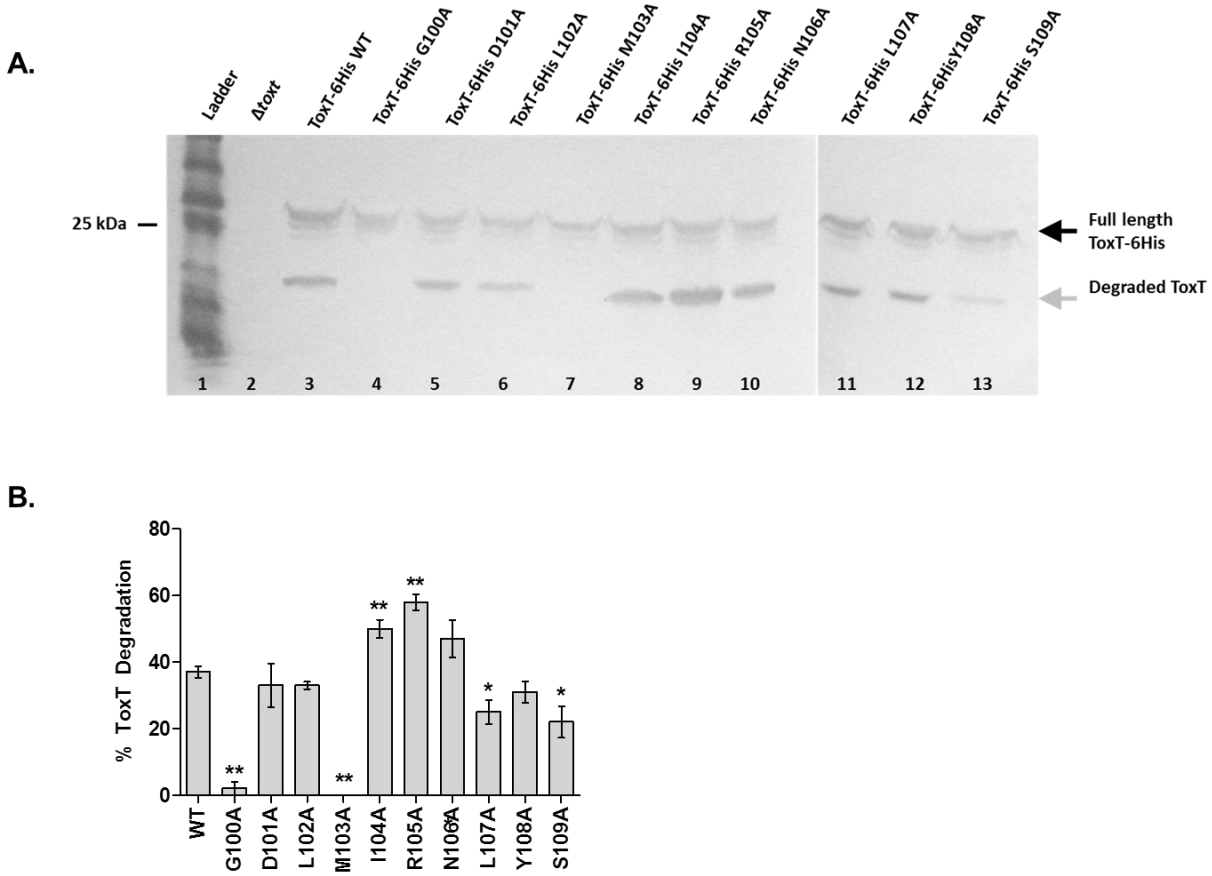

FIGURE 9. Effect of mutations to unstructured region of ToxT on proteolysis. (A) *V. cholerae* was grown under virulence-repressing conditions (LB pH 8.5, 37°C, shaking) for 4 hours producing a degradation product of ToxT (gray arrow). Full length ToxT-6His expressed from pBAD33 migrates to ~32 kDa (black arrow). ToxT mutants G100A and M103A lacked degradation product (lanes 4 and 7). I104A and R105A mutants had increased degradation of ToxT (lanes 8 and 9). Blots were probed with mouse monoclonal anti-His tag antibody. Samples were normalized by OD_{600} and gap between gels indicates two separate gels. Experiments were performed a minimum of three times and representative data are shown. (B) Quantification of band intensity was performed using ImageJ software. Graph represents percent ToxT intermediate compared to total ToxT protein. Statistical significance of percent ToxT mutant degradation compared to WT percent degradation was determined by Student's *t*-test (*,$P<0.05$; **,$P<0.01$). Error bars represent +/-standard error of the mean (SEM).

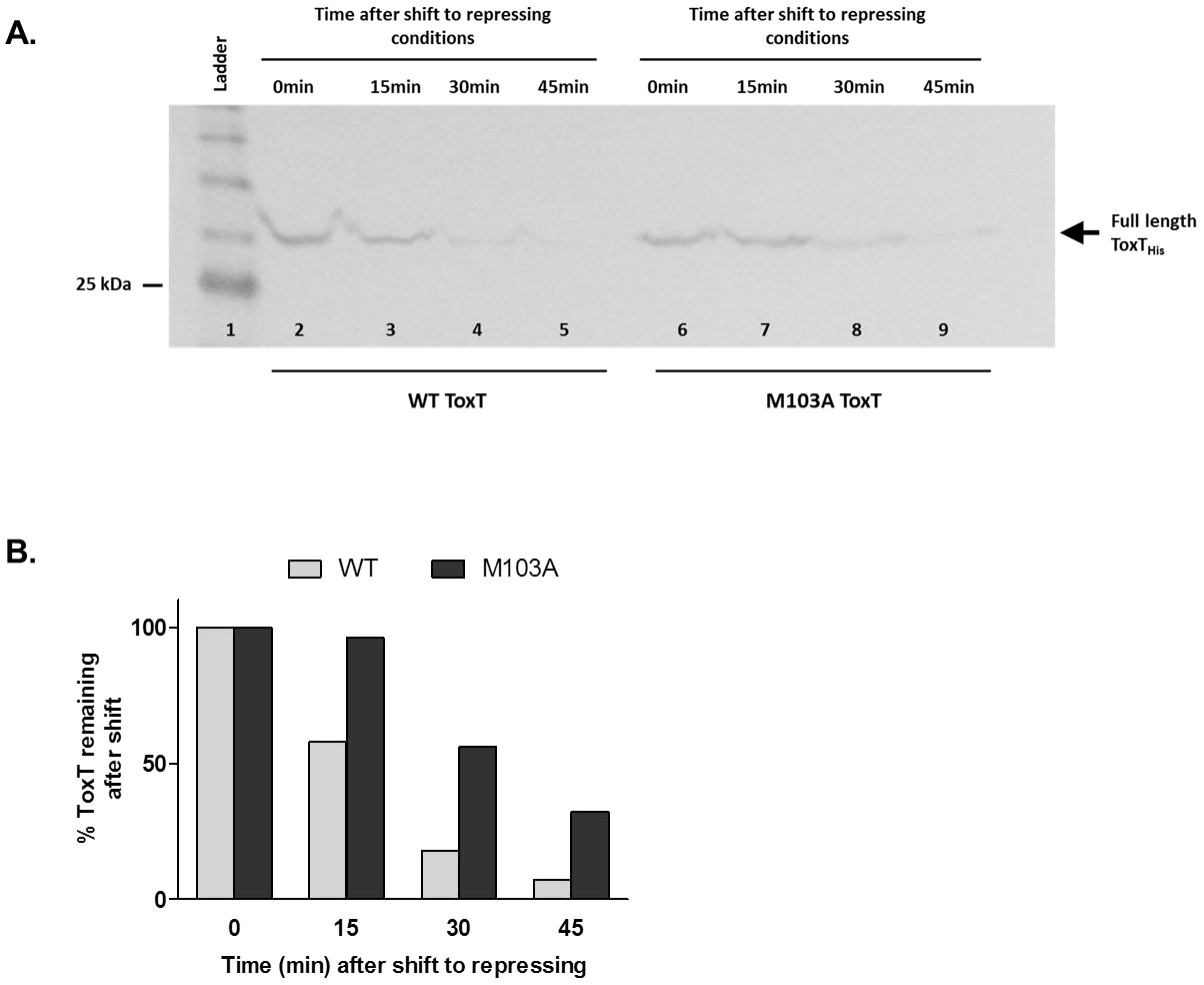

FIGURE 10. Full length WT and M103A ToxT degradation after shift to virulence repressing conditions. (A) *V. cholerae* was grown under virulence inducing conditions (LB pH 6.5, 30° C, shaking) for 3 hours while inducing WT ToxT or ToxT M103A from pBAD33. Samples were resuspended in virulence repressing growth conditions (LB pH 8.5, 37° C, shaking). Samples were normalized by OD_{600} and equivalent culture volumes were taken at each time point to monitor degradation of full length ToxT. Full length ToxT migrates to ~32 kDa (black arrow). WT ToxT degradation over time is shown in lanes 2-5; ToxT M103A degradation over time is shown in lanes 6-9. (B) Quantification of band intensity was performed using ImageJ software. Graph represents percent ToxT remaining compared to T=0 after shift to virulence repressing conditions. WT ToxT was almost completely degraded 30 minutes after shift to virulence repressing growth conditions, while ToxT M103A persisted to a greater extent.

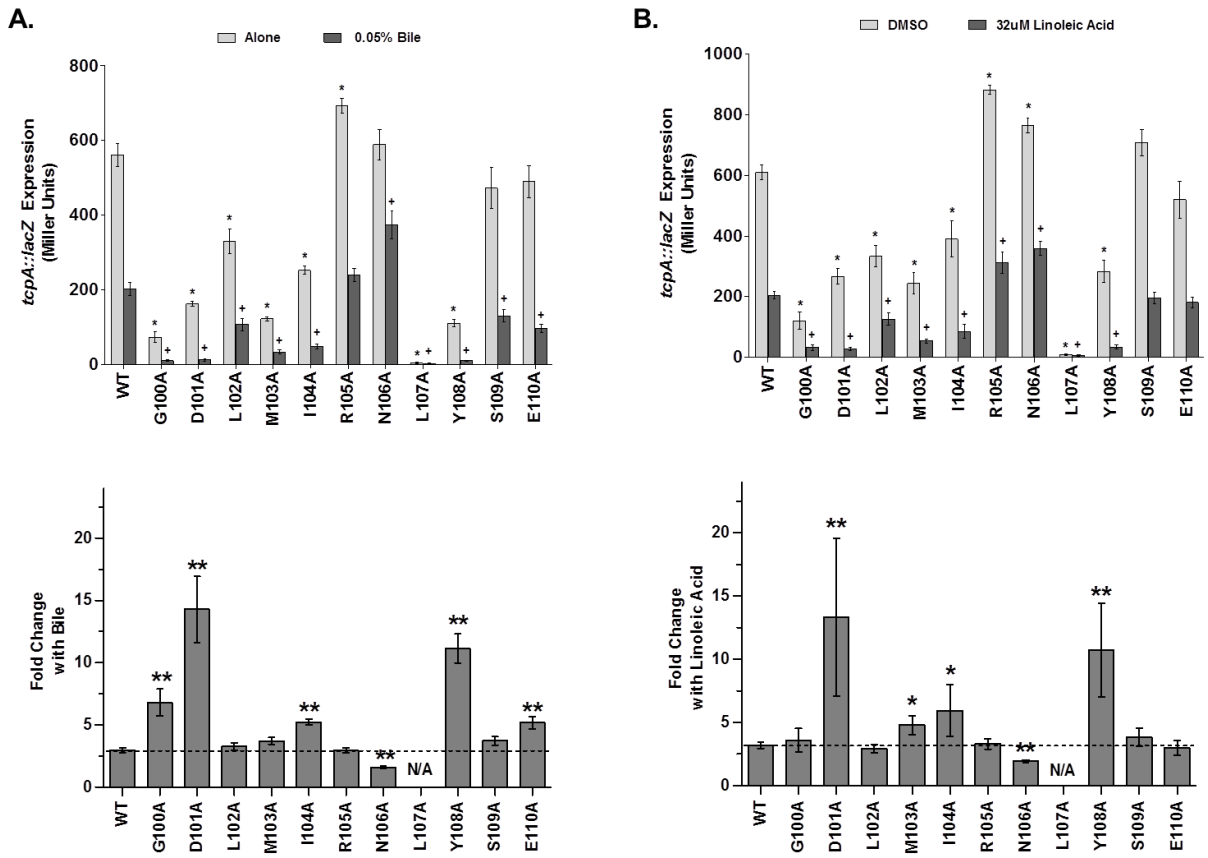

FIGURE 11. Effect of MBP-ToxT alanine mutagenesis on response to negative effectors, bile and linoleic acid. *V. cholerae* O395 Δ*toxT* mutant with plasmid-borne WT or MBP-ToxT mutants were grown under virulence-inducing conditions (LB pH 6.5, 30°C, shaking) with or without the addition of negative effectors for 3 hours. (Top) Light gray bars, *V. cholerae* grown (A) without bile or (B) with dimethyl sulfoxide (DMSO) alone; dark gray bars, grown with (A) 0.05% bile or (B) 32μM linoleic acid dissolved in DMSO. β-galactosidase produced from chromosomal *tcpA::lacZ* in classical strain O395 Δ*toxT*. Statistical significance of ToxT mutant activation of *tcpA::lacZ* in each condition compared to WT ToxT activation of *tcpA::lacZ* in the same condition was calculated using Student's *t*-test. Statistical significance of activation by ToxT mutants without effector denoted by *, with effector by $^{+}$ (*, $^{+}$,$P<0.05$). (Bottom) Mean fold-decrease in activation of *tcpA::lacZ* upon addition of (A) 0.05% Bile or (B) 32 μM linoleic acid for each ToxT mutant. Statistical significance of fold change MBP-ToxT mutant compared to WT MBP-Toxt was determined by Student's *t*-test (*,$P<0.05$; **,$P<0.01$). Horizontal dashed line represents fold-change with addition of effector of WT ToxT. ToxT mutants above line have increased sensitivity to effector, while below line represents decreased sensitivity to effector. WT, wild type. Error bars represent +/- standard error of the mean (SEM).

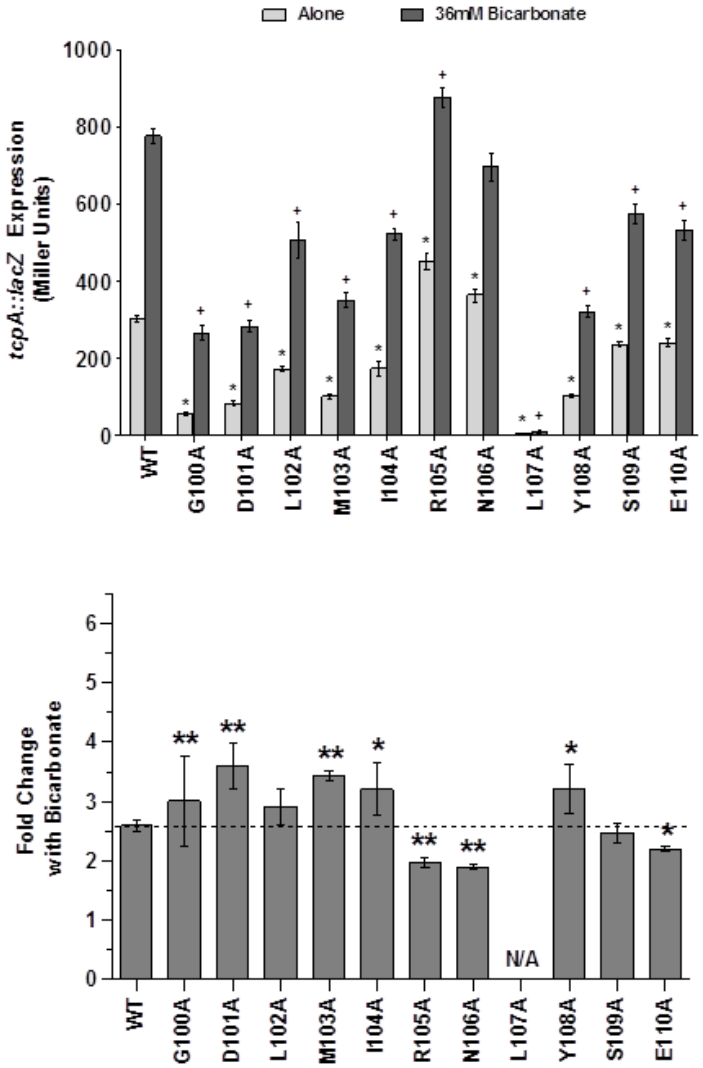

FIGURE 12. Effect of alanine mutagenesis of unstructured region on ToxT response to positive effector, bicarbonate. *V. cholerae* O395 Δ*toxT* mutant with plasmid-borne WT or mutant MBP-ToxT were grown under bicarbonate virulence inducing conditions (AKI medium, 37°C, static) for 4 hours. (Top) White bars, grown without bicarbonate; dark gray bars, grown with 36mM bicarbonate (0.3%). β-galactosidase produced from chromosomal *tcpA::lacZ* in classical strain O395 Δ*toxT*. Statistical significance of ToxT mutant activation of *tcpA::lacZ* in each condition compared to WT ToxT activation of *tcpA::lacZ* in the same condition was calculated using Student's *t*-test. Statistical significance of activation by ToxT mutants without effector denoted by *, with effector by $^{+}$ (*, $^{+}$,P<0.05). (Bottom) Mean fold-increase in activation of *tcpA::lacZ* upon addition of bicarbonate shown for each ToxT mutant. Statistical significance of fold change MBP-ToxT mutant compared to WT MBP-Toxt was determined by Student's *t*-test (*,P<0.05; **,P<0.01). Horizontal dashed line represents fold-change with addition of effector of WT ToxT. ToxT mutants above line have increased sensitivity to effector, while below line represents decreased sensitivity to effector. WT, wild type. Error bars represent +/- standard error of the mean (SEM).

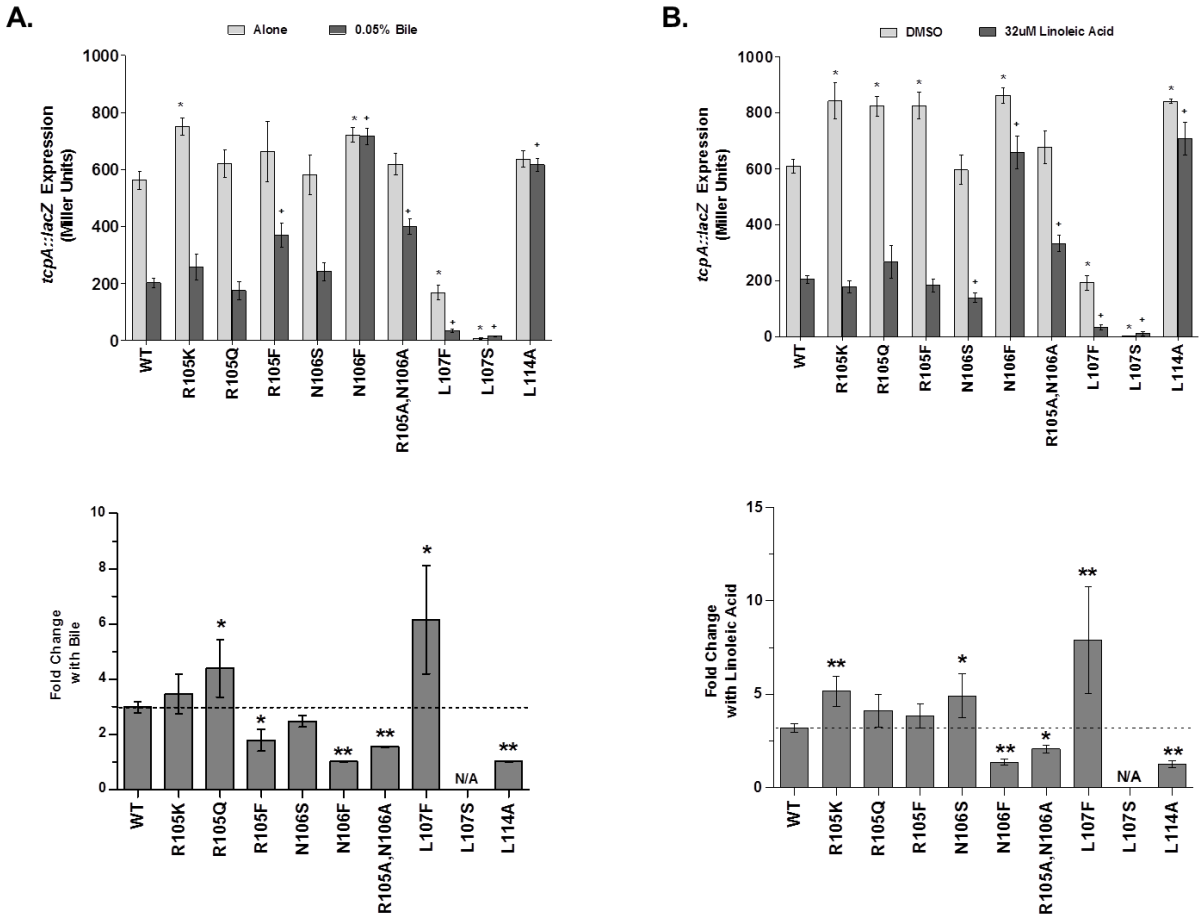

FIGURE 13. Alternate substitutions of unstructured region of ToxT affect response to negative effectors. *V. cholerae* O395 Δ*toxT* mutant with plasmid-borne WT or mutant MBP-ToxT were grown in the absence and presence of negative effectors. (Top) (A) β-galactosidase produced in absence and presence of 0.05% bile. (B) β-galactosidase produced in dimethyl sulfoxide (DMSO) or 32μM linoleic acid dissolved in DMSO. β-galactosidase produced from chromosomal *tcpA::lacZ* in classical strain O395 Δ*toxT*. Statistical significance of ToxT mutant activation of *tcpA::lacZ* in each condition compared to WT ToxT activation of *tcpA::lacZ* in the same condition was calculated using Student's *t*-test. Statistical significance of activation by ToxT mutants without effector denoted by *, with effector by $^{+}$ (*, $^{+}$,P<0.05). (Bottom) Mean fold-decrease in activation of *tcpA::lacZ* upon addition of effector shown for each ToxT mutant. Statistical significance of fold change MBP-ToxT mutant compared to WT MBP-Toxt was determined by Student's *t*-test (*,P<0.05; **P,<0.01). Horizontal dashed line represents fold-change with addition of effector of WT ToxT. ToxT mutants above line have increased sensitivity to effector, while below line represents decreased sensitivity to effector. WT, wild type. Error bars represent +/- standard error of the mean (SEM).

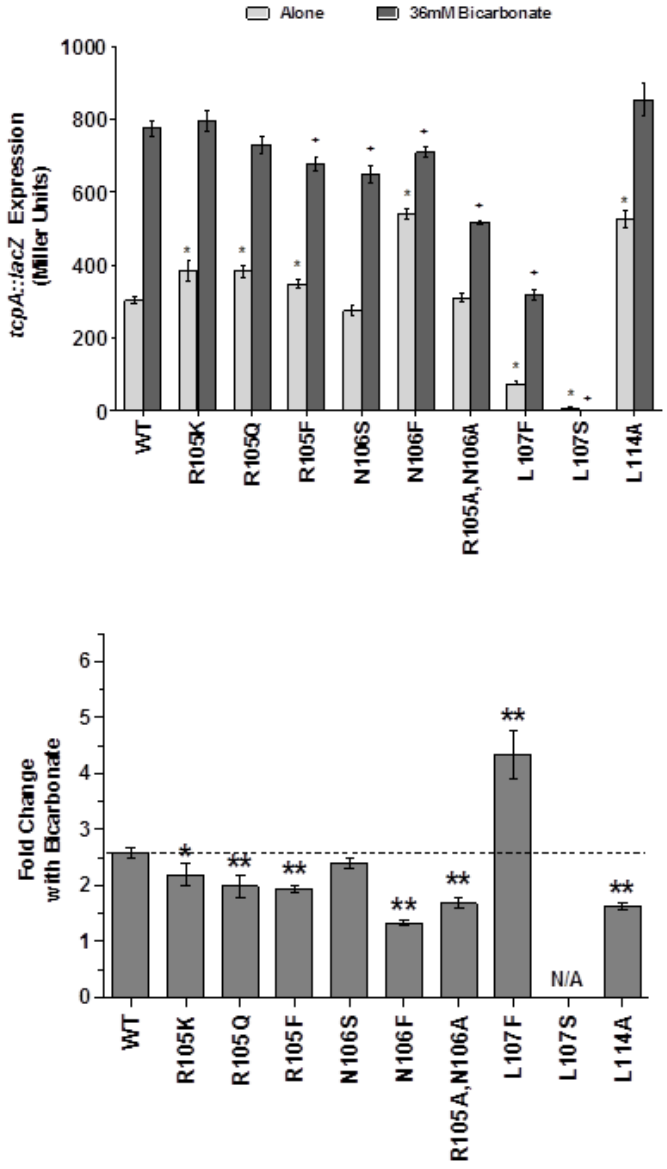

FIGURE 14. Alternate substitutions of ToxT unstructured region affect response to bicarbonate. *V. cholerae* O395 Δ*toxT* mutant with plasmid-borne WT or mutant MBP-ToxT were grown in the absence or presence of 36 mM bicarbonate (0.3%). (Top) β-galactosidase from chromosomal *tcpA::lacZ* in classical strain O395 Δ*toxT* produced in absence or presence of bicarbonate. Statistical significance of ToxT mutant activation of *tcpA::lacZ* in each condition compared to WT ToxT activation of *tcpA::lacZ* in the same condition was calculated using Student's *t*-test. Statistical significance of activation by ToxT mutants without effector denoted by *, with effector by $^{+}$ (*, $^{+}$,P<0.05). (Bottom) Mean fold-increase in activation of *tcpA::lacZ* upon addition of bicarbonate shown for each ToxT mutant. Statistical significance of fold change MBP-ToxT mutant compared to WT MBP-ToxT was determined by Student's *t*-test (*,P<0.05; **P,<0.01). Horizontal dashed line represents fold-change with addition of effector of WT ToxT. ToxT mutants above line have increased sensitivity to effector, while below line represents decreased sensitivity to effector. WT, wild type. Error bars represent +/- standard error of the mean (SEM).

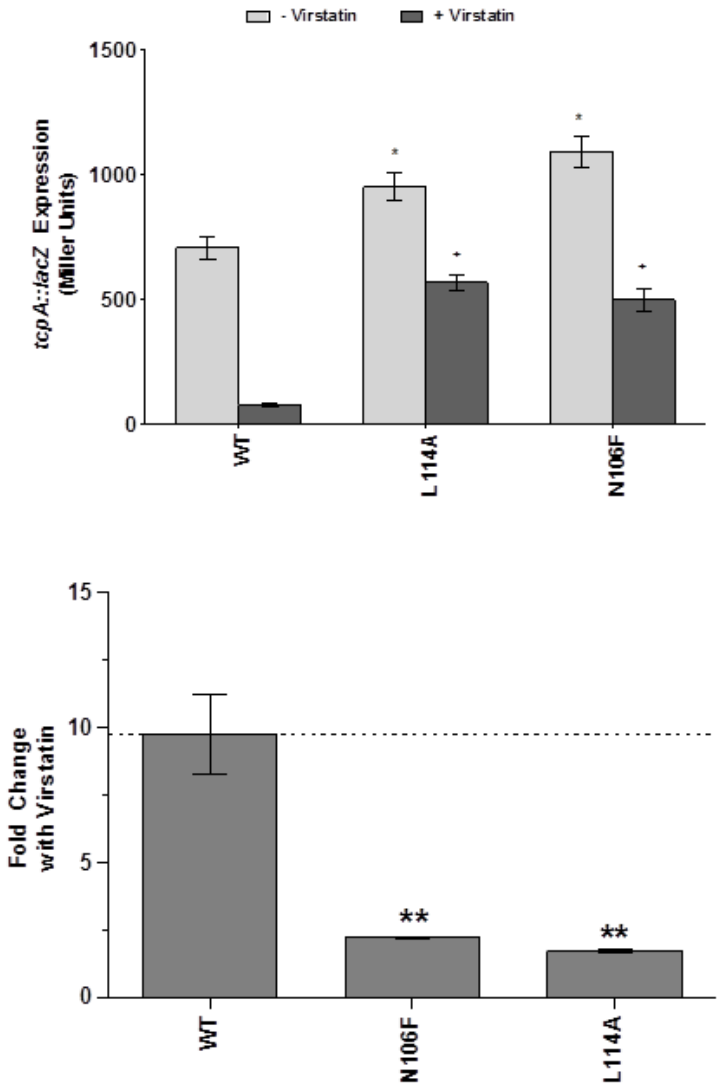

FIGURE 15. MBP-ToxT mutant N106F is insensitive to the negative effector virstatin. (Top) *V. cholerae* O395 Δ*toxT* mutant with plasmid-borne WT or mutant MBP-ToxT in dimethyl sulfoxide (DMSO) alone or 50μM virstatin dissolved in DMSO. β-galactosidase produced from chromosomal *tcpA::lacZ* in classical strain O395 Δ*toxT*. Statistical significance of ToxT mutant activation of *tcpA::lacZ* in each condition compared to WT ToxT activation of *tcpA::lacZ* in the same condition was calculated using Student's *t*-test. Statistical significance of activation by ToxT mutants without effector denoted by *, with effector by $^{+}$ (*, $^{+}$,P<0.05). (Bottom) Mean fold-decrease in activation of *tcpA::lacZ* upon addition of virstatin shown for each ToxT mutant. Statistical significance of fold change MBP-ToxT mutant compared to WT MBP-Toxt was determined by Student's *t*-test (*, P<0.05; **,P<0.01). Horizontal dashed line represents fold-change with addition of effector to WT ToxT. ToxT mutants above line have increased sensitivity to effector, while below line represents decreased sensitivity to effector. WT, wild type. Error bars represent +/- standard error of the mean (SEM).

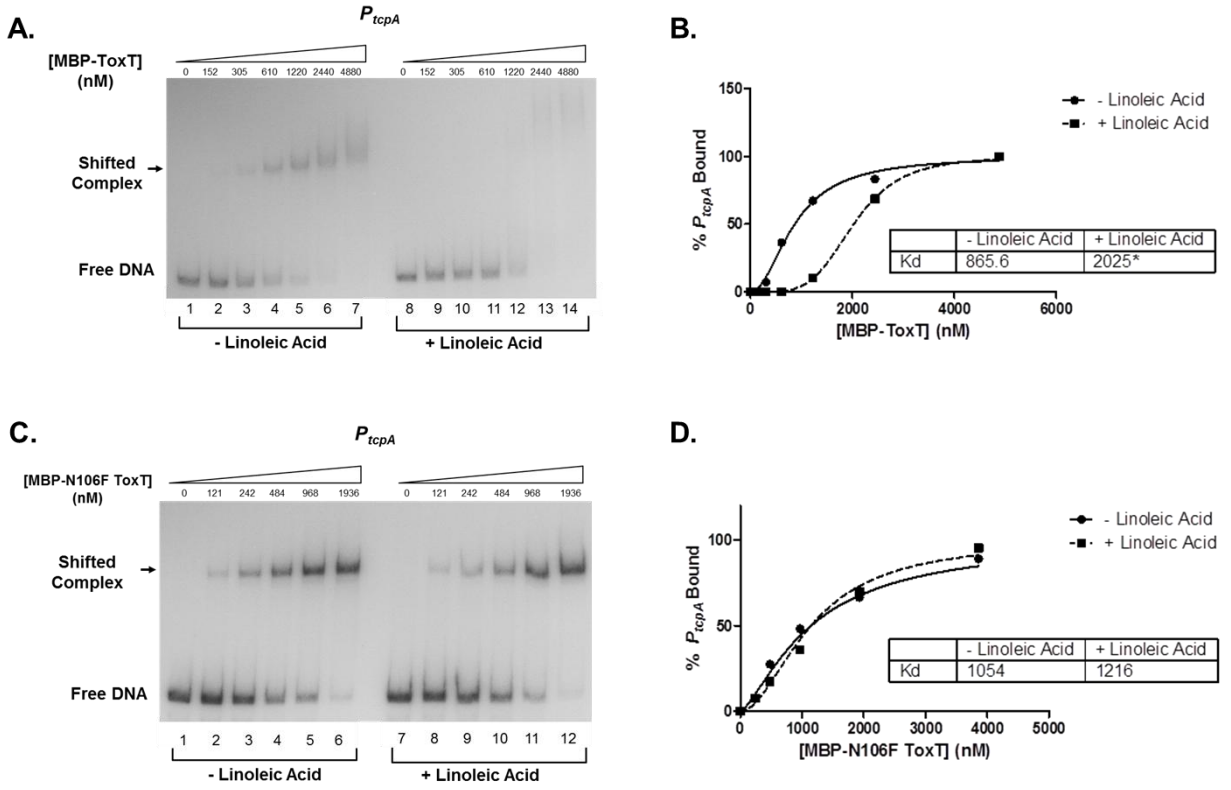

FIGURE 16. MBP-ToxT N106F has no change in binding affinity to P_{tcpA} after addition of linoleic acid. MBP-ToxT WT and N106F binding to P_{tcpA} was analyzed using EMSA. Autoradiographs of EMSAs presented are representative of three or more independent experiments. (Left) Binding reactions between (A) MBP-ToxT WT or (B) MBP-ToxT N106F and P_{tcpA} in lanes 1-7 took place with the addition of DMSO. Lanes 8-14 were incubated in the presence of 32 μM linoleic acid. Lanes 1 and 8 contained P_{tcpA} DNA in the absence of MBP-ToxT. Subsequent lanes contained a titration of MBP-ToxT with concentrations labeled in the figure. (Right) Binding curve for the autoradiograph shown to the left. Densitometry of autoradiograph was performed with ImageJ software. Circles represent percent P_{tcpA} bound by MBP-ToxT in the absence of linoleic acid. Solid line corresponds to the binding curve for MBP-ToxT to P_{tcpA} determined by the equation $\%Bound=B_{max}*[Protein]h/(K_dh + [Protein]h)$ with B_{max} constraint set to 100 using Graphpad Prism 5 software. Squares and dashed line represent percent bound and binding curve, respectively, in the presence of linoleic acid. K_d for each condition is inset and significant difference between the best-fit values of each data set is denoted by * ($P < 0.00025$).

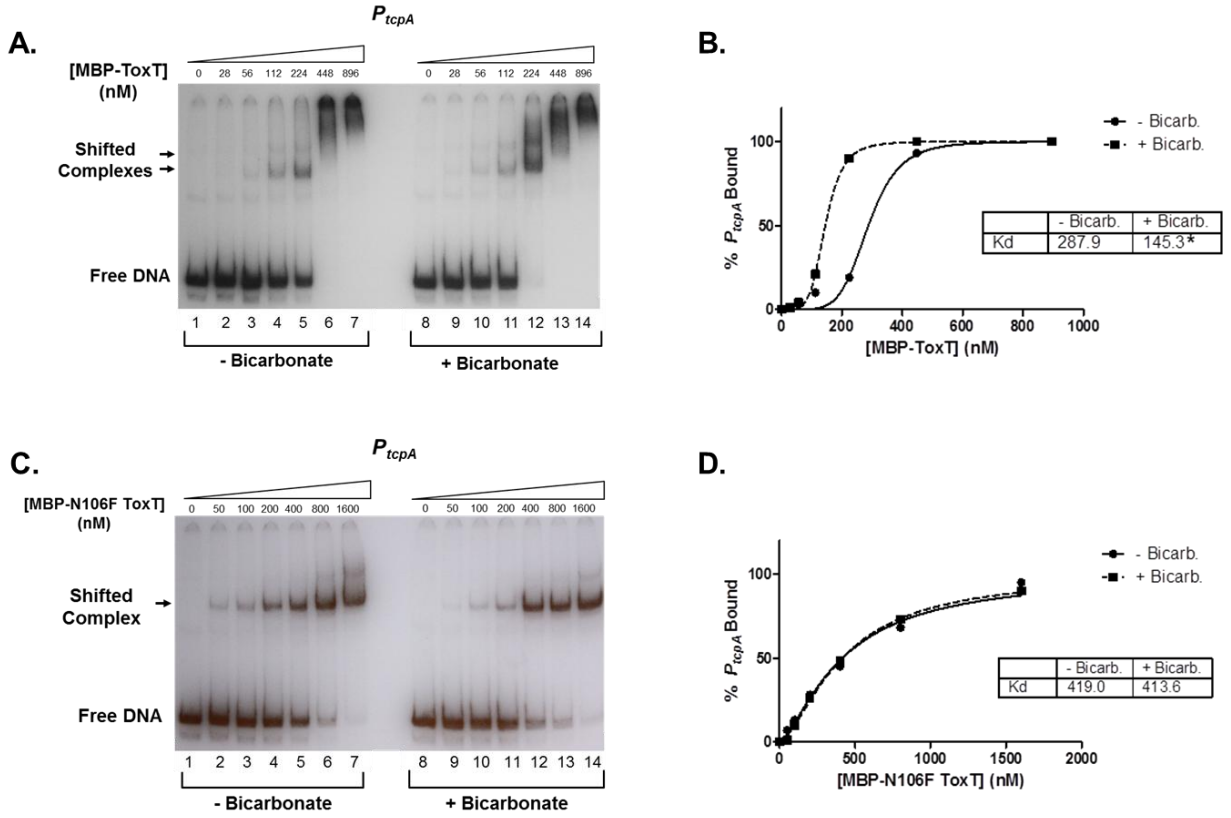

FIGURE 17. MBP-ToxT N106F has no change in binding affinity to P_{tcpA} after addition of bicarbonate. MBP-ToxT WT and N106F binding to P_{tcpA} was analyzed using EMSA. Autoradiographs of EMSAs presented are representative of three or more independent experiments. (Left) Binding reactions between (A) MBP-ToxT WT or (B) MBP-ToxT N106F and P_{tcpA} in lanes 1-7 took place in the absence of $NaHCO_3$. Lanes 8-14 were incubated in the presence of 36 mM $NaHCO_3$. Lanes 1 and 8 contained P_{tcpA} DNA in the absence of MBP-ToxT. Subsequent lanes contained a titration of MBP-ToxT with concentrations labeled in the figure. (Right) Binding curve for the autoradiograph shown to the left. Densitometry of autoradiograph was performed with ImageJ software. Circles represent percent P_{tcpA} bound by MBP-ToxT in the absence of bicarbonate. Solid line corresponds to the binding curve for MBP-ToxT to P_{tcpA} determined by the equation $\%Bound=B_{max}*[Protein]h/(K_dh + [Protein]h)$ with B_{max} constraint set to 100 using Graphpad Prism 5 software. Squares and dashed line represent percent bound and binding curve, respectively, in the presence of bicarbonate. K_d for each condition is inset and significant difference between the best-fit values of each data set is denoted by * ($P < 0.00025$).

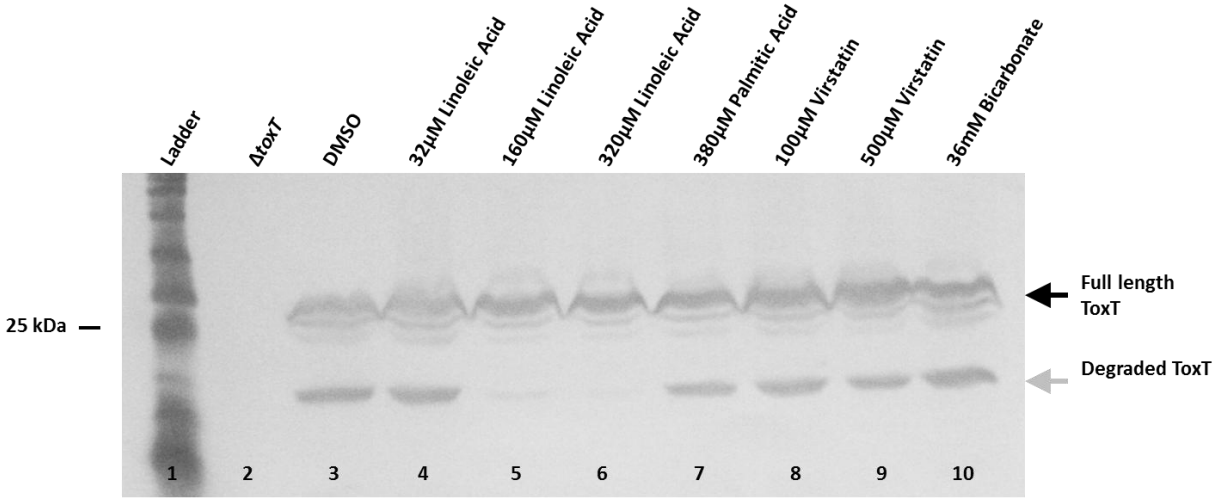

FIGURE 18. Linoleic acid inhibits ToxT proteolysis. (A) WT ToxT was produced from pBAD-*toxT* (2) under virulence repressing conditions (LB pH 8.5, 37° C, shaking) for 4 hours producing the proteolytic intermediate (gray arrow). Full length WT ToxT (black arrow) is produced under each condition. Control DMSO does not affect proteolysis (lane 3). The addition of increasing linoleic acid blocks formation of the ToxT degradation product (lanes 5 and 6). Palmitic acid, virstatin and bicarbonate did not affect ToxT proteolysis (lanes 7-10). Blots were probed with mouse monoclonal anti-His tag antibody.

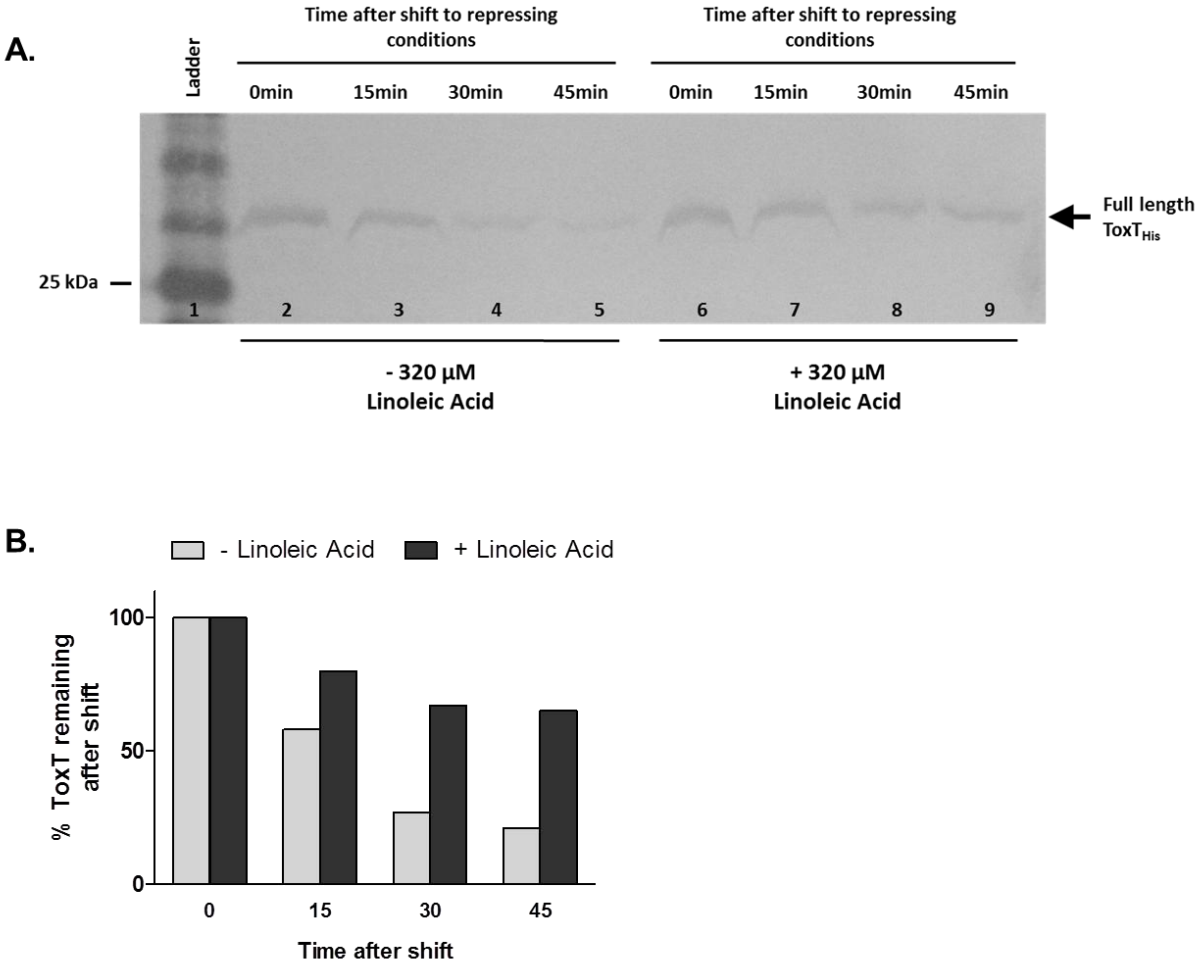

FIGURE 19. Full length WT ToxT degradation inhibited with addition of linoleic acid after shift to virulence repressing conditions. (A) *V. cholerae* was grown under virulence inducing conditions (LB pH 6.5, 30°C, shaking) for 3 hours while inducing WT ToxT from pBAD33. Samples were resuspended in virulence repressing growth conditions (LB pH 8.5, 37° C, shaking) with the addition of DMSO or 320 µM linoleic acid. Equivalent culture volumes were taken at each time point to monitor degradation of full length ToxT. Full length ToxT migrates to ~32 kDa (black arrow). WT ToxT degradation in the absence of linoleic acid over time is shown in lanes 2-5; WT ToxT degradation with the addition of linoleic acid over time is shown in lanes 6-9. (B) Quantification of band intensity was performed using ImageJ software. Graph represents percent ToxT remaining compared to T=0 after shift to virulence repressing conditions. WT ToxT was almost completely degraded 30 minutes after shift to virulence repressing growth conditions, while ToxT in the presence of linoleic acid persisted to a greater extent.

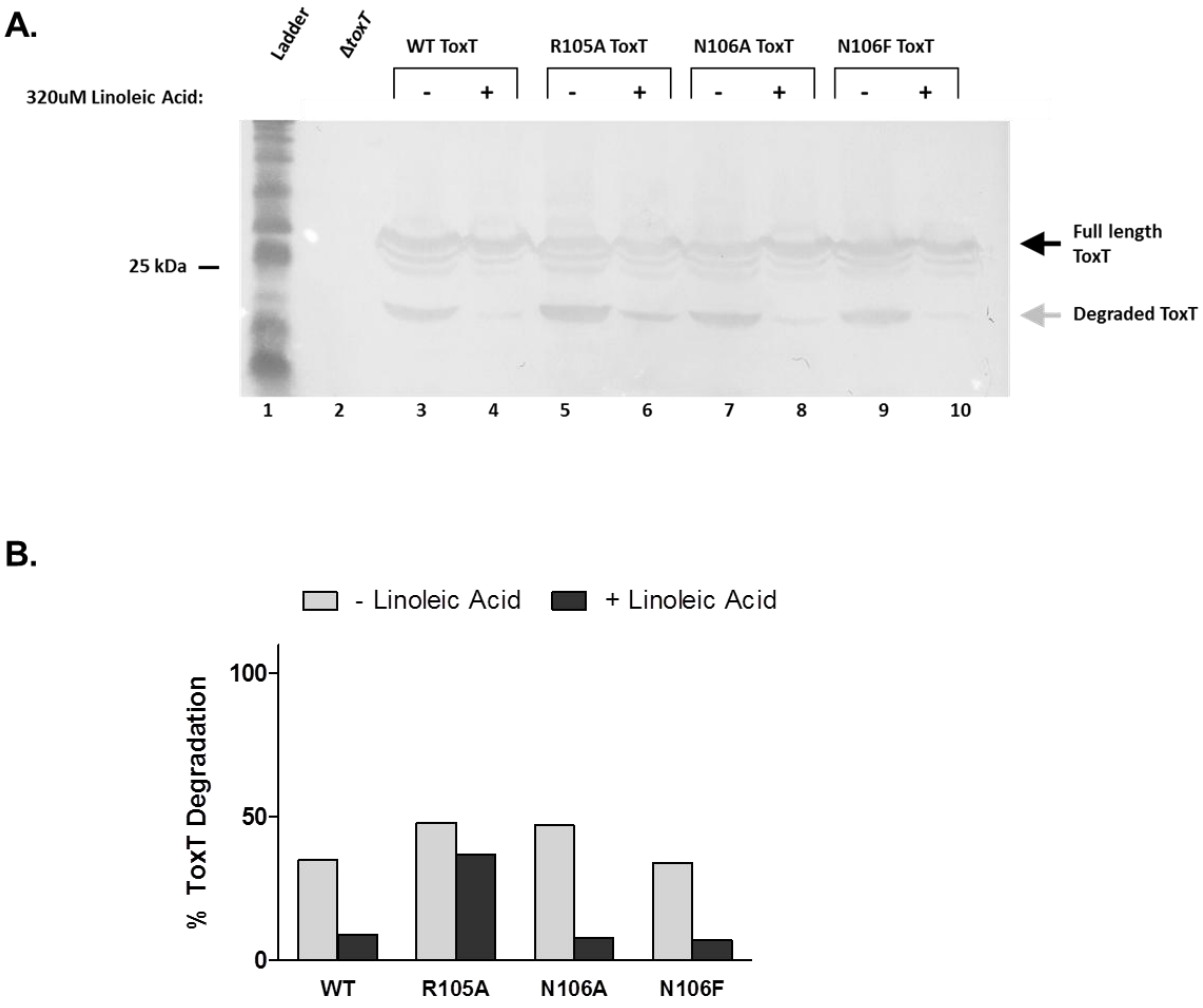

FIGURE 20. Linoleic acid inhibits proteolysis of effector-insensitive ToxT mutants. (A) Degradation of WT ToxT and mutants R105A, N106A, N106F is shown in DMSO (-) and in the presence of 320 μM linoleic acid (+). Linoleic acid inhibits proteolysis with each of the mutations (lanes 4,6,8,10). R105A still showed a small band of degradation in the presence of linoleic acid (lane 6). Samples were normalized by OD_{600} and experiments were performed a minimum of three times and representative data are shown. (B) Quantification of the band intensity was performed by densitometry using ImageJ software. Graph represents percent ToxT intermediate compared to total ToxT protein.

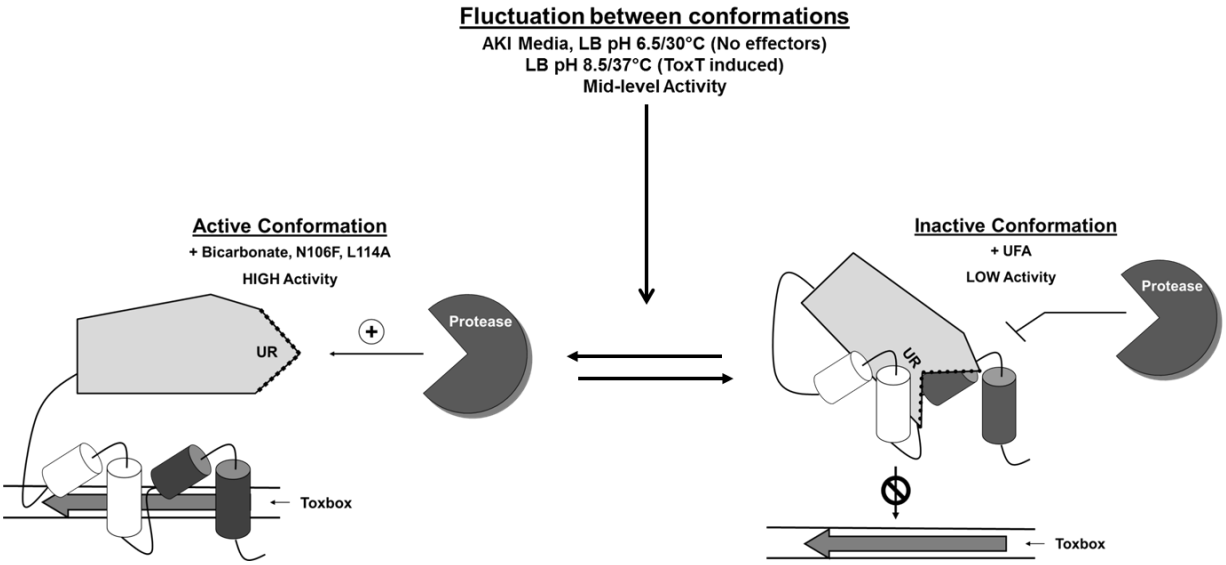

FIGURE 21. Model for effector control of ToxT activity (A) High-level transcriptional activation by ToxT with addition of positive effector, bicarbonate. ToxT undergoes conformational change with addition of bicarbonate that allows increased DNA binding affinity to toxbox and leaves unstructured region (UR) accessible by protease. Active conformation resembles that of ToxT mutants N106F and L114A. (B) Low-level transcriptional activation by ToxT with addition of unsaturated fatty acids (UFA). Upon binding UFA, ToxT takes on an inactive conformation resulting in low toxbox binding affinity and decreased proteolysis, due to unexposed unstructured region. (C) Mid-level transcriptional activation by ToxT in the absence of effector molecules. ToxT is in a fluctuation between inactive and active conformations, leading to potential ToxT proteolysis. Media conditions: AKI without added bicarbonate, virulence inducing growth conditions (LB pH 6.5, 30° C, shaking) without negative effectors, or ToxT induced *in trans* in virulence repressing growth conditions (LB pH 8.5, 37° C, shaking).

CHAPTER THREE

Bicarbonate increases expression of multiple categories of genes associated with *Vibrio cholerae* virulence and host survival

ABSTRACT

RNA deep sequencing (RNA-Seq) has become a technique that allows accurate assessment of the transcriptome of bacteria under different conditions. Whole cell gene expression in microbes can change rapidly in response to environmental signals. *Vibrio cholerae*, the enteric pathogen responsible for causing the severe diarrheal illness cholera, persists in aquatic environments and transitions to colonization of a human host after consumption of contaminated water or food. The ability of the bacteria to recognize and sense this change in environment enhances its chances of survival. Many studies of *V. cholerae* have shown various subsets of genes that are expressed in the human host and animal models. However, the signals for activation of many of these genes remain unclear. In this study, we performed RNA-Seq to monitor the changes in the *V. cholerae* transcriptome in response to external bicarbonate, a signal present in the human upper small intestine where the bacterium colonizes. Bicarbonate has previously been shown to increase virulence gene expression in a ToxT-dependent manner. Here we show that *V. cholerae* utilizes bicarbonate as a signal to up-regulate genes involved in host survival and pathogenesis, while down-regulating some genes that are not essential for colonization of the intestine.

INTRODUCTION

Enteric pathogens use signals within the human gut to initiate changes in gene expression profiles to adapt to the new environment and increases chances of survival (85). Upon entering the stomach from the external environment, the bacteria encounter a shift in temperature to 37°C, as well as extremely low pH and high chloride concentrations due to gastric acid. Once the bacteria have exited the stomach into the duodenum of the small intestine, there is an increase in pH as stomach acid is neutralized. This region of the intestine is anaerobic and the change in O_2 can initiate changes in transcription within a bacterium (20). The gut microbiota and innate immune defenses can also trigger changes within the pathogen. Additional potential signals exist in this region of the intestine where many bacteria colonize, including bile in the lumen, the mucus layer itself, and bicarbonate secreted by epithelial cells into the mucus layer (104). The ability to rapidly change gene expression in response to these various signals allows the pathogen to produce factors that increase its chance of survival in the new environment (162).

Vibrio cholerae, the enteric pathogen that causes the severe, watery diarrheal illness cholera, is one such bacterium that uses signals from the host to initiate gene transcription changes. *V. cholerae* is largely found in the aquatic environment in coastal regions. Bacteria are ingested by humans from contaminated water or food and must transition from a gene expression profile essential for survival in the environment to one suited for the host intestinal environment (24, 143). The infection caused by the consumption of bacteria leads to severe dehydration due to extreme fluid loss from diarrhea. The disease affects up to 5 million people annually and poses a constant threat as outbreaks continue to affect areas that have been free of cholera (27, 46, 219, 252).

The O1 and O139 serogroups of *V. cholerae* are known to have epidemic potential, while non-O1 strains are considered non-toxigenic and, although they can cause diarrhea, cannot cause cholera disease (16, 51, 102). The O1 serogroup is divided into two biotypes: classical and El Tor (200). The classical biotype is currently rare in the environment, leaving El Tor the predominant biotype in nature (57, 205). The O139 strains that have caused epidemics are derived from El Tor strains (59). Interestingly, variants of El Tor that phenotypically resemble classical strains have been isolated from cholera patients in recent years (183, 187, 207).

Strains that have epidemic potential have acquired two distinctive elements: the cholera toxin phage (CTXΦ) and the *Vibrio* pathogenicity island (VPI) (128, 246). The CTXΦ encodes three enterotoxins, most importantly cholera toxin (CT), which is essential for causing the characteristic watery diarrhea of cholera (60, 123, 243, 246). CT toxin is a canonical AB_5 toxin and is encoded by *ctxAB (49)*. The VPI carries the genes that encode the other essential virulence factor for causing cholera: the toxin co-regulated pilus (TCP) (128). The major pilin subunit of TCP, as well as other genes necessary for pilus assembly, is encoded on the 12 gene *tcp* operon (57). In addition to the genes for TCP, the VPI carries genes encoding other accessory virulence factors that are co-regulated with CT and TCP and are important for colonization and disease, including *acfA-D, tagA, aldA, tcpI,* and *tarAB* (21, 47, 55, 90, 91, 192, 194, 202, 247, 249).

The major virulence factors, CT and TCP, are under control of a transcriptional regulatory cascade (194). The AraC/XylS family transcription regulator, ToxT, is directly responsible for activation of CT and TCP, as well as the accessory virulence genes mentioned above (29, 47, 101, 115, 247-249, 262). Transcription of *toxT* is activated by the coordinated efforts of the integral membrane transcription regulators ToxR/ToxS and TcpP/TcpH (95, 100,

144). ToxR is constitutively produced while AphA and AphB positively control production of *tcpPH* (139, 222).

Upon entering the human host from the marine environment, *V. cholerae* must alter gene expression to increase survival in the host. Approximately 10^8-10^{11} bacteria must be consumed in order for the disease to initiate, primarily to overcome the low pH of the stomach (127). Acid tolerance genes are up-regulated in response to the low pH in the stomach (165, 166, 168) and eventually the bacteria will transit to the small intestine, where they colonize. *V. cholerae* also initiates changes in gene expression in response to intestinal signals such as anerobiosis, and temperature changes. Furthermore, each level of the virulence cascade can be influenced by signals from the gut environment. AphB increases activity in response to low pH and anaerobiosis (135, 236), which can be seen in transition from the stomach to the duodenum. Repression of *aphA* by HapR is absent under low cell density, potentially increasing *tcpPH* transcription in the host (138). Transcription of *tcpPH* is repressed by the aminopeptidase PepA under non-permissive pH (11). TcpP activity is increased in response to a bile salt, taurocholate (261), and ToxR activates production of CT as well as the protective outer membrane protein OmpU in response to bile (116, 170).

Additionally, the activity of ToxT, the major transactivator of CT and TCP, is altered based on signals from the intestinal environment. ToxT activates transcription of ToxT-dependent genes by binding to 13bp degenerate sequences in the promoters, called toxboxes (248). Activity of ToxT can be inhibited by the addition of bile or the unsaturated fatty acid components of bile, found in the lumen of the upper small intestine (31, 86, 212). Additionally, the red chili component capsaicin represses activity of ToxT (32). Conversely, ToxT activity is enhanced by bicarbonate (2), which is secreted from intestinal epithelial cells. We have shown

that transcription of each known ToxT-dependent gene increases with the addition of bicarbonate (Chapter 2).

Much work has been done to elucidate the genes that are differentially expressed in the host. However, there is little overall gene expression profiling in response to individual host signals. In this study, we performed RNA-Seq to assess the overall changes in the *V. cholerae* transcriptome in response to bicarbonate. Bicarbonate is present at high concentration in close proximity to intestinal epithelial cells and in the mucus layer of the intestine. This is the location where *V. cholerae* colonizes and bicarbonate could provide the essential signal for virulence gene activation, as well as genes critical for protection and survival.

MATERIALS AND METHODS

Strains and Growth Conditions. All strains used in this study were maintained at -70° C in LB containing 20% glycerol. *V. cholerae* El Tor strain N16961 was used for this study. For *in vitro* RNA preparations, the *V. cholerae* El Tor strain N16961 was grown overnight in LB and subcultured 1:1000 into AKI medium (1.5% peptone, 0.3% yeast extract, 0.5% NaCl) in the presence or absence of freshly prepared 0.3% sodium bicarbonate (36 mM). Cultures were grown statically for 4 hours at 37° C and analyzed. Media for growth of *V. cholerae* N16961 was supplemented with 100 µg/mL streptomycin. In strains containing variants of the pTL61T plasmid, 100 µg/mL ampicillin was added.

RNA preparation and RNA-Sequencing. *V. cholerae* El Tor strain N16961 was grown in the presence and absence of 36 mM sodium bicarbonate for 4 hours statically at 37° C in AKI medium. RNA from three biological samples in each condition was extracted using the RNeasy Bacteria Protect Mini Kit (Qiagen) and manufacturer's protocols were followed. DNA

contamination was removed using an on-column RNase-free DNase kit (Qiagen). RNA quality assessment was performed by the Wayne State University Applied Genomics Technology Center (AGTC). 2-3 µg total RNA was used by Expression Analysis for RNA-Sequencing. 50 bp paired end libraries were prepared for sequencing.

RNA-Seq Data Analysis. Data analysis was performed by Expression Analysis. Differential gene expression with the addition of sodium bicarbonate was analyzed. A t-test was performed on each gene, comparing the mean of the log normalized expression of the two groups. Upper quartile normalization was used. p-value and the $\log_2$ fold-change were reported. Annotations of protein-encoding genes are from PATRIC (PAThoSystems Resource Integration Center), on *V. cholerae* serogroup O1 biovar E1 Tor strain N16961.

Quantitative Real-Time PCR (qRT-PCR). RNA extraction was performed similar to RNA-Seq. To test for DNA contamination after extraction, logarithmic PCR was performed. The absence of bands confirmed the samples were free of DNA. RNA was normalized to 100 ng/µL. To measure the relative mRNA levels of tcpA, almE, and cadA using the following primers: forward tcpA (5'-ACGCAAATGCTGCTACACAG-3') reverse tcpA (5'-CCCCTACGCTTGTAACCAAA-3'), forward almE (5'-GAGCTATTTGGGCGGTATGA-3') reverse almE (5'-GACGTAACGGTTCCACATCC-3'), forward cadA (5'-CCGTGAAGTGATTGCAGAGA-3') reverse cadA (5'-CCGCACTTGCCTTCATAAAT-3') qRT-PCR was performed using one-step SYBR green Mastermix (Invitrogen) and the program: cDNA synthesis for 10min. at 55° C, denaturing step for 5min. at 95° C, followed by 35 cycles of 95° C for 10 s, then 55° C for 30 s. The level of each mRNA was normalized to the level of rpoB using primers forward rpoB (ACCTGAAGGTCCAAACATCG) and reverse *rpoB*

(CAAAACCGCCTTCTTCTGTC). Relative levels of transcript with the addition of bicarbonate were calculated using $2^{-\Delta\Delta CT}$. Standard deviation between experiments was calculated.

RESULTS AND DISCUSSION

Differential gene expression in *V. cholerae* cells in response to external HCO_3^-. To identify genes whose expression is up or down regulated in response to the presence of bicarbonate in culture media, we performed RNA-Seq. RNA-Seq was used as it can establish a more accurate assessment of the transcriptome than other methods such as microarrays (244). The strain of *V. cholerae* used for this study was the El Tor biotype strain N16961. We used this strain due to the importance of the El Tor biotype in the current pandemic as well as its overwhelming prevalence of the in the environment compared to the classical biotype (57, 205). *V. cholerae* was grown under bicarbonate virulence-inducing conditions in the presence and absence of 36mM bicarbonate for 4 hours (2). Total RNA was extracted from the cultures and RNA-Seq was performed by Expression Analysis. A two-group comparison of three biological replicates from each condition was performed and statistically significant differential gene expression was determined. A volcano plot showing the distribution of gene expression in response to bicarbonate is shown in Fig. 22. The x-axis of the volcano plot represents the cutoffs for up or down regulated genes in the presence of bicarbonate, which were established using the $\log_2$ fold change of *toxT*, which was 1.78. *toxT* mRNA has previously been shown to be unaffected by the addition of bicarbonate to the culture medium (2). Therefore, we chose a $\log_2$fold change of 2 for a gene to be considered differentially regulated by bicarbonate. The upper and lower cutoffs are shown by vertical dashed lines (Fig. 22). The y-axis represents the statistical significance of genes regulated by bicarbonate. Each circle represents an individual gene and if they are above

the horizontal dashed line they are considered significant. Over 200 genes were differentially regulated with the addition of bicarbonate: 94 genes up-regulated, 111 genes down-regulated.

ToxT-dependent transcription of virulence genes in response to bicarbonate. Previous work in our lab has shown that the activity of the transcription regulator, ToxT, is enhanced in the presence of bicarbonate. Increased activity of ToxT leads to increased expression of downstream gene products including the major virulence factors, CT and TCP, as well as the accessory virulence factors TagA, AldA, TcpI, AcfA, and AcfD (2) (Chapter 2). As a comparison control, we examined the differential expression of these pathogenesis genes. An overview of the VPI, showing genes that are up-regulated in response to bicarbonate is shown in Fig. 23. The RNA-Seq confirmed that transcription of the entire *tcp* operon was up-regulated, as well as the genes encoding CT, *ctxAB* (Table 3). Furthermore, expression of the other known ToxT-dependent genes was up-regulated. *aldA* transcription had the largest $\log_2$fold change of any of the genes in the study.

The *tagA-orf2-orf3-mop-tagD* (VC0820-VC0824) region (Fig. 23) has been shown to be involved in many aspects of virulence and colonization (265). *tagD* contains a domain with homology to thiol peroxidase of *Escherichia coli*, which plays a role in alleviating membrane damage by reactive oxygen species (28, 114). Mutations to the protein encoded by *mop* have been shown to cause pleiotropic effects including decreased colonization of the infant mouse, decreased CT, and increased biofilm and motility (266, 267). This region of the VPI was very highly up-regulated in response to bicarbonate (Table 3). *tagA* has a promoter that has been shown to be ToxT-dependent (249). However, as previously shown, this region can be divided into several potential operons (265). A polycistronic transcript beginning at *tagA* can read through all the way to *tagD*. Also, *tagD* can be transcribed monocistronically. Additionally, a

transcript encoding *orf3-mop-tagD* or *orf3-mop* can be produced, with a potential ToxT-dependent promoter upstream of *orf3* (265). Every gene in this region was up-regulated in response to bicarbonate. We have shown that transcription beginning at *tagA* can be enhanced with the addition of bicarbonate (Chapter 2), but have not attempted to determine if the other promoters in the region are involved as well.

Another ToxT-dependent region located on the VPI and important for colonization and disease is the *acf* region (Fig. 23). All of the accessory colonization factors (ACF) were up-regulated by bicarbonate, as well as *tagE*, which is located in the middle of the operon. *tagE* encodes a gene with unknown function and its importance in pathogenesis is under investigation (134). Another gene located in the region, *VC0842*, was also up-regulated; however the role of this gene in pathogenesis is unclear. *acfA* and *acfD* are both ToxT-dependent genes and we have shown them to be up-regulated in response to bicarbonate (247). The increase in expression of *acfB* and *acfC* is most-likely due to being transcribed along with the *tcp* operon.

Virulence and survival genes up-regulated by external bicarbonate. The results of the RNA-Seq recapitulated results from our previous studies, showing increased transcription from every ToxT-dependent promoter in response to bicarbonate (Chapter 2). Additionally, there were categories of other genes that were up-regulated in the presence of bicarbonate. The overwhelming category of genes up-regulated by bicarbonate encompassed genes involved in pathogenesis, colonization, and survival. Most of these genes were represented by the ToxT-dependent genes; however, many other genes were up-regulated that have been implicated in colonization and survival in the host (Table 4).

One locus that was very highly up-regulated by bicarbonate in the RNA-Seq, *almEFG* (*VC1579-1577*), has been shown to be involved in the modification of lipid A. In its transition to

colonization of the human intestine, *V. cholerae* encounters cationic antimicrobial peptides (CAMPs) located in crypts of the intestinal villi. CAMPs are positively charged peptides that bind to the negatively charged outer membrane of gram-negative bacteria, leading to permeabilization of the membrane and death to the bacterium (215). The lipid A modification locus of *V. cholerae*, containing the genes *almE*, *almF*, and *almG*, acts to modify the bacterial cell membrane to lower the net negative charge, reducing the action of CAMPs. This locus is transcribed as a polycistron and contributes to the resistance of the El Tor biotype to polymyxin B (89). *V. cholerae* may use bicarbonate as an environmental signal to modify its cell membrane charge before reaching the highest concentration of CAMPs closer to the epithelium, leading to increased survival in the presence of innate immunity.

To confirm that this locus was up-regulated in response to bicarbonate, we performed quantitative real-time PCR (qRT-PCR). First, we cultured the bacteria and extracted RNA with the same methods used for the RNA-Seq. qPCR was performed using primers for *tcpA*, *almE*, and *cadA*, with the gene *rpoB* as the housekeeping gene control. *tcpA* was used as a positive control for activation by bicarbonate, while *cadA* was a down-regulated gene isolated in our RNA-Seq screen that will be discussed later. The qRT-PCR results showed that transcription of *almE* was more highly up-regulated in response to bicarbonate than *tcpA*, confirming the results of the RNA-Seq (Fig. 24). The down-regulated gene in response to bicarbonate, *cadA*, was also decreased in the qRT-PCR results.

Another subset of genes that were up-regulated by bicarbonate is involved in polyamine transport. Polyamines are organic, cationic molecules that are ubiquitous in nature. Polyamines mediate cell functions in both prokaryotes and eukaryotes (119). In the lumen of the intestine, they exist at concentrations in the mid-high micromolar range and are in millimolar

concentrations in the epithelial cells of the intestine (13, 71). The polyamines putrescine, spermidine, and spermine have all been shown to inhibit auto-agglutination of TCP, a process required for efficient colonization (79). The RNA-Seq results revealed three genes involved in polyamine uptake that were up-regulated by bicarbonate: *potB* (*VC1427*), *potC* (*VC1426*), and *potE* (*VCA1062*). *potB* and *potC* are in an operon that is involved in spermidine import in both *E. coli* and *V. cholerae* (160). Mutations of the substrate binding protein, *potD*, in this operon cause an increase in biofilm formation (160). The two *potD* homologs in *V. cholerae* were not up-regulated with bicarbonate, while *potB* and *potC* had increased transcription. Further work will be needed to identify the role that bicarbonate plays in regulation of this operon and pathogenesis in the host. *potE* shares homology with putrescine transporters and has not been studied in *V. cholerae*.

Another transporter, *fadL-2*, was up-regulated in response to bicarbonate. *fadL-2* encodes a long-chain fatty acid transporter, which plays a role in pathogenesis and remodeling the membrane of *V. cholerae* (199). In particular, *fadL-2* transports fatty acids into the cell where they are then processed into precursors for phospholipid synthesis (149). This particular gene has been shown to be important in the transition from the human host back into the environment (126). Bicarbonate could be acting as a signal to initiate changes in the bacterial membrane that is needed upon exposure to a new environment.

The outer membrane porin encoded by *VC1565*, which shares homology with *tolC* from *E. coli*, was also up-regulated by bicarbonate. *ΔtolC* strains of *V. cholerae* show reduced resistance to bile and antimicrobials, as well as reduced secretion of the RTX toxin. *ΔtolC* strains are also deficient for intestinal colonization (18). Additionally, genes involved in purine and pyrimidine biosynthesis were up-regulated in response to bicarbonate. Mutations of these genes

have been shown to cause defects in colonization of the infant mouse and are important for fitness in the intestine (126, 169).

Other genes that were up-regulated by bicarbonate but have unknown effects on host survival include *ompV, carS, carR,* and a methyl-accepting chemotaxis protein encoded by *VC0514*. *ompV* (*VC1318*) encodes an outer membrane protein that is involved in osmoregulation (253) and also has increased transcription in response to antimicrobial peptides (36). *ompV* resides upstream of *carS* (*VC1319*) and *carR* (*VC1320*). All four genes from *VC1317-1320* were up-regulated by bicarbonate. *carS/carR* are a calcium sensing two-component system that is involved in responding to the changing Ca^{2+} concentrations in the aquatic environment as well as in the host. The CarRS system negatively regulates expression of *vps* (*Vibrio* polysaccharide) genes and biofilm formation (17). Both genes in this system are down-regulated by external Ca^{2+}, which is at different concentrations in the environment, and we report here that they are up-regulated by the intestinal signal bicarbonate. We currently do not know if there is a link between this two-component system and the other adjacent genes that were up-regulated: *ompV* and *VC1317*. However, *V. cholerae* using bicarbonate as a signal to up-regulate genes involved in the repression of biofilm formation is novel.

Genes down-regulated by external bicarbonate. We have shown that genes involved in pathogenesis and survival in the host are up-regulated by bicarbonate. Therefore, we hypothesized that bicarbonate down-regulates genes that are detrimental to survival in the host or were up-regulated before the bacterium reached the colonization site in the intestine. Interestingly, the RNA-Seq showed 6 chemotaxis genes that were down-regulated as well as 3 genes involved in fatty acid degradation (Table 5). Virulence and motility are known to be inversely regulated. Bicarbonate is a signal for virulence in the host and may potentially be a

signal for down-regulation of chemotaxis genes. Fatty acid degradation is a mechanism for energy production by β-oxidation (70). Bicarbonate may be a signal to reduce the amount of fatty acid degradation because the need for energy is lower once the bacterium reaches the region of colonization in the intestine.

After *V. cholerae* is ingested, it eventually will reach the stomach where there is an extremely low pH due to gastric acid. It is believed that the low pH of the stomach is the reason for the high infectious dose of *V. cholerae.* This was shown by the addition of buffers that would neutralize stomach acid to inocula in human volunteers, which greatly reduced the infectious dose (110, 206). *cadA* and *cadB* are genes that are induced by *V. cholerae* in response to high lysine and low pH, and were also induced in the infant and adult mouse intestines (167). These genes are involved in the acid tolerance response (ATR) and help with pH homeostasis. Our RNA-Seq showed that both of these genes were greatly reduced in response to bicarbonate (Table 5). This down-regulation was confirmed by the qRT-PCR experiments described earlier (Fig. 24). We hypothesize that bicarbonate reduces transcription of this locus as the pH in the intestine where bicarbonate is located is not as extreme as the stomach. The effect on transcription of *cadBA* could be due to the slight pH change with the addition of bicarbonate. However this remains unclear.

One other gene down-regulated by bicarbonate is *clpB-1*. *clpB-1* encodes an ATP-dependent protease that is involved in the stress response to degrade protein aggregates. *V. cholerae* encodes two homologs, *clpB-1* and *clpB-2*. ToxT, the direct activator of CT and TCP, is degraded by an ATP-dependent protease that is active or induced *in vitro* at pH 8.5 and 37° C (3). *clpB-1* fills the description of the ToxT protease (181). Bicarbonate, which enhances ToxT

activity, may act to down-regulate this protease to further the activation capability of ToxT in the host.

The overwhelming majority of genes differentially regulated by the addition of bicarbonate are related to pathogenesis. The main up-regulated genes were ToxT-dependent as bicarbonate enhances the activity of ToxT. However, other genes with potential roles in virulence and survival in the host, as well as some genes with defined roles, were up-regulated as well. The genes that were down-regulated in response to bicarbonate were mostly involved in stress response and starvation. This could be due to the slight growth advantage that *V. cholerae* exhibits when grown in bicarbonate. However, the addition of bicarbonate could act as a signal to down-regulate genes that are expressed in the gut before the bacterium reaches the intestine. The results of this study show that *V. cholerae* uses bicarbonate as a signal to alter gene expression. The up-regulation of genes known to be involved in host survival and virulence add to the importance of bicarbonate as a host signal for temporal and spatial expression of genes crucial for *V. cholerae* pathogenesis.

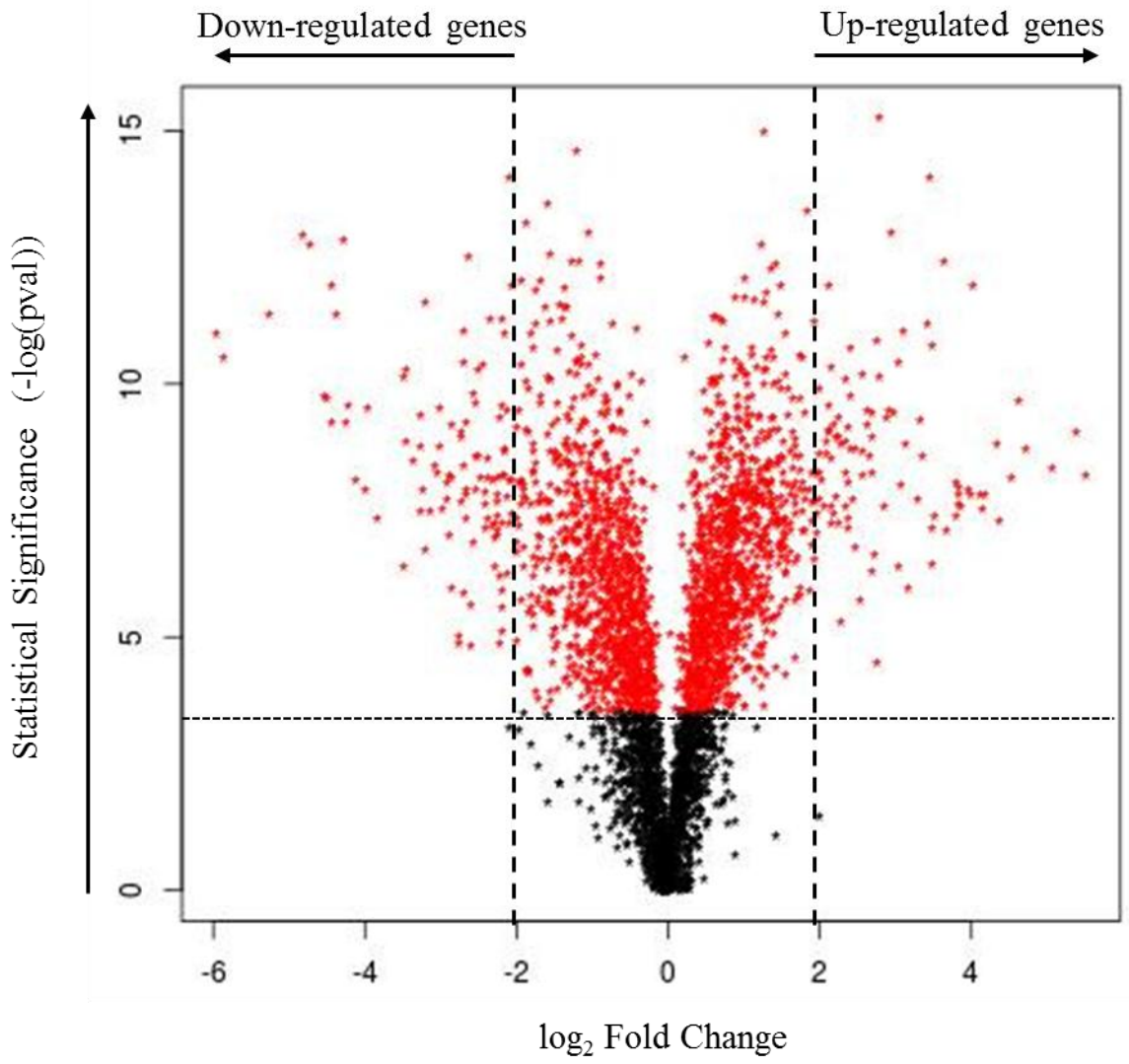

Figure 22. Distribution genes differentially expressed with the addition of external bicarbonate. The x-axis represents the $\log_2$fold change of transcript level of genes from *V. cholerae* cultured in the presence of bicarbonate compared to the levels in cultures in the absence of bicarbonate. Vertical dashed lines signify the upper and lower cut-off for genes to be up- or down- regulated in the presence of bicarbonate. Statistical significance is represented by the y-axis. Red dots are statistically significant and the horizontal dashed line is the cut-off statistical significance. 94 genes are up-regulated by bicarbonate while 111 are down-regulated.

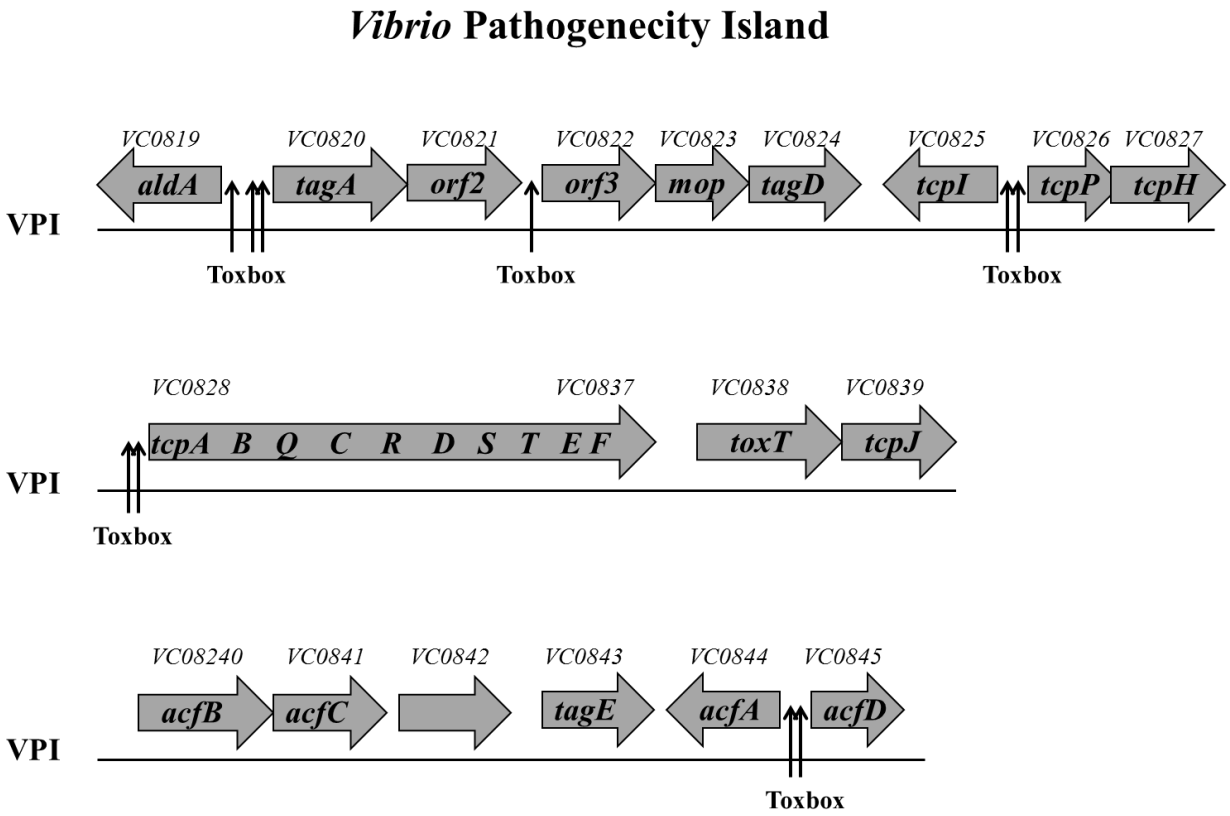

Figure 23. Genetic map of the *Vibrio* pathogenicity island (VPI). Gray arrows signify genes located on the VPI. Arrows facing right are in the 5'-3' direction and arrows facing right are genes encoded on the 3'-5' strand. Black arrows represent ToxT binding sites (Toxboxes). Gene IDs are labeled above the arrows and the gene symbols are labeled within the arrow.

Table 3. VPI and CTXΦ Linked Genes Up-regulated by Bicarbonate

Gene ID	Gene Symbol	Gene Product	log2 Fold Change +HCO3/-HCO3
VC_0819	*aldA*	aldehyde dehydrogenase	5.51
VC_0820	*tagA*	ToxR-activated gene A protein	5.08
VC_0821	*orf2*	hypothetical protein	5.38
VC_0822	*orf3*	inner membrane protein, putative	4.63
VC_0823	*mop*	Mop Protein	2.65
VC_0824	*tagD*	ToxR-activated gene D protein	3.51
VC_0825	*tcpI*	toxin co-regulated pilus biosynthesis protein I	3.07
VC_0826	*tcpP*	toxin co-regulated pilus biosynthesis protein P	0.46 [a]
VC_0827	*tcpH*	toxin co-regulated pilus biosynthesis protein H	1.09
VC_0828	*tcpA*	toxin co-regulated pilin	3.80
VC_0829	*tcpB*	toxin co-regulated pilus biosynthesis protein B	3.68
VC_0830	*tcpQ*	toxin co-regulated pilus biosynthesis protein Q	3.99
VC_0831	*tcpC*	toxin co-regulated pilus biosynthesis outer membrane protein C	3.96
VC_0832	*tcpR*	toxin co-regulated pilus biosynthesis protein R	3.82
VC_0833	*tcpD*	toxin co-regulated pilus biosynthesis protein D	3.85
VC_0834	*tcpS*	toxin co-regulated pilus biosynthesis protein S	4.11
VC_0835	*tcpT*	toxin co-regulated pilus biosynthesis protein T	4.54
VC_0836	*tcpE*	toxin co-regulated pilus biosynthesis protein E	4.71
VC_0837	*tcpF*	toxin co-regulated pilus biosynthesis protein F	3.80
VC_0838	*toxT*	TCP pilus virulence regulatory protein	1.79 [a]
VC_0839	*tcpJ*	leader peptidase TcpJ	2.84
VC_0840	*acfB*	accessory colonization factor AcfB	3.30
VC_0841	*acfC*	accessory colonization factor AcfC	3.49
VC_0842		hypothetical protein	3.85
VC_0843	*tagE*	tagE protein	3.83
VC_0844	*acfA*	accessory colonization factor AcfA	2.53
VC_0845	*acfD*	hypothetical protein	3.36
VC_1457	*ctxA*	cholera enterotoxin, A subunit	3.03
VC_1456	*ctxB*	cholera enterotoxin, B subunit	2.69

[a] Not up-regulated with the addition of bicarbonate.

Table 4. Selected Genes Up-regulated by Bicarbonate

Gene ID	Gene Symbol	Gene Product	log2 Fold Change +HCO3/-HCO3
Lipid A modification			
VC_1577	*almG*	Aminoacyl lipid modification protein G	4.17
VC_1578	*almF*	Aminoacyl lipid modification protein F	4.35
VC_1579	*almE*	Aminoacyl lipid modification protein E	4.16
Transport and Binding Proteins			
VC_1426	*potC*	Spermidine/putrescine ABC transporter membrane protein	2.23
VC_1427	*potB*	Spermidine/putrescine ABC transporter membrane protein	2.39
VC_A1062	*potE*	Putrescine transporter	3.49
VC_1043	*fadL-2*	Long-chain fatty acid transport protein	2.76
Cell Envelope Proteins			
VC_1565		Outer membrane protein TolC homolog	2.17
VC_1318	*ompV*	Outer membrane protein V	4.37
Purines/ Pyrimidine Biosynthesis			
VC_0869	*purL*	Phosphoribosylformylglycinamidine	4.03
VC_0051	*purK*	Phosphoribosylaminioimidazole, ATPase subunit	2.93
VC_1190	*purC*	Phosphoribosylaminioimidazole	2.79
VC_2389	*carB*	Carbamoyl phosphate synthase large subunit	2.56
VC_1004	*purF*	Amidophosphoribosyltransferase	2.34
VC_2390	*carA*	Carbamoyl phosphate synthase small subunit	2.13
Regulatory Proteins			
VC_1319	*carS*	Calcium-regulated Sensor	1.99
VC_1320	*carR*	Calcium-regulated Regulator	2.41
Chemotaxis/Motility			
VC_0514		Methyl-accepting chemotaxis protein	2.39

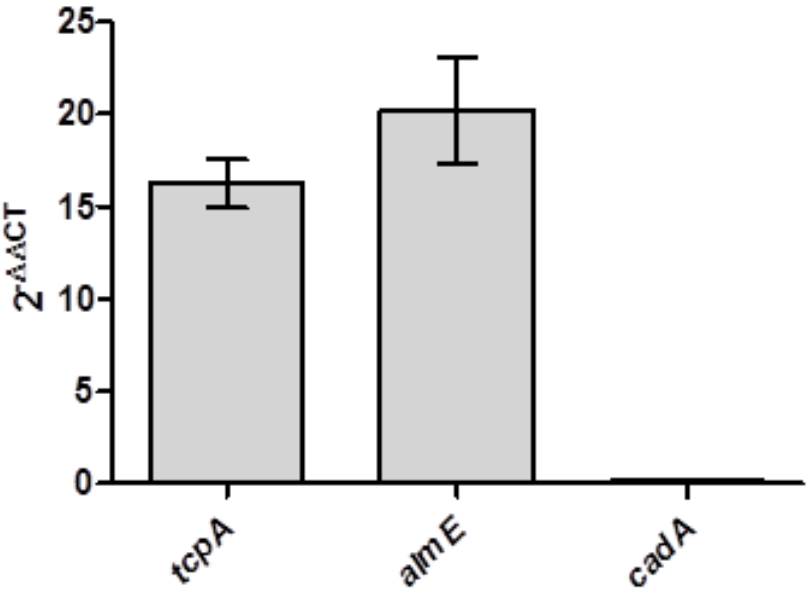

Figure 24. Quantitative real-time PCR (qRT-PCR) confirming results of the RNA-Seq. *tcpA* and *almE* are up regulated in response to bicarbonate, while *cadA* is down-regulated. Fold change gene expression is expressed on the y-axis and was determined using the $2^{-\Delta\Delta CT}$ equation.

Table 5. Genes Down-regulated by Bicarbonate

Gene ID	Gene Symbol	Gene Product	log2 Fold Change +HCO3/-HCO3
Chemotaxis			
VC_1248		methyl-accepting chemotaxis protein	-2.67
VC_A0008		methyl-accepting chemotaxis protein	-2.06
VC_A0923		methyl-accepting chemotaxis protein	-2.16
VC_A1093	*cheW*	purine-binding chemotaxis protein CheW	-2.07
VC_A1094	*cheW*	purine-binding chemotaxis protein CheW	-2.26
VC_A1096	*cheY*	chemotaxis protein CheY	-2.50
Fatty Acid Degradation			
VC_2231	*fadE*	acyl-CoA dehydrogenase	-2.70
VC_2758	*fadB*	multifunctional fatty acid oxidation complex subunit alpha	-2.98
VC_2759	*fadA*	3-ketoacyl-CoA thiolase	-2.71
Other			
VC_0280	*cadB*	lysine/cadaverine antiporter	-4.20
VC_0281	*cadA*	lysine decarboxylase	-3.01
VC_0711	*clpb-1*	clpB protein	-2.16

CONCLUSIONS

Vibrio cholerae causes the diarrheal disease cholera as a result of the production of many virulence factors but, in particular, cholera toxin (CT) and the toxin co-regulated pilus (TCP). The production of these virulence factors is directly controlled by the transcription regulator ToxT. Production of ToxT is dependent on a complex regulatory cascade including the transcription regulators ToxR and TcpP. Activity of many of the transcription regulators in the cascade can be post-transcriptionally controlled by environmental signals. Recent work has shown that ToxT can also be both negatively and positively controlled by intestinal signals including bile and components of bile, as well as bicarbonate. These effectors are normal constituents of the human intestine and potentially help to guide transcription of virulence factors, so the bacteria are able to occupy the most favorable niche for infection. Much work has been done to characterize the mechanisms by which bile and the unsaturated fatty acid (UFA) components of bile affect ToxT activity. The results of the work described in these studies have elucidated some of the mechanisms of bicarbonate-mediated enhancement of ToxT activity.

Bicarbonate enters the bacterial cell where it can interact with ToxT to increase transcription of virulence genes. Further work is required to identify the mechanism of transport of bicarbonate into the cell. Determining this mechanism could elucidate a potential therapeutic target to limit disease in the host. Evidence is accumulating that bicarbonate is an important signal that *V. cholerae* uses to initiate disease in the host and blocking the pathway of bicarbonate transport could provide a species-specific therapy.

Bicarbonate has been shown to increase production of CT and TCP in a ToxT-dependent manner. The work described in this dissertation has shown that the effect of bicarbonate occurs at every ToxT-dependent promoter, each of which directs transcription of virulence genes

important for colonization and full virulence of *V. cholerae.* Bicarbonate enhances the activity of ToxT by increasing its binding affinity for the promoters of the genes it activates. The binding site for bicarbonate within ToxT remains unclear; however, we have found mutations that affect the response of ToxT to both bicarbonate and its negative counterpart, UFAs. The ToxT N106F mutant showed increased transcriptional activity compared to WT ToxT, and this increase in activity was unable to be negatively modulated by the UFA linoleic acid. Additionally, transcription by ToxT N106F was unable to be increased further by the addition of bicarbonate. Therefore, we conclude that the conformation of ToxT N106F resembles the conformation of a maximally active ToxT having bound bicarbonate. The bulky amino acid side chain of phenylalanine at the 106 position is similar in size to bicarbonate and may mimic the conformation of ToxT in its bound state with bicarbonate.

Proteolysis of ToxT is another post-transcriptional mechanism that controls the ability of ToxT to activate virulence genes. Once ToxT is produced through the concerted efforts of ToxR and TcpP, it autoregulates through a long transcript beginning at the *tcpA* promoter. ToxT proteolysis provides the mechanism to shut down this autoregulatory loop. We have identified residues that are essential for recognition by the ToxT protease. Through mutation of the unstructured region in the N-terminal domain of ToxT, we have developed a model for ToxT activity and proteolysis. In this model. the addition of UFAs blocks proteolysis, while the addition of the positive effector bicarbonate results in normal proteolysis. We hypothesize in this model that two conformations of ToxT exist in nature, an active conformation and an inactive conformation. With no effector molecules, ToxT fluctuates between the two conformations, resulting in a low to moderate level of transcriptional activity. The positive effector bicarbonate, when present, locks ToxT in an active conformation and results in maximal transcriptional

activity. In this active conformation, the protease is able to cleave ToxT due to an exposed unstructured region. The negative effectors, UFAs, lock ToxT in an inactive conformation with a buried unstructured region, resulting in significantly reduced proteolysis. The two conformations of ToxT and the ability of the protease to cleave them based on availability of access to the unstructured region could play a role the recognition of environmental signals by *V. cholerae.*

Furthermore, we have shown that bicarbonate is not only a signal for increased transcription of ToxT-dependent genes. The addition of bicarbonate to culture media increases transcription of genes that are associated with host colonization and survival within the host intestine. The mechanism of transcription activation of ToxT-independent genes, and/or possible ToxT involvement at some level in indirectly regulating expression of these genes, requires further work. Chromatin immunoprecipitation combined with deep sequencing has ostensibly identified all DNA binding sites for ToxT on the *V. cholerae* chromosomes. However, with our finding that bicarbonate increases the binding affinity of ToxT, it is possible that other toxboxes that require a higher binding affinity may exist. Additionally, bicarbonate acts as a signal to down-regulate genes that are not essential in colonization of the human intestine and could possibly be a detriment to survival of the bacterium. The finding that bicarbonate could be acting through other signaling pathways outside of the regulatory cascade in *V. cholerae* is novel and furthers the correlation and importance of this molecule with the pathogenesis of the bacterium.

FUTURE DIRECTIONS

The work presented in the dissertation advances the understanding of the impact that bicarbonate has on virulence gene transcription in *V. cholerae.* Much of the work was performed to elucidate the mechanism of bicarbonate-mediated enhancement of ToxT's ability to initiate

transcription of virulence genes. We have determined that bicarbonate enhances ToxT activity within the bacterial cytoplasm, as well as in close proximity with ToxT in *in vitro* assays. However, we were unable to show an actual binding event of bicarbonate to ToxT and the subsequent conformational change that we hypothesized based on our work in this dissertation. Further work is needed to show that there is an actual interaction between ToxT and bicarbonate. This can be achieved using nuclear magnetic resonance (NMR). The result of an interaction with bicarbonate should result in two different spectra in these experiments. Determination of an interaction between bicarbonate and ToxT could lead to potential drug development to antagonize the effect of bicarbonate binding and limit cholera disease progression.

Additionally, we have proposed that ToxT is able to exist in two different conformations: active and inactive. This was deduced from monitoring ToxT proteolysis with the addition of effectors. Additional work needs to be done to confirm the different conformations of ToxT and determine if there is a difference between the conformation of bound linoleic acid compared to bound virstatin. This could be achieved by crystallizing Toxt with each of the effector molecules.

Finally, in this dissertation we showed that bicarbonate enters the cell cytoplasm where it could potentially interact with ToxT. However, we are unclear as to the mechanism of import of bicarbonate. Import could be carried out through the action of bicarbonate transporters. Additionally, carbonic anhydrases could convert bicarbonate to carbon dioxide which could enter the cell. Identifying the mechanism of transport into the cell could be beneficial for the development of therapeutics that is specific for aiding to limit cholera disease by blocking bicarbonate import.

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ABSTRACT

MECHANISMS FOR BICARBONATE-MEDIATED VIRULENCE IN *VIBRIO CHOLERAE*

by

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August 2014

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Major: Immunology and Microbiology

Degree: Doctor of Philosophy

Vibrio cholerae is the etiologic agent of the severe diarrheal disease cholera. The aquatic bacterium is ingested by humans through contaminated water or food. Disease initiation depends on the production of the major virulence factors: cholera toxin (CT) and the toxin co-regulated pilus (TCP). The bacterium responds to signals in the human host that activate a virulence regulatory cascade termed the "ToxR Regulon". The ToxR regulon consists of various transcription regulators whose activity culminates in the production of the major virulence regulator, ToxT. ToxT directly activates transcription of CT and TCP, as well as many other gene products involved in disease. ToxT is a 276 amino acid AraC/XylS family member that binds to 13 base pair degenerate sequences, called toxboxes, to initiate transcription. Post-transcriptional modulation of ToxT activity occurs via negative and positive effector molecules present in the human upper small intestine where *V. cholerae* colonizes. Negative effectors of ToxT activity include bile and the unsaturated fatty acid (UFA) components of bile. Conversely, the positive effector, bicarbonate, enhances ToxT activity. These positive and negative effectors provide *in vivo* signals to the bacterium to initiate transcription of virulence genes in the

appropriate location. This dissertation is focused on identifying the molecular mechanism of positive modulation of ToxT activity by bicarbonate. Additionally, the role of bicarbonate in controlling transcription of ToxT-independent genes associated with colonization and host survival is explored.

Chapter one elucidates the mechanism for bicarbonate-mediated enhancement of ToxT activity. The ToxT-dependent promoters contain toxboxes that exist in various orientations, including direct and inverted repeat configurations. Additionally, the promoter of the ToxT-dependent gene *aldA*, contains only a single toxbox. We have shown that bicarbonate enters the *V. cholerae* cell where it can interact with ToxT in the cytoplasm. Furthermore, we have shown that bicarbonate can enhance the activity of ToxT regardless of toxbox orientation. This is achieved through an increase in ToxT binding affinity for the promoters it activates.

Chapter two characterizes an unstructured region in the N-terminal domain of ToxT that controls the response of ToxT to effectors, as well as mediating proteolysis. Through mutagenesis of this region we have discovered a ToxT mutant that has a decreased response to both the negative and positive effectors of ToxT activity. The highly active ToxT N106F mutant can no longer be activated by bicarbonate. Similarly, activity of the mutant is unresponsive to negative modulation of activity by bile and UFAs. In addition to its importance in responding to effector molecules, this region of ToxT is essential for normal proteolysis. Many mutations in this region alter proteolysis, with the ToxT M103A mutant completely abolishing proteolysis.

Chapter three discusses alterations in the transcriptome of *V. cholerae* with the addition of external bicarbonate. RNA-Seq was performed to assess these changes. Bicarbonate mediated the up-regulation of all the known ToxT-dependent genes, as well as other genes important for host survival and colonization. Conversely, bicarbonate caused down-regulation of genes that are

unnecessary or detrimental for host colonization and survival. The findings in this chapter signify the importance of the host molecule bicarbonate to initiate essential changes in the *V. cholerae* transcriptome.

AUTOBIOGRAPHICAL STATEMENT

My career in research began during my undergraduate studies at the University of Detroit Mercy in the lab of Dr. Gregory Grabowski exploring the effects of heavy metals on the development of *Xenopus laevis.* I graduated from the University of Detroit Mercy with a B.S. degree in Biology in 2009, receiving the Biology Award for highest overall GPA. Upon completion of my undergraduate degree, I was accepted into the Ph.D. program at Wayne State University School of Medicine in the Department of Immunology and Microbiology. I joined the lab of Dr. Jeffrey Withey in June of 2010. My dissertation work consisted of the determination of the molecular mechanisms of bicarbonate-mediated enhancement of ToxT activity. During my time in the Withey lab I have prepared three manuscripts for submission. I will be joining the lab of Dr. Eric Krukonis at the University of Detroit Mercy Dental School in August 2014 as a post-doctoral fellow.