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The Mechanism For Inhibition Of Vibrio Cholerae Virulence Gene Expression By Bile And Its Fatty Acid Components

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THE MECHANISM FOR INHIBITION OF *VIBRIO CHOLERAE* VIRULENCE GENE EXPRESSION BY BILE AND ITS FATTY ACID COMPONENTS

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General Introduction

Cholera Disease

Vibrio cholerae is a gram-negative, curved rod-shaped bacterium that causes the severe diarrheal illness cholera. When a host ingests large quantities of fecal-contaminated water or raw shellfish colonized with *V. cholerae*, disease can occur within a short incubation period from as little as two hours to up to five days. Cholera is characterized by severe dehydration and shock caused by voluminous watery diarrhea, colloquially known as “rice-water stool” as its appearance is similar to water left over from washing rice. Other symptoms include vomiting, leg cramps, and decreased skin turgor due to the dehydration (1, 2). Treatment includes oral rehydration solution (ORS) as specified by the World Health Organization (WHO) to contain strict amounts of various salts including sodium chloride, trisodium citrate dihydrate, and potassium chloride, as well as glucose (3). For severe patients, additional IV fluids containing sodium and glucose are administered to relieve dehydration. As cholera patients can lose up to one liter of water per hour in the early stages of the disease, ORS not only hydrates the patient, but also insures that cholera patients have their sodium levels replenished in the small intestine (4, 5). In addition, antibiotics also may be used conjointly to slightly shorten the disease symptom duration and reduce the period of time that there is bacterial shedding into the environment.

Approximately 75% of people show no symptoms when infected with *V. cholerae*; however, these carriers can still shed bacteria for 7-14 days, albeit at lower amounts than a person showing symptoms. In addition, carriers can still infect other people (6, 7). For the children or adults who develop symptoms, approximately 80% experience mild or moderate

symptoms, with the remaining 20% experiencing severe symptoms that include massive dehydration and diarrhea. Without treatment, survival is approximately 50% and death can occur within a few hours after the onset of diarrhea; however, with treatment survival is approximately 99% (8).

Global Impact of Cholera

Although scholars disagree on when the first instances of cholera occurred, there is historical evidence of a disease that resembles cholera dating back thousands of years in the Indian subcontinent. The origins of the word cholera likely come from the Greek words “chole,” meaning bile, and “rein,” meaning to flow, first used in Hippocrates writing around 400 BC. Written accounts of cholera also appear in Sanskrit around 500 BC (9). The first recorded pandemic of cholera occurred in 1817, as traders visited India and carried the disease with them, and since then a total of seven pandemics have occurred, with the most recent, seventh pandemic still ongoing (10). As the world became more industrialized, cholera likely spread following trade and immigration routes, and first reached the United States in 1832 (11). It was initially thought cholera spread through inhalation of a foul-smelling pollution known as miasma, but this myth was dispelled during the third pandemic that occurred in the 1850s (9). John Snow, a practicing physician in London, showed in 1854 that the source of a large and very deadly outbreak of cholera was a town water pump in the Soho region of London (12). Once the pump was decommissioned, the epidemic ended in this region of London (12). Around this same time, Filippo Pacini isolated “comma-like” shaped bacteria from the intestinal lumens of deceased cholera patients and described these as both infectious and the causative agent of

the disease (13). However, his publication was written in Italian and was not widely disseminated. It was not until 1884, one year after Pacini's death, that Robert Koch rediscovered *V. cholerae* from patients and publicized the information, therefore getting credit for many years as the initial discoverer of *V. cholerae* (13).

In regards to recent cases of cholera, the WHO reported that the incidence of cholera had increased steadily from 2005-2012, and although the number of reported cases has decreased since then, it continues to be a concern (14). This is especially true in the developing world, where access to clean water and adequate sanitation is a problem. In 2013, 129,064 cases of cholera were officially reported to WHO, resulting in 2,102 deaths, but the number of cholera cases each year is actually estimated to be between 1.4-4.3 million cases with 28,000-142,000 deaths worldwide (14, 15). This large discrepancy between reported cases and estimated cases may in part be due to underreporting because of lack of consistency in case definitions, but also because of the negative political and economic implications on travel and trade resulting from a publicized cholera epidemic.

Cholera is currently endemic in approximately 50 countries, mainly within Asia and Africa. From the mid-1990s until 2010, the continent of Africa reported the greatest number of cases, but in 2010 an earthquake hit Haiti, resulting in a massive increase in cholera cases in the Americas (14). As a result of the earthquake, there was infrastructure breakdown and, within a month, a cholera outbreak occurred, spreading throughout the entire country and eventually to the Dominican Republic and Cuba. This ongoing outbreak has led to over 700,000 cases and over 8,000 deaths (2).

V. cholerae is classified based on the O antigen of its lipopolysaccharide, and despite the fact there are over 200 serogroups in the environment, all seven pandemics have been attributed to one serogroup, O1 (1, 8, 16). The O1 serogroup can be further divided into two biotypes: classical and El Tor. Historically, the classification difference has been based upon different phenotypes such as hemolysis, hemagglutination, phage lysis and polymyxin B resistance, but more recently, it has been shown that the two biotypes are genotypically different as well (17, 18). The classical biotype was the causative agent of the first six pandemics, while the El Tor biotype has caused the most current pandemic (8, 16). The classical biotype also causes a more severe disease, but El Tor is thought to survive better in the environment, although the reasons for this are not understood (16, 17). One additional serogroup, O139, emerged in 1992 and is the causative agent of more recent outbreaks (18-21). O139 is genetically very similar to O1 EL Tor biotype, suggesting horizontal gene transfer of the O antigen biosynthetic genes, which in turn, caused the conversion (18, 22, 23). As such, some refer to these outbreaks as an eighth pandemic (18).

Even though cholera is a huge problem worldwide and much work has been done to make effective vaccines, there are no current vaccines that offer long-term or complete protection. Two oral cholera vaccines are available: the first is called Dukoral and is composed of killed whole cells in a mixture of the two biotypes of O1 *V. cholerae*, plus a recombinant B subunit of the cholera toxin (to be discussed later), and another called Shanchol that has both O1 and O139 serogroups. Dukoral is manufactured by SBL Vaccines in Sweden and prequalified and licensed in over 60 countries, but not the United States (24). Shanchol is manufactured by Shantha Biotec in India and in 2011 acquired WHO prequalification and was used in a 2012 pilot

study to look at feasibility, not efficacy, of mass vaccination in Haiti (25-27). Disappointingly, both of these vaccines need to be taken in multiple doses and have been shown only to be about 50-60% effective in the first year (28, 29). Injectable vaccines of whole cells are no longer used. Making an effective vaccine is currently a top priority as stated in the WHO Model List of Essential Medicines (30). The Centers for Disease Control and Prevention (CDC) currently does not recommend receiving a cholera vaccination for travelers, and, as stated, neither vaccine is available in the United States. This is due to the fact that the vaccines only offer partial protection for a short period of time.

V. cholerae Life Cycle

V. cholerae is found predominantly in the aquatic environment as normal inhabitants of freshwater, coastal and brackish waters as either free-swimming bacteria or in association with marine organisms including shellfish, copepods, chironomid egg masses, and vertebrate fish (31-37). Changes in weather that affect surface water temperature and therefore phytoplankton blooms have been correlated with cholera outbreaks (38, 39). In nature, humans are generally the only host of *V. cholerae* to get cholera disease, but a very high infectious dose of around 10^6-10^{11} bacteria is necessary to infect healthy individuals (17). In the environment, both motility and chemotaxis genes are expressed, which are also important at early time points after human ingestion. Expression of these genes allows the bacteria to pass through the host's defenses such as the low pH of the stomach, bile from the duodenum, and the innate immune system (40, 41). Using its single polar flagellum, *V. cholerae* is able to traverse the stomach and enter the small intestine, where it is then able to pass into the protective mucus

layer of the intestine and colonize the surface of the epithelium in the intestinal crypts and villi surfaces (42-44). At this point, the bacteria downregulate motility genes and become immotile, while genes involved in virulence are upregulated (40, 41). Colonization occurs for approximately 12-72 hours before symptoms occur (6). Once a person begins to shed the bacteria through either vomit or diarrhea, termed the "mucosal escape response", *V. cholerae* is in a hyperinfectious state where the infectious dose can be as low as 10-100 bacteria needed to infect a new host (45-48). Gene expression is now reversed, such that there is downregulation of genes involved in virulence and upregulation of genes required for motility, as well as upregulated protease expression that allows detachment from the epithelium (49). The hyperinfectious state lasts for at least five hours, and seems to be an effective way that the bacteria can pass from person-to-person, particularly in heavily crowded areas such as a household, potentially causing an epidemic due to the rapid rate of infection (46, 47). Bacteria shed from humans have been shown to be in two forms: a more infectious biofilm-like form with small aggregates of bacteria and a planktonic form (47, 48, 50).

When not in a human host, *V. cholerae* naturally lives in an aquatic environment. In aquatic environments, the bacteria live as either free-swimming planktonic cells or attached to various types of surfaces that can be living or non-living (31, 36, 37, 51-53). As part of the free-swimming state, *V. cholerae* can persist in a condition traditionally known as viable but non-culturable (54). In this state, the bacteria cannot be cultured on standard media but are still able to perform some metabolic processes such as protein synthesis and maintain membrane integrity (55). This state is thought to occur when the bacteria are not associated with chitin, and therefore are nutrient-deprived (55). The term "viable" has been questioned, as it is

unclear if the bacteria are actually alive in this state as they cannot be cultured, therefore a more conservative term of “active but non-culturable” (ABNC) has also been used (6). These bacteria can regain the ability to multiply when put into a human once again, or are grown in the presence of eukaryotic cells, and can be cultured after such exposures (56, 57).

When not free-swimming or ABNC, *V. cholerae* can associate with marine organisms in a biofilm either on the organism, or on chitin (such as the shell of crustaceans) where chitin can then be used as a carbon and nitrogen source (58-60). In general, a biofilm is a micro-community of bacteria that is attached to some surface and is composed of a matrix of DNA, proteins, and polysaccharides that holds the micro-community together. In *V. cholerae*, biofilm formation is mediated by the *Vibrio* polysaccharide (VPS) regulon, which encodes extracellular polysaccharide and leads to increased resistance to chlorine (61-63). Bacteria found in association with a biofilm, as opposed to free-swimming bacteria, display hyperinfectivity and it has been postulated that this form is the most likely source of human infections (64-66). Potentially, this could be at least in part because bacteria associated with a biofilm have decreased acid susceptibility, which in turn allows the bacteria to better survive throughout the stomach (67).

V. cholerae **Pathogenesis Genes**

In order to initiate disease, and potentially cause a pandemic, production of two virulence factors is necessary: cholera toxin (CT) and toxin co-regulated pilus (TCP). These are acquired from the mobile genetic elements cholera toxin bacteriophage (CTXϕ) and the *Vibrio* pathogenicity island (VPI), respectively. CTXϕ is a filamentous bacteriophage with a 6.9 kb

single-stranded DNA genome and is found in all pandemic strain environmental isolates, but rarely found in non-O1/O139 environmental strains (16). CTXϕ is separately inserted in each of the two *V. cholerae* chromosomes in the classical strain, but is found tandemly arranged on the larger chromosome of El Tor (68-71). Uptake of this prophage is dependent on TCP as its receptor to enter *V. cholerae* cells and to convert nontoxigenic strains into toxigenic ones (72). As TCP is expressed in the intestine, this lysogenic conversion occurs inside the host (72). In addition to encoding the genes for CT, CTXϕ also carries genes that encode the zona occludens toxin (Zot) and accessory enterotoxin (Ace); however, the role in pathogenesis of these additional toxins remains unclear (71, 73-75). VPI is 40 kb long, and, besides containing the genes required for TCP production, also encodes a number of other accessory virulence factors. Like the accessory toxins encoded by CTXϕ , most of these accessory factors have poorly defined roles in pathogenesis (76, 77).

CT, the cause of the voluminous diarrhea in cholera, was first discovered to act as an enterotoxin by S.N. De in 1959, but it was not isolated until 1969 by Finkelstein and LoSpalluto (77-79). De discovered that cell-free supernatants for the classical biotype could cause fluid accumulation in rabbit ligated ileal loops in a similar manner to when *V. cholerae* cultures were injected in the loops (78). Since that initial discovery, much research has been conducted on CT. CT is an 84 kDa protein, part of the large family of bipartite A-B toxins consisting of five smaller subunits, collectively called the B subunit, responsible for binding the toxin to its target and one larger subunit, the A subunit, responsible for the toxin action (77, 80-82). The 6 subunit holotoxin is assembled in the periplasm, and then secreted by a type II secretion system (83). The B subunit pentamer binds to the receptor of cholera toxin, ganglioside GM_1, found

associated in lipid rafts on the intestinal epithelial cell surfaces (81, 84-86). This bound complex is endocytosed and then continues through retrograde transport to the endoplasmic reticulum (ER) (87-89). Once in the ER, the toxin is able to use the ER-associated protein degradation pathway to release the A1 subunit into the cytosol of the host (90, 91). Inside the host cell, the A subunit dissociates from the B subunit, and it is then cleaved by host proteases into two fragments: A1 and A2. The A1 subunit contains the active site, while the disulfide bond-attached A2 subunit functions as a tether to the B subunit pentamer (92). The A1 subunit then ADP-ribosylates the $G_{s\alpha}$ subunit of adenylate cyclase causing adenylate cyclase to be locked in its GTP-bound active state (93). This in turn causes an increased concentration of cAMP, leading to a rapid secretion of chloride ions from crypt cells and therefore decreased sodium uptake from the surrounding villus cells (94, 95). This electrolyte imbalance causes an osmotic gradient which in turn causes massive secretion of water from the cells into the intestinal lumen (96, 97). This fluid accumulation in the small intestine is the source of the profuse watery diarrhea associated with the disease.

TCP is the other major virulence factor produced by *V. cholerae* that is essential to cause cholera disease (76, 98). TCP is a type IV bundle-forming pilus that is expressed under the same conditions as CT, thus leading to the term “toxin co-regulated” (99-101). The exact role of TCP in colonization is not yet defined, as it has not been shown to act as an adhesin for cell-bacteria interactions, but it has been shown to mediate microcolony formation on the surface of epithelial cells and therefore its required role may be for inter-bacterial interactions (99). Assembled TCP bundles are between 5-7nm in diameter and are composed of polymers of the first protein encoded by the *tcp* operon, TcpA (99). The 12 gene *tcp* operon is located on the VPI

(16). In *V. cholerae* strains in which *tcpA* have been deleted, the bacteria are no longer able to colonize the small intestine, as shown in healthy human volunteer studies (98). TCP is also involved in the secretion of the colonization factor TcpF, but is not clear how this required factor is used for colonization (102). It is known that *tcpJ* is involved in the processing of TcpA during secretion by encoding a leader peptidase (103). Most of the *tcp* operon is either involved in or part of the actual pilus.

Other genes important for host colonization that are located on the VPI have also been identified, including *acfABCD, aldA, tagA,* and *tcpI*, but their roles are largely unknown (1, 104-110). The *acf* genes make up the accessory colonization factor (ACF) and are required for enhanced intestinal colonization in mice, but the functions of the proteins are still largely unknown (108). AcfB and TcpI share homology with methyl-accepting chemotaxis proteins, and when mutated together cause decreased colonization in mice (106, 111, 112). Decreased colonization does not occur when each gene is mutated individually, suggesting redundancy in the chemotactic properties of these proteins (106, 111, 112). Two small, non-coding RNAs encoded by *tarA* and *tarB* are also located within the VPI (113, 114). TarA regulates *ptsG*, a major glucose transporter, while TarB regulates the secreted TcpF before penetration into the intestinal epithelial layer (113, 114). When mutated, the aldehyde dehydrogenase *aldA* does not show a defect in colonization; however it is coordinately regulated along with the *tcp* operon (115). TagA is a metal-dependent mucinase that acts to modify mucin proteins by cleaving them and potentially leading to enhanced binding of *V. cholerae* to the cell surface (116). More work is needed to elucidate the complete role of these and other VPI genes in disease.

Motility and Chemotaxis

Motility and chemotaxis have been extensively studied in regard to their roles in pathogenesis. However, their roles in disease are highly debated as different strains, biotypes, mutations and animal models can show different, and sometimes opposite results (117-124). As mentioned previously, *V. cholerae* is a highly motile bacterium that has a single, polar flagellum. The flagellum is driven by sodium motive force. When genes involved in flagellum sodium uptake are mutated, under non-virulence inducing conditions, there CT and TCP production increased. This suggests a mechanism in which sodium concentration affects virulence gene expression (lower sodium levels lead to increased virulence gene expression), which would be an indirect effect of the flagellar action (125). At the strain level using the classical biotype with the infant mouse model, it has been shown that non-motile strains, in which the genes involved in flagellar production have been mutated, are able colonize to similar levels as wild-type, motile strains (120). Similar experiments have been carried out with the El Tor biotype and have shown opposite results: non-motile mutants were unable to colonize the infant mouse (126-128). Even with the conflicting data, the current hypothesis is that motility is necessary in order to penetrate the mucus layer of the small intestine, which, in turn, gives *V. cholerae* access to the epithelial cells (40).

Chemotaxis has also been shown to be important for colonization and disease. In the most general terms, chemotaxis is the movement of an organism either towards or away from a chemical stimulus. There are three chemotaxis operons in the *V. cholerae* genome, but only one that is essential for chemotaxis (129, 130). Like motility, the importance of chemotaxis in *V. cholerae* is controversial. In one study, various genes of the El Tor biotype involved in

chemotaxis were deleted and colonization of the infant mouse intestine was determined. Non-chemotactic mutants colonized the intestine 70-fold better than WT (127). However, the bacteria were found throughout the intestine, as opposed to only the lower half of the small intestine corresponding the lower jejunum and ileum, suggesting *V. cholerae* uses chemotaxis as a means to find a preferred site for colonization (40).

ToxR Regulon

Transcription of the complex network of virulence genes from both the CTXϕ and VPI is tightly coordinated by a regulatory cascade termed the "ToxR Regulon" (108). Although the virulence regulon is still often referred to as the ToxR regulon for historical reasons, ToxR is not the transcriptional regulator of CT and TCP expression, but was simply the first regulator identified (108, 131). Instead, the coordinated expression of virulence genes is directly regulated by the transcriptional activator ToxT, and multiple regulatory proteins and factors affect the cascade to ensure that ToxT is only expressed under optimal environmental conditions (104, 132-134). ToxR and another transcriptional regulator, TcpP, control transcription of *toxT* (135-137). *toxR* is found in all *Vibrio* species as part of the ancestral genome and is constitutively expressed, while *tcpP* is located on the VPI, and thus found only in pandemic strains and expressed only under virulence inducing conditions (138-140).

ToxR is an integral membrane regulatory protein homologous with the OmpR family of proteins that contain a cytoplasmic winged helix-turn-helix DNA-binding domain (141, 142). ToxS, another integral membrane protein, acts to increase dimerization of ToxR, which is needed for full ToxR activity in order to then initiate *toxT* transcription (143, 144). At the *toxT*

promoter, it is thought that ToxR acts as an enhancer of TcpP binding as evidence suggests that although TcpP can interact directly with the RNA polymerase, ToxR is necessary to activate *toxT* transcription (145). ToxR/S bind to the *toxT* promoter between -100 and -69 with respect to the transcriptional start site as shown by DNase I footprinting in this region (137). ToxR also regulates transcription of the outer membrane porins OmpU and OmpT. ToxR upregulates expression of *ompU*, which encodes a porin that is protective against the negative effects of bile (146-148). *ompU* transcription by ToxR is also increased in the presence of bile (149). In contrast, ToxR downregulates expression of *ompT*. OmpT is expressed in the environment and under nutrient limited conditions (150).

TcpP interacts with another membrane protein, TcpH, to regulate transcription of *toxT* in combination with ToxR/S (135). TcpP is also a member of the OmpR family of proteins, with a homologous cytoplasmic domain (142). TcpH is required for the stability of TcpP, as in the absence of it, TcpP is rapidly degraded by the metalloprotease YaeL (151, 152). In conditions not permissive for virulence, degradation of TcpP occurs even when TcpH is present (151). YaeL targets the periplasmic domain of TcpP and is also present in conditions not permissive for virulence (151). Taurocholate, a bile salt found in the intestine, has been shown to induce dimerization of TcpP leading to increased activation potential of *toxT* (153). TcpP/H binds to the *toxT* promoter between -51 and -32 relative to the transcriptional start site, and TcpP alone can activate expression of *toxT* when over-expressed by relying on its DNA-binding specificity to locate the promoter (137, 154).

As mentioned previously, *tcpPH* is not expressed constitutively. Instead, expression of these genes is dependent on the transcription regulators AphA and AphB (155, 156). AphA

binds to the *tcpPH* promoter between -101 and -71, while AphB binds between -78 and -43 (157). AphA is a dimer that is a member of the winged helix transcription factor superfamily (158), while AphB is a LysR-type regulator protein (155). AphB interacts directly with AphA on the DNA to activate *tcpPH* and changes conformation in response to pH and oxygen level changes (159-161). *tcpPH* is also negatively regulated by cAMP receptor protein (CRP) and PepA (131, 157, 162, 163). CRP, normally important in metabolism, can bind to the promoter and repress expression by competing at the same binding sites as AphA and AphB (157). The mechanism of PepA repression is still unclear; however it is known that PepA responds to changes in pH and temperature (162). Under virulence non-permissive conditions, PepA represses transcription of *tcpPH* (162).

Signaling Systems

Quorum sensing is a system used to allow communication between bacteria in order to change gene expression in response to cell density through the production of small molecules called autoinducers (164). These autoinducers are constitutively produced so that at low cell density quorum sensing is turned off due to low concentration of the autoinducers and at high cell density bacteria sense the presence of autoinducers and alter their gene expression patterns. *V. cholerae* uses the system to influence virulence gene expression by downregulating CT and TCP at high cell density (64, 157, 165, 166). There are three quorum sensing systems in *V. cholerae* that control HapR, which in turn, represses transcription of *aphA* (165, 166). Two systems are similar, each employing an autoinducer and a sensor kinase. The first system uses the autoinducer CAI-1 and sensor kinase CqsS, and the second system uses the autoinducer AI-2

and the sensor kinase LuxQ, with its periplasmic partner LuxP (167). Under low cell density the kinases phosphorylate LuxU, which in turn transfers the phosphate group to LuxO, and then this initiates transcription of four small RNAs called *qrr1-4* (168, 169). These sRNAs bind to the 5'UTR of HapR mRNA along with Hfq in order to initiate its degradation (170, 171). Under this condition, there is no HapR and therefore *aphA* is expressed and virulence gene expression can occur. Conversely, at high cell density, the autoinducers bind to their respective sensor kinases, which then act as phosphatases to remove the phosphates from LuxU and LuxO. LuxO now can no longer activate transcription of *qrr1-4* and HapR can then made. With HapR produced, *aphA* is now repressed and virulence gene expression is downregulated (165). The third quorum sensing system uses VarS/VarA proteins in order to activate LuxO (170). VarS/VarA is a two-component signaling system that activates CsrB, CsrC, and CsrD, while deactivating CsrA. When CsrA is inactivated, LuxO can become phosphorylated and act as it does in the other two systems (170). Even though the direct correlation between quorum sensing and virulence gene expression seems straightforward, there are some epidemic El Tor strains that have a mutated *hapR* that leaves it non-functional, suggesting that quorum sensing is not important for causing human disease (64). Therefore, more research is needed to fully determine the role that quorum sensing plays in virulence gene regulation. HapR also controls other genes involved in virulence expression in addition to *aphA* such as HapA and VPS operon (172). HapR increases expression HapA, a hemagglutin/protease involved in mucosal escape in El Tor strains, while in turn, represses the VPS operon, which is involved in biofilm formation. At low cell density, biofilms are activated as HapR is not present (173).

In addition to quorum sensing, *V. cholerae* is also able to use the second messenger 3',5'-cyclic diguanylate (c-di-GMP) as part of a signaling system to change gene expression (174). With this system, c-di-GMP levels are modulated in response to some first messenger and these different c-di-GMP levels directly correspond to changes on the cell surface (175). C-di-GMP production is controlled by diguanylate cyclase, and increased levels of c-di-GMP lead to increased expression of the biofilm VPS genes, extracellular protein secretion system and mannose sensitive hemagglutinin type IV pilus biogenesis genes (176, 177). Increased levels of c-di-GMP also correlate to decreased expression of the *fla* genes involved in the production of flagella (176, 177). The levels of c-di-GMP are controlled directly by the VieSAB system (178). VieA is a phosphodiesterase that acts to decrease the levels of intracellular c-di-GMP. Decreased c-di-GMP expression correlates with maximal CT production (174).

The Virulence Gene Transcription Activator ToxT

ToxT is a 32 kDa, 276 amino acid protein that acts as the main virulence gene regulator for *ctxAB* and *tcp* (104, 132-134). It is a member of the AraC/XylS family of proteins, sharing homology in the C-terminal domain (CTD) (179-181). The CTD is about 100 amino acids and contains two helix-turn-helix DNA-binding motifs (179, 181). The N-terminal domain (NTD) spans amino acids 1-160 and the CTD consists of amino acids 170-276 with a small linker region of ten amino acids between the two domains (182). The NTD does not share sequence similarity to other AraC/XylS family members, but the ToxT crystal structure does show some secondary structural homology to AraC despite having very little amino acid identity (182).

ToxT not only activates transcription of the genes encoding CT and TCP, but also activates transcription of other virulence genes including *acfA, acfD, aldA, tagA, tcpI,* and the RNAs *tarA* and *tarB* (109, 110, 113, 114, 183). To activate these genes, ToxT binds to 13 base pair degenerate sequences termed toxboxes, each containing a poly-T tract at the 5' end of the binding site. Toxboxes are located upstream of the -35 promoter element that is recognized as part of the RNA polymerase binding site (183). This toxbox location suggests that ToxT must directly interact with the RNA polymerase α subunits to stimulate transcription (184). Depending on the particular virulence gene in question, the toxboxes can have different arrangements. At the promoters of *tcpA, ctxAB, and tarA* there are two toxboxes arranged as direct repeats (114, 183). For *acfA, acfD, tagA,* and *tcpI* there are two toxboxes arranged as inverted repeats, with the divergently transcribed *acfA* and *acfD* sharing the same toxboxes (110, 183, 185). In contrast, *aldA* only contains one toxbox (109). ToxT can also activate its own transcription through its role in activating transcription of the *tcp* operon, within which the *toxT* gene is located (133, 134). The ability of ToxT to regulate its own expression is thought to allow for finer control of virulence gene expression.

Members of the AraC/XylS family include some proteins that act as dimers, including AraC, RhaS, and RegA and some member that act only as monomers, including MarA, SoxS, and Rob (185-188). The ability of ToxT to act as either a monomer or a dimer at the promoters it activates has been quite controversial. As most ToxT-activated promoters contain two toxboxes, it is hypothesized that ToxT acts as a homodimer, but, *aldA* only has one toxbox suggesting that ToxT does not require dimerization in order to activate the virulence gene promoters (109). The NTD of ToxT has been shown to be involved in dimerization through

studies using the LexA dimerization assay as well as bacterial two-hybrid systems (182, 189-192). Interestingly, full length, intact ToxT has never been observed to dimerize and the ToxT crystal structure is of a monomeric protein. Virstatin, a small molecule inhibitor of virulence gene expression, has been proposed to act by decreasing ToxT dimerization (192, 193). Other studies, however, have shown that ToxT can bind to promoters as a monomer, such as at the *tcpA* promoter (110, 183). In this experiment, the spacing between the toxboxes was increased and using DNA-footprinting it was shown that ToxT was still able to protect the DNA (110, 183). However, when the spacing was greatly altered, ToxT was not able to activate transcription indicating that the distance between the sites is important for full activation. Even though there has been extensive work on the dimerization ability of ToxT, more work still needs to be done to understand its role in ToxT function.

Effectors of ToxT

There are both positive and negative effectors that affect the ability of ToxT to activate transcription. The histone-like protein H-NS is a global repressor protein of transcription and is found in many gram-negative bacteria, including *V. cholerae* (194). H-NS is able to oligomerize and occupy multiple binding sites near promoters such as *ctxAB, toxT and tcpA*, where it acts to repress transcription (195, 196). H-NS has also been shown to compete with ToxT at the *ctxAB* promoter, suggesting a mechanism by which H-NS must be displaced by ToxT in order to de-repress transcription of the virulence gene it is acting upon (197).

To induce virulence *in vitro*, different growth conditions are used for the two different *V. cholerae* biotypes. For the classical biotype, 30°C, Luria-broth (LB) at pH 6.5, and aeration are

sufficient for maximal activation of *ctxAB* and *tcp* (198). In contrast, for the El Tor biotype, a biphasic culture condition termed AKI conditions is used for maximal production of CT and TCP. Under AKI conditions, the culture is grown statically at 37°C in AKI peptone medium for several hours and then switched to aeration conditions for an additional two hours (199-201). Although the two conditions differ, they each allow for maximal ToxT production and therefore maximal CT and TCP production. Another growth condition that was found to result in CT production in both biotypes was in rich medium containing fresh sodium bicarbonate statically at 37°C (202). This will be described more below.

While the laboratory growth conditions described above are artificial and do not resemble conditions that would be present in the human small intestine, effectors of ToxT activity can be found in the environment that *V. cholerae* inhabits. Using *in vitro* conditions has allowed the elucidation of some of the chemical signals necessary for virulence induction, but as the small intestine is at 37°C and under static conditions, it is obvious that there must be certain *in vivo* signals that allow for virulence production to occur. These include temperature, pH, and various host chemicals such as bicarbonate and bile (160, 202-205). The chemical bicarbonate has been shown to be a positive effector of ToxT activation of CT and TCP (202). Bicarbonate is found in the same place where *V. cholerae* colonizes to act as a protective buffer in the small intestine and is secreted by the intestinal epithelial cells (206). Although it does not increase the production of ToxT, bicarbonate has been shown to increase the ability of ToxT to activate virulence gene transcription by enhancing DNA binding (202, 207).

Bile, conversely, is a negative regulator of ToxT activity (203-205). Secreted by the gall bladder, bile contains many different components, including bile salts, saturated and

unsaturated fatty acids, cholesterol, proteins, and phospholipids that normally act together to emulsify fats in the lumen of the small intestine and act as a bactericide. When added *in vitro* to *V. cholerae,* bile is shown to cause decreased production of TCP and CT, and increased motility, biofilm formation, and outer membrane protein quantities (203-205). In one study, bile was fractionated to determine the components responsible for decreased virulence activation by ToxT (204). From this information, it was shown that only the unsaturated fatty acid (UFA) components are responsible for decreased virulence (204). Of the UFA components, oleic, linoleic, and arachidonic acids are the most abundant UFA components of bile (204). Linoleic acid, the UFA primarily used in the studies described in this dissertation, is an 18-carbon fatty acid that contains two cis-double bonds. Upon resolution of the crystal structure of ToxT, *cis*-palmitoleic acid, another UFA, was found buried in the NTD (182). In one study, *cis*-palmitoleic acid was only ~0.5% of the total fatty acid content in bile, as compared to oleic acid, which accounted for ~23.5%, linoleic acid accounted for ~14%, and arachidonic acid accounted for approximately ~3% of the total UFA content in bile (204). However, this fractionation was done with ox bile. A similar study in 1987 fractionated the fatty acid components in 24 healthy, adult human volunteers and found slightly different amounts: ~2.68% for palmitoleic acid, ~12.09% for oleic acid, ~32.83% for linoleic acid and ~5.64% for arachidonic acid with the rest composed of saturated fatty acids (208). In this study, linoleic acid composed the largest proportion of UFA, as opposed to the previous study in which oleic acid accounted for the largest proportion.

The aim of this work is to better understand how *V. cholerae* responds to a change from environmental to host conditions. This includes work aimed at identifying the mechanism by which linoleic acid decreases virulence gene expression by acting through ToxT. Conjugated

linoleic acid is currently on the market as a weight loss supplement proposed to inhibit fat absorption and this is also studied as it could potentially be used as either a prophylactic or as a therapy to reduce CT production and thus disease duration and intensity. In current era in which antibiotic resistance is becoming a severe problem, therapeutics that inhibit pathogenesis but do not affect bacterial survival are likely to become much more attractive as the bacteria are unlikely to develop resistance against them.

A list of all strains used is included in Table 1. Together, this dissertation enhances both our basic understanding of virulence gene regulation and host signals during cholera and translates this newly acquired knowledge into a potential cholera therapeutic that is safe for human consumption and is inexpensive.

Strain	Plasmid	Description	Parent Strain	Source	Chapter
JW 467	pMAL-c2e-ToxT	*Escherichia coli* BL21(DE3)		New England Biolabs	1-4
JW 1560	pJW 407	MBP-ToxT for protein purification	JW 467	Lab Collection	1-4
JW 9		*Vibrio cholerae* classical strain O395		Lab Collection	1-4
JW 150		*Δtoxt*	JW 9	Lab Collection	1
VJ740		Δ*toxt*-helix-turn-helix	O395	(132)	1
JW 18	pJW 54	P*tcpA::lacZ* in pTL61t	JW 9	Lab Collection	1
JW 441	pJW 211	P*ctxAB::lacZ* in pTL61t	JW 9	Lab Collection	1
JW 87	pJW 82	P*aldA::lacZ* in pTL61t	JW 9	Lab Collection	1
JW 97	pJW 89	P*tagA::lacZ* in pTL61t	JW 9	Lab Collection	1
JW 86	pJW 81	P*acfA::lacZ* in pTL61t	JW 9	Lab Collection	1
JW 89	pJW 84	P*acFD::lacZ* in pTL61t	JW 9	Lab Collection	1
JW 169	pJW 91	P*tcpI::lacZ* in pTL61t	JW 9	Lab Collection	1
JW 666	pJW 308	P*toxT::lacZ* in pTL61t	VJ740	Lab Collection	1
JW 329	pJW 179	P*tcpP::lacZ* in pTL61t	JW 9	Lab Collection	1
JW 515	pJW 54	P*tcpA::lacZ* in pTL61t	JW 150	Lab Collection	1
JW 1809	pJW 211	P*ctxAB::lacZ* in pTL61t	JW 150	Lab Collection	1
JW 167	pJW 82	P*aldA::lacZ* in pTL61t	JW 150	Lab Collection	1
JW 168	pJW 89	P*tagA::lacZ* in pTL61t	JW 150	Lab Collection	1
JW 165	pJW 81	P*acfA::lacZ* in pTL61t	JW 150	Lab Collection	1
JW 166	pJW 84	P*acFD::lacZ* in pTL61t	JW 150	Lab Collection	1
JW 169	pJW 91	P*tcpI::lacZ* in pTL61t	JW 150	Lab Collection	1
CRP4	pCRPhis	CRPhis in pBAD33	K2294	Lab Collection	1
JW 714		O395*tcpA::lacZ Δtoxt*	JW 150	Lab Collection	2
JW 1066	pJW 407	MBP-ToxT	JW 714	Lab Collection	2
JW 1414	pJW 501	MBP-ToxT (G100A)	JW 714	Lab Collection	2

Table 1. List of strains used in this work.

Strain	Plasmid	Description	Parent Strain	Source	Chapter
JW 1415	pJW 502	MBP-ToxT (D101A)	JW 714	Lab Collection	2
JW 1416	pJW 503	MBP-ToxT (L102A)	JW 714	Lab Collection	2
JW 1417	pJW 504	MBP-ToxT (M103A)	JW 714	Lab Collection	2
JW 1418	pJW 505	MBP-ToxT (I104A)	JW 714	Lab Collection	2
JW 1419	pJW 506	MBP-ToxT (R105A)	JW 714	Lab Collection	2
JW 1420	pJW 507	MBP-ToxT (N106A)	JW 714	Lab Collection	2
JW 1421	pJW 508	MBP-ToxT (L107A)	JW 714	Lab Collection	2
JW 1422	pJW 509	MBP-ToxT (Y108A)	JW 714	Lab Collection	2
JW 1423	pJW 510	MBP-ToxT (S109A)	JW 714	Lab Collection	2
JW 1424	pJW 511	MBP-ToxT (E110A)	JW 714	Lab Collection	2
JW 1469	pJW 520	MBP-ToxT (L107F)	JW 714	Lab Collection	2
JW 1470	pJW 521	MBP-ToxT (R105K)	JW 714	Lab Collection	2
JW 1471	pJW 522	MBP-ToxT (R105Q)	JW 714	Lab Collection	2
JW 1472	pJW 523	MBP-ToxT (L107S)	JW 714	Lab Collection	2
JW 1474	pJW 525	MBP-ToxT (N106S)	JW 714	Lab Collection	2
JW 1475	pJW 526	MBP-ToxT (N106F)	JW 714	Lab Collection	2
JW 1556	pJW 536	MBP-ToxT (L114A)	JW 714	Lab Collection	2
JW 1582	pJW 538	MBP-ToxT (R105A, N106A)	JW 714	Lab Collection	2
JW 690		O395tcpA::lacZ	JW 9	Lab Collection	3
JW 75		*E. coli* SM10 λpir		Lab Collection	4
JW 751	pJW316	P*tcpA::gfp*	JW 690	Lab Collection	4
JW 422	pFD1	*mariner* transposon	JW 75	Lab Collection	4
JW 947		Δ*pckA*	JW 690	Lab Collection	4
JW 939		Δ*pepcase*	JW 690	Lab Collection	4
JW 940		Δ*icd*	JW 690	Lab Collection	4
JW 1125	*pckA*	*pckA* in pBAD33 in Δ*pckA*	JW 947	Lab Collection	4

Table 1 cont. List of strains used in this work.

CHAPTER ONE

The mechanism for inhibition of ToxT activity by the unsaturated fatty acid components of bile in *Vibrio cholerae*

ABSTRACT

The gram-negative curved bacillus *Vibrio cholerae* causes the severe diarrheal illness cholera. During host infection, a complex regulatory cascade results in production of ToxT, a DNA-binding protein that activates the transcription of major virulence genes that encode cholera toxin (CT) and toxin-coregulated pilus (TCP). Previous studies have shown that bile and its unsaturated fatty acid (UFA) components reduce virulence gene expression. Bile is likely an important chemical recognized by *V. cholerae* upon entering the host. However, the mechanism for the bile-mediated reduction of TCP and CT expression has not been defined. There are two likely hypotheses for this effect: 1) UFAs decrease DNA binding by ToxT or 2) UFAs decrease dimerization of ToxT. To determine whether bile/UFAs affect DNA binding by ToxT, we first determined that UFAs, specifically linoleic acid, can enter *V. cholerae* when added exogenously. We then performed electrophoretic mobility shift assays with ToxT on various virulence promoters in the presence or absence of UFAs. The results indicate that UFAs can enter the bacteria, where they can then interact with ToxT in the cytoplasm to decrease ToxT binding to DNA even at a promoter having only one ToxT binding site. This suggests a mechanism in which UFAs do not affect dimerization but can affect monomeric ToxT binding to DNA.

INTRODUCTION

V. cholerae, a gram-negative curved bacillus possessing a single polar flagellum, is the causative agent of cholera. Cholera is a diarrheal disease contracted by consuming contaminated food or water and is characterized by severe diarrhea that leads to dehydration, and can ultimately cause death if left untreated. Each year, there are an estimated 1.4-4.3 million cholera cases and 20,000-142,000 deaths from the disease (14, 15). In order to cause disease, *V. cholerae* must colonize the duodenum, where it is able to express its virulence genes, including the most important of these: toxin co-regulated pilus (TCP) and cholerae toxin (CT). TCP is required for intestinal colonization, while CT is responsible for the massive secretion of electrolytes and water into the lumen, causing diarrhea. These virulence genes and others are regulated by the major virulence transcription activator ToxT.

Virulence gene expression is regulated by what is historically known as the ToxR regulon (108, 131). *toxT* transcription is activated by two sets of inner membrane proteins, ToxR/ToxS and TcpP/TcpH, that bind upstream of *toxT*, as well as by a ToxT positive feedback loop (133-137). ToxT is a 32-kDA member of the AraC/XylS transcriptional regulator family, having a conserved 100-amino-acid DNA binding domain, consisting of two helix-turn-helix motifs, in its C-terminal domain (CTD). Located upstream of all ToxT-activated genes are 13-bp degenerate DNA sequences called toxboxes that vary in configuration at individual genes (110, 114, 183-185). ToxT directly controls transcription of genes encoding not only TCP and CT, but also other accessory virulence genes including *acfA, acfD, aldA, tagA*, *tarA, tarB,* and *tcpI* (109, 110, 113, 114, 183). Except for the *aldA* promoter, in which there is a single toxbox, there are two toxboxes present at ToxT-activated promoters (109, 110, 114, 183, 185). ToxT can bind to single

toxboxes as a monomer but it is thought that full activation only occurs upon ToxT dimerization on the DNA, at least at some genes (110, 183). The ToxT N-terminal domain (NTD), does not share significant sequence homology with other proteins, but has some structural similarity to the AraC NTD, which is the domain necessary for AraC dimerization and arabinose binding (182). NTDs of ToxT have been shown to interact when separated from the CTD, although the true role of dimerization in ToxT function is not fully understood (109, 182, 189-193).

A number of host and environmental factors affect ToxT activity, including temperature, pH, bile and bicarbonate (160, 202-205). Bile is produced by the liver and then subsequently stored in the gallbladder. Upon eating, bile is released into the duodenum where it acts to solubilize lipids. Bile itself is a complex, heterogeneous mixture that includes bile salts, cholesterol, bilirubin, and saturated and unsaturated fatty acids (UFAs). *V. cholerae* encounters bile early during infection and it is proposed to be a natural effector of ToxT as it is found in the same places that *V. cholerae* colonizes (203-205). *V. cholerae* has reduced virulence gene expression in the presence of bile and/or UFAs, with increased motility gene expression, biofilm formation, the induction of efflux pumps, and increased amounts of outer membrane proteins OmpU, OmpT and TolC (146, 203, 204, 209-211). Bile and its UFA components have been previously shown to decrease CT and TCP production, but the mechanism of this effect on virulence gene expression levels is not understood (203, 204). Bile also has another role, in that it is able to act as a bactericide for the benefit of the host, but enteric bacteria, such as *V. cholerae*, are not only adapted to live in the presence of bile, but potentially can recognize bile as a signal to ensure survival in the host (203, 205). Another negative regulator of ToxT is virstatin, which is a small, synthetic molecule that has been previously shown to decrease ToxT

activity (192, 193). The mechanism for this has been proposed to be by inhibiting ToxT dimerization (192). Whether UFAs and virstatin inhibit ToxT activity via the same mechanism is unknown.

In this study we show that UFAs, specifically the unsaturated fatty acid linoleic acid, are able to enter the bacterial cell where ToxT is also present in the cytoplasm. We report that linoleic acid and virstatin cause decreased transcription of ToxT-controlled virulence genes and determined that the mechanism by which this happens is decreased binding affinity at ToxT-activated promoters, regardless of the number or configuration of the toxboxes. Our results indicate that UFAs decrease ToxT binding to DNA even at promoters having one ToxT binding site, suggesting a mechanism in which UFAs do not only affect the ability of ToxT to dimerize, but also affect the ability of monomeric ToxT to bind to DNA. These results give us a clearer understanding of the regulatory networks controlling virulence gene expression during human infection.

MATERIALS AND METHODS

***V. cholerae* strains and growth conditions.** All *V. cholerae* strains used in the study are derived from classical biotype O395. Strains were maintained in Luria Broth (LB) containing 20% glycerol and stored at -70°C. All promoter::*lacZ* fusions used for β-galactosidase assays were made in plasmid pTL61T as described in previous studies (202, 207). Overnight cultures were grown for ~16 hours at 37°C in LB and then diluted 1:40 into LB pH 6.5 at 30°C for virulence inducing conditions in the presence or absence of freshly prepared .05% bile (sodium choleate), 160μM linoleic acid, or 100μM virstatin. *V. cholerae* strains were grown with

antibiotic concentration of 100µg/mL streptomycin and strains with the plasmid pTL61T were grown with 100µg/mL ampicillin.

Western blot analysis of ToxT. Immunodetection of ToxT was performed using the same procedure as previously described (202). Briefly, bacteria were harvested after inducing conditions either DMSO only, 160µM linoleic acid, or with 100µM virstatin. Cells were normalized at an optical density of 600nM and resuspended in 2x protein buffer. Samples were then boiled, separated by 14% SDS-PAGE, blotted, and blocked for 30 minutes in TBS buffer. After blocking, the blots were incubated overnight in a 1:2,500 dilution of rabbit polyclonal anti-ToxT serum, washed three times in TBS buffer, and secondary goat anti-rabbit IgG conjugated to alkaline phosphatase (AP) was used at a dilution of 1:5000 (Southern Biotech). Blots were washed again and then developed using immune-BCIP (MP Biomedicals).

^{14}C linoleic acid uptake and fractionation. *V. cholerae* classical biotype strain O395 was grown overnight in LB at 37°C, subcultured 1:40 in LB pH 6.5, and grown for two hours in the absence of linoleic acid. At two hours, 0.1µCi ^{14}C-radiolabeled linoleic acid (58.2mCi/mmol) (Perkin-Elmer) was added for each milliliter of the subculture. Upon addition of the radiolabeled linoleic acid, 1 mL of the culture was immediately centrifuged, and the supernatant saved to compare to cell pellet cpms. The cell pellet was washed 3 times with 1 mL of PBS and centrifuged each time. Cell pellets were resuspended in 100µL PBS and added to 5mL Scintillation cocktail (Fisher Scientific). The same procedure was followed for other aliquots of the subculture at times 5, 15 and 30 minutes. After uptake, cpm was measured for each time point using an LS6000IC liquid scintillation counting system (Beckman).

^{14}C linoleic acid fractionation assay. After overnight growth, *V. cholerae* O395 classical biotype was subcultured 1:40 in the absence of linoleic acid. After 2 hours, 0.1µCi ^{14}C-radiolabeled linoleic acid (58.2mCi/mmol) (Perkin-Elmer) was added to one milliliter of the subculture and incubated at room temperature. After one hour, the bacteria were harvested by centrifugation and washed three times in PBS. Bacteria were then resuspended in 20mM Tris-HCl, pH 8.5 and 500 mM NaCl) and freeze-thawed in an ethanol and dry ice bath, then thawed at 37°C. This process was repeated for a total of three freeze-thaw cycles. The bacteria were then fractionated by centrifugation for 10 min at 15,000 x g to separate the periplasm/membrane and cytoplasm. A Western Blot was performed to ensure efficient fractionation (data not shown). The cytoplasm was taken as the supernatant and the pellet was resuspended in PBS. Each fraction was then measured for cpm of each fraction using a LS6000IC liquid scintillation counting system (Beckman).

β-galactosidase assays. Strains to be analyzed were grown overnight in LB at 37°C and then subsequently subcultured 1:40 into LB pH 6.5 in the presence or absence of 160µM linoleic acid dissolved in DMSO. Cultures were grown under virulence inducing conditions (aeration at 30°C) for 3 hours and then analyzed. The β-galactosidase assay was then performed following the same established protocol (212).

qRT-PCR. As in previous assays, after overnight growth, *V. cholerae* classical biotype O395 was subcultured 1:40, either with or without 160µM linoleic acid. 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) and confirmed free of DNA by the absence of

bands using logarithmic PCR. To measure the relative mRNA levels of *tcpA* and *aldA* the following primers were used: forward *tcpA* (5'-ACGCAAATGCTGCTACACAG-3') reverse *tcpA* (5'-CCCCTACGCTTGTAACCAAA-3'), and forward *aldA* (5'-TTGGTGGGCATCCTAACAAT-3') reverse *aldA* (5'-ACACCGGCACCTAAACCATA-3'). qRT-PCR was performed using one-step SYBR green Mastermix (Invitrogen) and the following 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* (5'-ACCTGAAGGTCCAAACATCG-3') and reverse *rpoB* (5'-CAAAACCGCCTTCTTCTGTC-3'). Relative levels of transcript with the addition of linoleic were calculated using $2^{-\Delta\Delta CT}$ and analyzed comparing the ΔΔCT values as previously described (213).

Protein Purification. Maltose binding protein-ToxT fusion (MBP-ToxT) and MBP-CRP purification was performed as previously described using *E. coli* strain BL21(DE3) with the plasmid pMAL-c2E carrying either MBP-ToxT or MBP-CRP (207, 214). Briefly, after MBP-ToxT or MBP-CRP induction by IPTG, cells were lysed by French press, run over an amylose column, and fractions containing MBP-ToxT or MBP-CRP were saved and dialyzed into 50mM Na_2HPO_4 (pH 8.0), and 100mM NaCl and then dialyzed into the same solution containing 20% glycerol to save as freezer stock at -80° C.

Electrophoretic mobility shift assays (EMSA) and binding curve analysis. EMSAs were performed as previously described (214). Purified MBP-ToxT, or MBP-CRP with cAMP (New England Biolabs), was incubated with DNA probes made by PCR from the promoter sequence of interest using one primer radiolabeled with γ-^{32}P (Perkin-Elmer) by T4 polynucleotide kinase (New England Biolabs). Binding reactions contained various amounts of MBP-ToxT with

constant 10 µg/mL salmon sperm DNA as nonspecific competitor, 10mM Tris-acetate (pH 7.4), 1mM Potassium EDTA (pH 7.0), 100mM KCl, 1mM dithiothreitol (DTT), 0.3mg/mL bovine serum albumin (BSA) and 10% glycerol in a volume of 30µL. To each reaction, a constant concentration of the labeled DNA probe was added. In reactions containing linoleic acid, the final concentration was 32µM for each reaction, and for reactions containing virstatin, the final concentration was 50µM, except at the *aldA* promoter where a higher concentration of 100µM was used. All other reactions contained 3.33% (1µL in 30µL) DMSO as a solvent control. Binding reactions were incubated for 30 minutes at 37°C and then loaded into a 6% polyacrylamide gel at 4°C. Gels were dried for 1 hour and then analyzed by autoradiography.

Binding curve analysis. Autoradiographs were analyzed using ImageJ software (NIH) as previously described with nonspecific binding omitted from further analysis (207). Briefly, to determine the K_d for samples containing either linoleic acid or virstatin and compared to protein bound to DNA without inhibitor, the percent of protein bound with labeled DNA was determined for each lane. This was then fit to the equation $\%Bound=B_{max}*[Protein]^h/(K_d^h + [Protein]^h)$ with B_{max} constraint set to 100 using Graphpad Prism 5 software. The K_d values for each condition were compared to each other using the extra sum of squares F test to determine if the two values were statistically different.

RESULTS

ToxT protein is produced in the presence of linoleic acid. Previous work has demonstrated that UFAs inhibit virulence gene expression by acting through ToxT (204). Oleic acid has been previously shown to negatively regulate TCP and CT expression levels (182). In preliminary experiments, we determined that linoleic acid had the strongest negative effect on

ToxT activity of several different UFAs tested (data not shown), and previous studies have shown that linoleic acid makes up the largest proportion of unsaturated fatty acid content in human bile (208). To date, direct interaction of linoleic acid and ToxT has not been observed, although a palmitoleic acid molecule was visualized within the ToxT NTD in the solved crystal structure (182). To begin our study, we investigated whether ToxT protein production was affected by the addition of linoleic acid in the media. It had been previously determined that there are comparable levels of *toxT* expression in cultures grown with and without linoleic acid (204). To confirm this, we analyzed the β-galactosidase production from a *toxT*::*lacZ* reporter plasmid in *V. cholerae* grown in the presence or absence of linoleic acid (Figure 1A). When *V. cholerae* was grown under virulence-inducing conditions (LB pH=6.5, aeration) in the presence of linoleic acid, the amount of β-galactosidase activity indicated that there was no statistically significant difference (P=.799) in *toxT* expression whether the culture had linoleic acid or lacked it. This is consistent with previous data confirming that linoleic acid does not affect *toxT* transcription.

To further examine the effect of linoleic acid on ToxT, and confirm that there were no effects of linoleic acid on translation, we carried out Western blot analysis to assess the ToxT protein levels (Figure 1B). *V. cholerae* was grown under virulence-inducing conditions in the presence or absence of linoleic acid and cell extracts were harvested. ToxT-specific polyclonal antibodies were used to detect protein levels. No differences in ToxT levels were observed regardless of the presence or absence of linoleic acid, as a ToxT-specific band was visible in Western blots at the same intensity under both conditions (Figure 1B, lanes 4 and 5). This band was not detected in the Δ*toxT* control strain (Figure 1B, lane 3). Thus, ToxT was stably

produced regardless of the presence of linoleic acid while bacteria were grown under virulence-inducing conditions.

Linoleic acid enters the *V. cholerae* cytoplasm. As ToxT protein levels were roughly equivalent with or without linoleic acid, we next assessed whether a direct interaction between linoleic acid and ToxT would be possible. Such an interaction would require that linoleic acid be imported into the bacterial cytosol. To determine whether this occurs, we performed an uptake assay using radiolabeled linoleic acid (Figure 2). *V. cholerae* was subcultured under virulence-inducing conditions for two hours and then ^{14}C-labeled linoleic acid was added to the culture. Aliquots of the culture were taken at times 0, 5, 15, and 30 minutes after addition of radiolabeled linoleic acid and radioactivity was quantified by a scintillation counter. The amount of radioactivity found in the cell pellet was compared to the supernatant. Results from this assay show that linoleic acid enters the bacteria at a linear rate with a best-fit line equation of $y = 3421.3x + 30974$ and an R^2-value of .969.

These data, however, do not indicate whether linoleic acid is present inside the bacterium or simply associated with the cell surface or outer membrane. To determine whether linoleic acid enters the cytosol, ^{14}C-labeled linoleic acid was added to *V. cholerae* cultures grown under virulence-inducing conditions and then the bacteria were fractionated into membrane and cytoplasmic portions. This allowed us to see where in the bacterium linoleic acid was localized: the membrane, the cytoplasm, or both. Cultures were incubated for one hour with the radiolabeled linoleic acid. These cultures were then pelleted and washed and the bacteria were separated into a cytoplasm and envelope fraction by multiple freeze-thaw cycles. The ^{14}C-linoleic acid in each fraction was then quantified using a scintillation counter. Results of

this experiment show that most of the linoleic acid is able to enter the cell (Figure 3). Directly comparing the amounts of linoleic acid in the two fractions showed that about 65% of the total ^{14}C-linoleic acid was present in the cytoplasmic fraction, while 35% was present in the membrane fraction. These data suggest that a significant fraction of extracellular linoleic acid can enter the cytosol, where it could then interact with ToxT.

Linoleic acid decreases ToxT activation of virulence gene transcription. *tcpA* and *ctxAB* expression have been previously shown to be inhibited by the addition of oleic acid to the media, but the effects of linoleic acid were not assessed in those studies (182). We assessed whether the inhibitory effect of linoleic acid that we observed extended to a variety of ToxT-activated promoters with various ToxT binding site (toxbox) configurations, including *tcpA, ctxAB, acfA, aldA, acfD, tagA,* and *tcpI*. All of these promoters contain two toxboxes in either direct (*tcpA, ctxAB*) (183) or inverted repeat configurations (*acfA, acfD, tagA, tcpI*) (110, 183, 185) except for *aldA*, which contains only one toxbox (109). Plasmid-borne promoter-*lacZ* fusions were added to the wild-type *V. cholerae* classical strain as well as an otherwise isogenic Δ*toxT* strain to measure transcriptional activity at these promoters with and without 160µM linoleic acid (Figure 4). At each of these promoters, linoleic acid had a statistically significant negative effect that prevented ToxT from fully activating transcription at the promoters in wild-type O395. No effect of linoleic was observed in the Δ*toxT* strain, confirming that linoleic acid acts through ToxT (Figure 4).

As the *aldA* promoter activity even in the absence of linoleic acid was much lower than at the other promoters, we performed quantitative real-time PCR (qRT-PCR) to compare expression levels of this promoter to expression levels of the *tcpA* promoter upon the addition

of linoleic acid. Bacteria were cultured using the same methods as the β-galactosidase assay and then the RNA was extracted. qPCR was then performed using primers for *tcpA*, and *aldA*, while using *rpoB* as the housekeeping gene control. The qPCR results showed that both *tcpA* and *aldA* were downregulated upon the addition of linoleic acid to the media (Figure 5). *tcpA* mRNA levels were lower when grown with linoleic acid, showing a mean fold decrease of 58.7 and *aldA* levels decrease by a fold change of 28.4.

There is decreased ToxT-DNA binding affinity with the addition of linoleic acid. To determine if linoleic acid is able to directly affect ToxT binding to DNA at the ToxT-activated promoters, we used EMSA. This method allows the binding pattern of ToxT to be characterized and give an estimation of the equilibrium dissociation constant, K_d, which represents the concentration of ToxT required for binding 50% of the DNA at equilibrium. The K_d values with and without linoleic acid will give a direct comparison of the effect of the fatty acid on the given promoter, as a higher K_d value indicates a lower binding affinity of ToxT for the DNA and a lower K_d value indicates higher binding affinity.

To begin, we used the primary promoter in the *tcp* operon, P_{tcpA}, as our ^{32}P-labeled DNA probe. Using purified MBP-ToxT, we added increasing amounts of protein to binding reactions with either DMSO alone (Figure 6A, lanes 1-7) or 32μM linoleic acid dissolved in DMSO (Figure6A, lanes 8-14) and a constant concentration of labeled DNA probe. Binding reactions were brought to equilibrium, and then run on a 6% polyacrylamide gel, followed by autoradiography. Densitometry analysis was performed using ImageJ software to calculate the percentage of bound DNA at each concentration of MBP-ToxT. A binding curve was made for each set of reactions to determine the percentage of bound DNA at each concentration of MBP-

ToxT using Graphpad software (Figure 6B). In the case of MBP-ToxT binding to P_{tcpA} DNA, the K_d without linoleic acid was 10.0nM and the K_d with linoleic acid was 33.1nM. These values are significantly different, and as the K_d with linoleic acid is greater than without, this indicates a lower ToxT binding affinity for P_{tcpA} in the presence of linoleic acid. These data suggest there is likely a direct interaction between linoleic acid and ToxT as exhibited by the decreased binding affinity.

To confirm that linoleic acid does not generally inhibit protein-DNA interactions, we used cyclic AMP receptor protein (CRP) as a control, together with the region upstream of *tcpP* as described above. We performed an EMSA comparing CRP binding with and without the addition of linoleic acid (Figure 6C) and performed densitometric analysis to determine the binding curves and K_d values. The binding curve and K_d values were statistically the same for CRP binding to P_{tcpP} with or without linoleic acid (Figure 6D).

We then wanted to determine the binding curves and K_d values at other ToxT-activated promoters, as there can be different toxbox orientations (Figure 7). As the equilibrium binding experiments on P_{tcpA} showed a decrease in DNA binding upon addition of linoleic acid, we wanted to determine whether this occurred at promoters having alternative toxbox configurations, particularly at P_{aldA}, which has only one toxbox (Figure 7.) If linoleic acid can decrease the binding affinity of ToxT for P_{aldA}, it could indicate a mechanism other than UFAs interrupting ToxT dimerization (189). The P_{ctxAB} contains two directly repeated toxboxes like P_{tcpA}, albeit in the opposite direction and having different spacing distance (214). The rest of the ToxT-activated promoters previously mentioned, P_{acfA}, P_{acfD}, P_{tagA}, and P_{tcpI} each contain

toxboxes oriented in inverted repeat configuration as well as various spacing between the toxboxes (110, 183, 185).

As linoleic acid was shown earlier to decrease promoter activity for all of these ToxT-activated promoters, we determined whether linoleic acid also affected the binding affinity using EMSA with each promoter region as described above. For P_{ctxAB}, containing direct repeats, the K_d without linoleic acid is 4.44nM, and with linoleic acid is 10.1nM, indicating decreased binding affinity of ToxT (Figures 7A,B). As an example of an inverted repeat promoter, we chose to look at P_{tagA}. Here, densitometry and binding curve analysis again revealed decreased binding affinity of ToxT for the promoter upon the addition of linoleic acid, with a K_d of 4.078nM without linoleic acid, and 27.8nM with linoleic acid (Figures 7C,D). Lastly, we examined ToxT binding to the single toxbox promoter of *aldA*. Figure 7E shows the autoradiograph of ToxT binding to P_{aldA} with and without linoleic acid. The K_d once again was increased going from 11.3nM without linoleic acid to 48.67nM with linoleic acid (Figure 7F). These data, along with the previously described decrease in promoter activity with the addition of linoleic acid as indicated by the β-galactosidase assays, suggest that linoleic acid decreases binding at each promoter regardless of toxbox configuration and spacing.

Another negative ToxT effector, virstatin, acts differently than linoleic acid. The results above suggest a direct negative effect of linoleic acid on ToxT binding to DNA. We next examined whether this was also true for another negative ToxT regulator, virstatin. Previous studies suggested that virstatin negatively affects the ability of ToxT to dimerize (192). To begin, we looked at ToxT levels using Western blot analysis to compare *V. cholerae* grown with or without virstatin (Figure 1, lanes 6 and 7). The ToxT levels were unchanged, confirming that

ToxT production is not affected by virstatin. We then performed β-galactosidase assays on the previously described ToxT-regulated genes to assess the effects of virstatin (Figure 8). These data show decreased ToxT activity at all promoters except for *aldA*, where virstatin had no significant effect. Previous work had suggested that the *aldA* promoter was affected by virstatin (192). We then performed EMSAs to determine whether virstatin affected the DNA binding ability of ToxT at *tcpA, ctxAB*, and *aldA*, as representatives of the different toxbox configurations (Figure 9). Virstatin decreased binding at *tcpA* and *ctxAB* through analysis of the binding curve and K_d, (Figures 9A-D) but did not decrease ToxT binding at the *aldA* promoter even at a higher concentration of virstatin (Figure 9E). In fact, the K_d significantly decreased from 21.83nM to 11.79nM with the addition of virstatin, indicating a higher binding affinity of ToxT at the *aldA* promoter when virstatin is present (Figure 9F).

DISCUSSION

The activation of virulence gene expression relies on the transcriptional activator, ToxT, active in mucus layer of the small intestine where *V. cholerae* colonizes. Previous studies have looked at the involvement of bile, UFAs, and virstatin on *V. cholerae* virulence gene expression and ToxT dimerization (182, 192, 193, 203-205). Most of this work with negative effectors has been done only on *tcpA* and *ctxAB*; here, in addition to these genes, we also look the effects of negative effectors on ToxT activity at *aldA, acfA, acfD, tagA,* and *tcpI*. All ToxT-controlled promoters showed a decrease in expression in the presence of linoleic acid. Prior to entry into the mucus layer of epithelial cells and colonization, *V. cholerae* must pass through the lumen of the small intestine where high concentrations of bile and UFAs are present. Linoleic acid needs to be taken up by *V. cholerae* and enter the cytoplasm in order to directly interact with ToxT.

Here, we show that linoleic acid is able to enter the cell and that the majority of it enters the cytoplasm.

Much work has been done previously on the effect of these negative effectors on virulence gene expression, but their direct effect on ToxT DNA binding affinity had not been previously assessed. It has been proposed that the mechanism of action for virstatin and UFAs is inhibition of ToxT dimerization (189, 192). The binding sites for ToxT, toxboxes, and their configurations have been extensively studied, but whether toxbox configuration factors into the inhibition of ToxT activity by linoleic acid had not been examined. Here we show that the decrease in binding affinity in response to linoleic acid can be seen at each ToxT-activated promoter that we examined, regardless of toxbox configuration. This suggests that linoleic acid has a general effect on DNA binding by ToxT monomers. Previous work strongly suggested that ToxT binds to DNA as a monomer (109, 110, 183) and that ToxT does not behave as a typical dimeric activator, such as AraC.

We also looked at virstatin and its method of action on ToxT. A previous study examined virulence gene promoter activity in the presence of virstatin; however, unlike this earlier study, the effect of virstatin in our hands was not as pronounced. We did observe that virstatin significantly decreased promoter activity to a lessened extent (Figure 8,(192)). While Shakhnovich et al. saw, at greatest, 20% promoter activity in the presence of virstatin at the *aldA, acfA,* and *tcpI* promoters, we observed a decrease, but not to that extent. Interestingly, we did not see any effect of virstatin on ToxT activation at the single toxbox, *aldA* promoter with the β-galactosidase assay (Figure 8). In this same regard, virstatin also did not have an

effect on the ability of ToxT to bind the *aldA* promoter (Figures 9E,F). In contrast, we did see a negative effect of linoleic acid at the *aldA* promoter on both ToxT-dependent virulence gene promoter activity (Figure 4) and ToxT binding affinity, where the K_d increased from 11.25nM to 48.67nM (Figures 7E,F).

These data suggest that virstatin and linoleic acid, although both negative effectors of virulence, do not have the same mechanism for downregulating gene expression. Another study performed bacterial two-hybrid assays to look at the effect of various UFAs on dimerization and the data suggest another UFA, oleic acid, does affect ToxT dimerization (204). However, linoleic acid was not tested and this study was done using only the NTD of ToxT instead of intact, full-length ToxT, whose dimerization has never been experimentally observed, at least in published reports. More testing using full-length ToxT in *V. cholerae* with this two-hybrid system in the presence of linoleic acid would confirm whether or not linoleic acid is involved in dimerization. As there was reduced binding of ToxT at the *aldA* promoter, it is likely that linoleic acid changes the structural conformation of ToxT, leading to inhibited DNA binding. This will be further explored in Chapter Two of this dissertation.

Understanding the effect of host signals on bacterial pathogenesis is useful for complete understanding of the *V. cholerae* virulence cascade, as many host signals are used by the bacteria to sense the appropriate location in which virulence gene transcription should be initiated. When passing through the small intestine, *V. cholerae* encounters many different chemical signals, such as bile and bicarbonate, which either inhibit or activate ToxT-dependent gene transcription. Bile and its UFA components are at high concentrations in the duodenal

lumen, in which ToxT is inactive (191, 204, 205, 215). Once the mucus layer is penetrated, UFA concentration decreases, while the bicarbonate concentration increases. At this point, along the epithelial surface, ToxT becomes active and *V. cholerae* can colonize. UFAs such as linoleic acid likely keep ToxT in a form unable to bind tightly to DNA in the lumen, because production of TCP would lead to bacterial aggregation and colonization of the epithelial surface would become impossible. In this model, *V. cholerae* is able to use both negative and positive effectors of virulence in order to colonize in its ideal niche. Our findings here show a direct effect of linoleic acid on ToxT, giving useful insight into the mechanisms of ToxT gene regulation.

a) b)

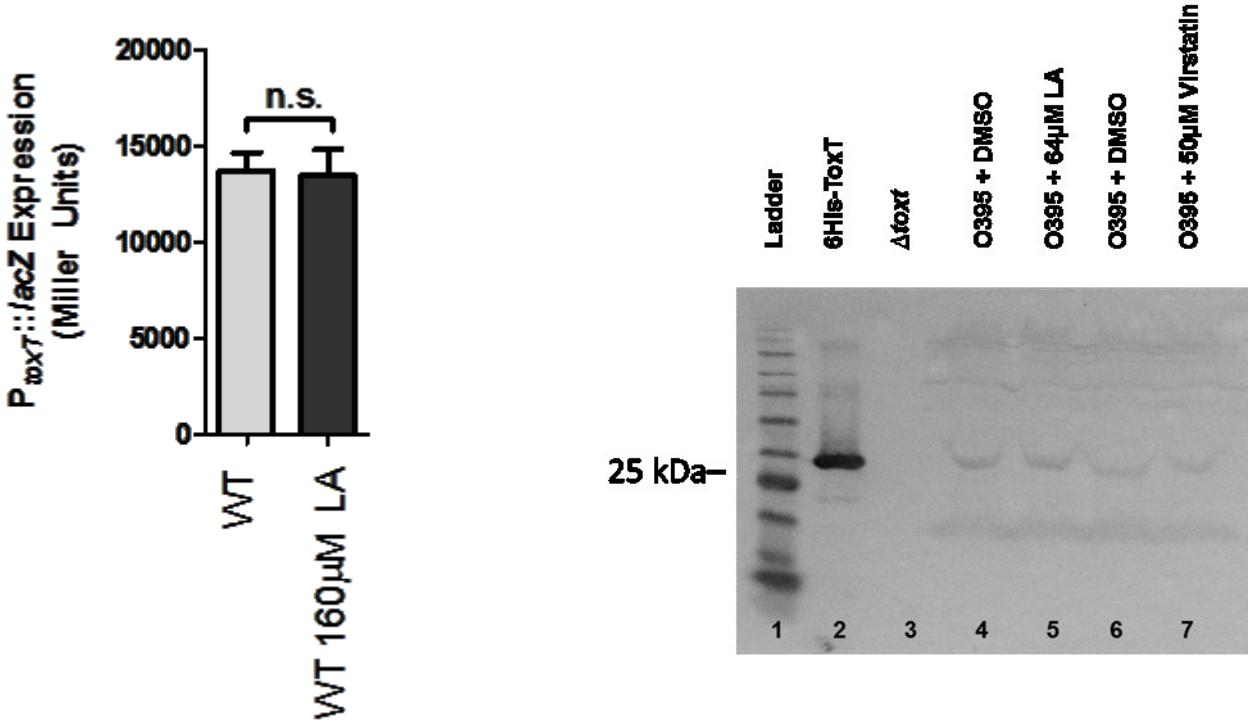


FIGURE 1. Effect of linoleic acid on P*toxT::lacZ* and ToxT expression. (A). Light gray bars, wild-type *V. cholerae* grown without linoleic acid; dark gray bars, wild-type *V. cholerae* grown with 64µM linoleic acid. Statistical significance was determined by Student's *t* test. (B). Effect of linoleic acid and virstatin on ToxT protein levels. *V. cholerae* was grown under virulence inducing conditions (LB pH 6.5, 30°C, aeration) with DMSO, linoleic acid or virstatin (lanes 4, 5, 6). Purified full length ToxT-6His migrates to ~32kDa (lane 2) and a *ΔtoxT* strain shows no band (lane 3) were used as controls. Samples were normalized by OD_{600}. The Western blot was probed with anti-ToxT antibody. This is representative of three separate experiments.

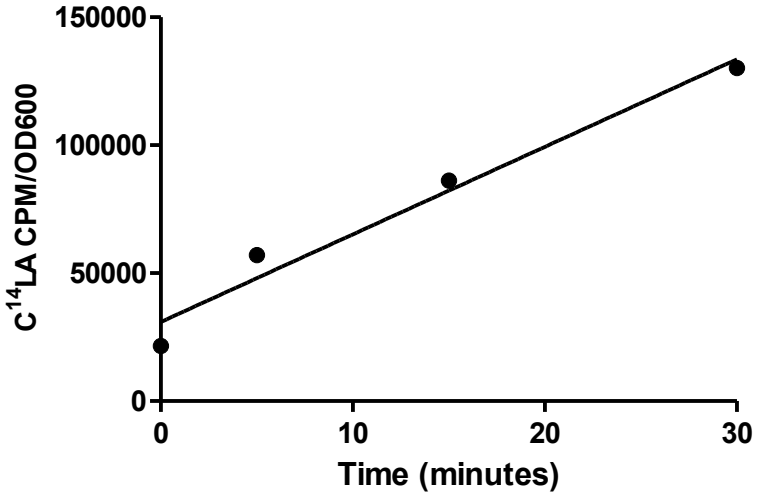


FIGURE 2. C^{14}-radiolabeled linoleic acid is taken up by *V. cholerae*. Classical strain O395 cells were grown under virulence-inducing conditions for two hours and then the radiolabeled linoleic acid was added at time 0 and analyzed at times 5, 15, and 30 minutes post-addition, measured as CPM per optical density unit at 600nm. The equation of best fit is y = 3421.3x + 30974 and an R^2-value of .969

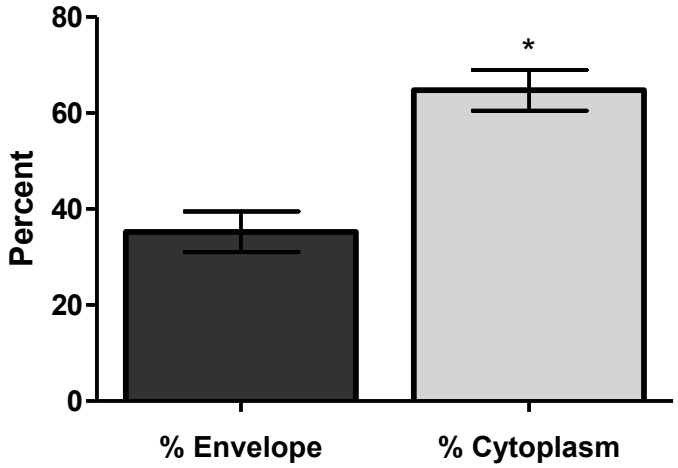


FIGURE 3. C^{14}-radiolabeled linoleic acid is able to enter the cell and enter the cytoplasm. After a 2-hour subculture in virulence-inducing conditions, linoleic acid was added to the culture and allowed to sit for one hour. After one hour, *V. cholerae* was fractionated into an envelope and cytoplasmic portion by multiple freeze-thaw cycles. There is significantly more linoleic acid in the cytoplasmic portion as compared to the envelope portion. Error bars represent +/- standard error mean and statistical significance was determined by Student's *t* test, *, $p<.05$

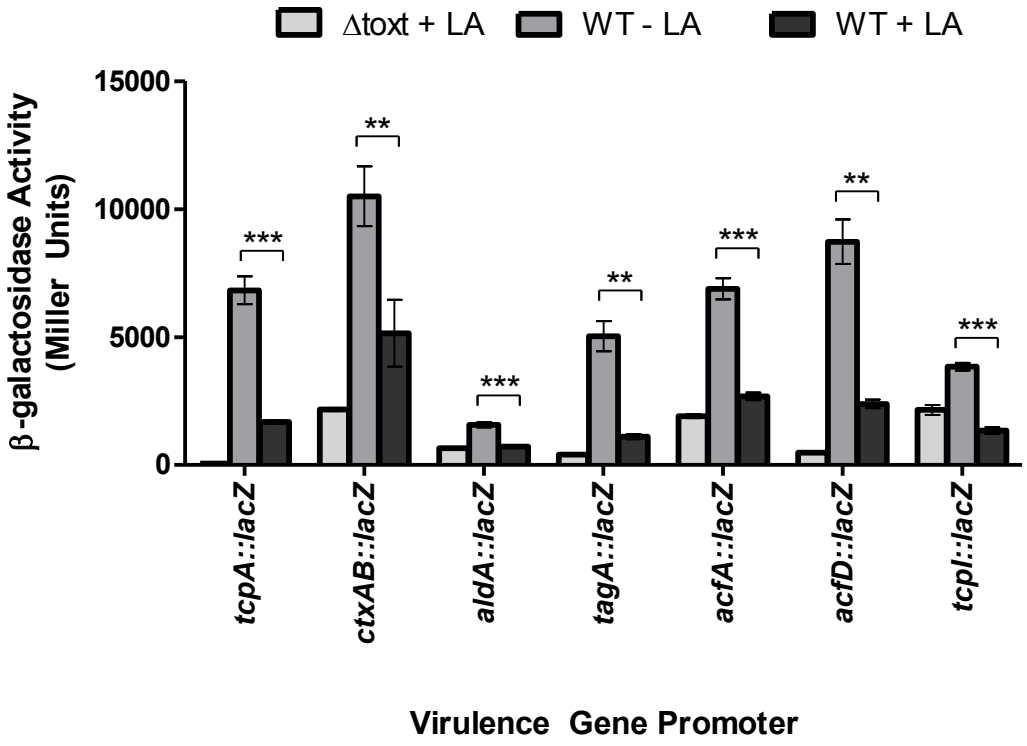


FIGURE 4. The negative effect of linoleic acid on various ToxT-activated promoters. Cultures were grown under virulence-inducing conditions with or without 160μM linoleic acid. β-galactosidase activity produced from plasmid-borne virulence gene promoter fusion constructs in either wild-type or isogenic Δ*toxT*. Statistical significance determined using Student's *t*-test (*, $p < 0.005$; **, $p<.0005$). Error bars represent +/- standard error mean.

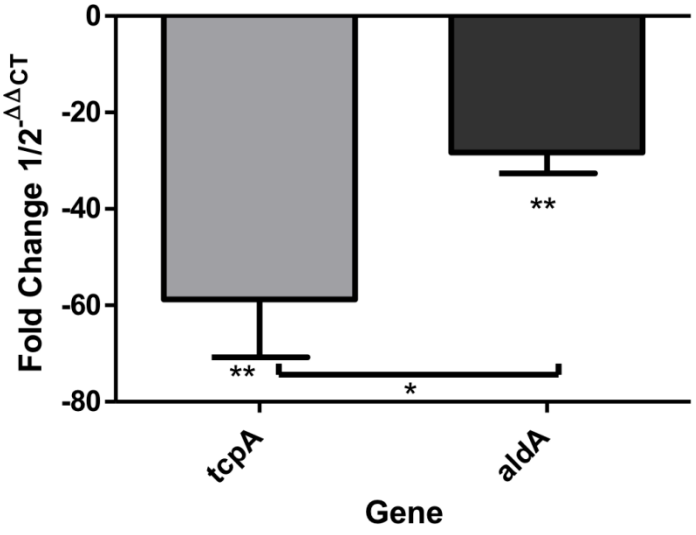


FIGURE 5. qRT-PCR data of *tcpA* and *aldA* comparing cultures grown with and without linoleic acid. Both transcripts were expressed less with the addition of linoleic acid. However there is a greater effect of linoleic acid on *tcpA* as compared to *aldA.* Data shown as the negative fold change of the inverse of the $2^{-\Delta\Delta CT}$ value. Statistical significance determined using Student's *t*-test (*, p<.05; **, p<.005).

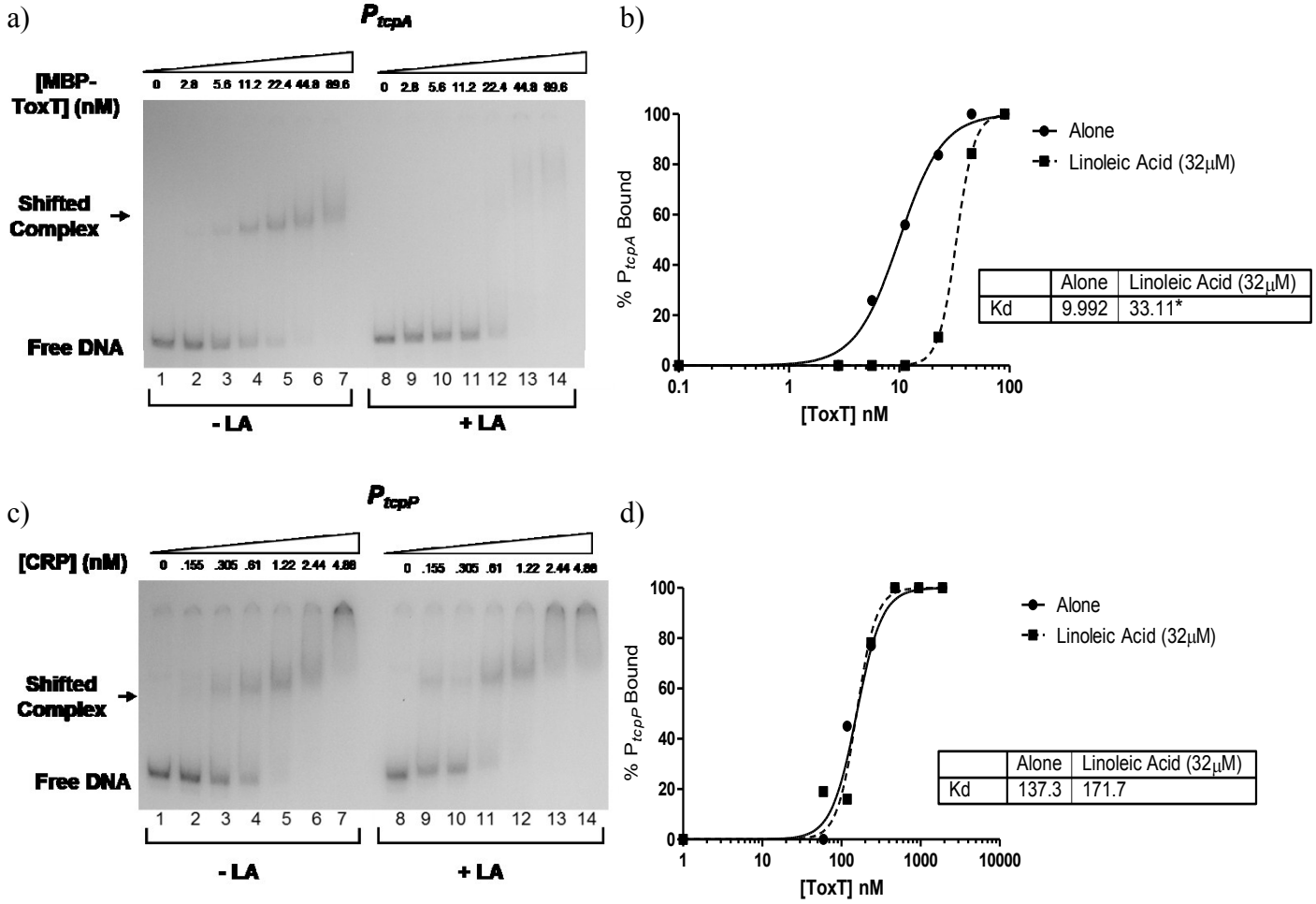


FIGURE 6. Linoleic acid decreases binding affinity of MBP-ToxT to P_{tcpA}. MBP-ToxT binding to P_{tcpA} was analyzed using EMSA. (A) Binding reactions between MBP-ToxT and P_{tcpA} in lanes 1-7 were conducted in the absence of linoleic acid. Lanes 8-14 were incubated in the presence of 32μM linoleic 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. (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 linoleic acid. Solid line corresponds to the binding curve for MBP-ToxT. Squares and dashed line represent percent bound and binding curve, respectively, in the presence of linoleic acid. (C) Autoradiograph of EMSA showing titration of MBP-CRP bound to P_{tcpP} with DMSO (Lanes 1-7) and linoleic acid (Lanes 8-14). (D) Binding curves with and without linoleic acid with K_d. Autoradiographs of EMSAs presented are representative of three or more independent experiments. K_d for each condition is inset and significant difference between the best-fit values of each data set is denoted by * ($p < 0.0001$).

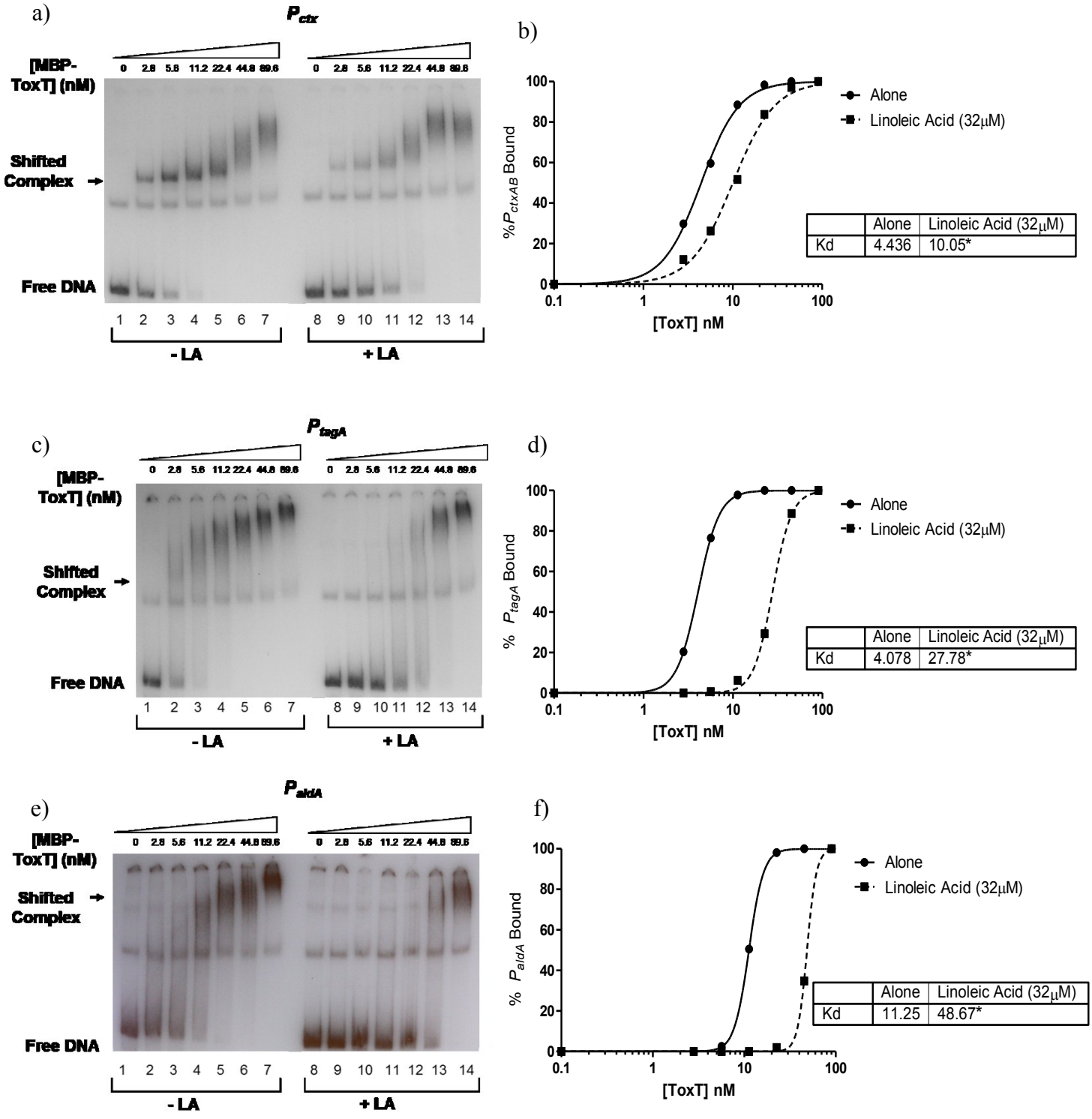

a)
P_{ctx}
[MBP-ToxT] (nM)
0 2.8 5.6 11.2 22.4 44.8 89.6
Shifted Complex
Free DNA
1 2 3 4 5 6 7 8 9 10 11 12 13 14
- LA
+ LA
b)
%P_{ctxAB} Bound
[ToxT] nM
Alone
Linoleic Acid (32μM)
Alone | Linoleic Acid (32μM)
Kd | 4.436 | 10.05*
c)
P_{tagA}
d)
% P_{tagA} Bound
Alone | Linoleic Acid (32μM)
Kd | 4.078 | 27.78*
e)
P_{aldA}
f)
% P_{aldA} Bound
Alone | Linoleic Acid (32μM)
Kd | 11.25 | 48.67*

FIGURE 7. Linoleic acid decreases binding affinity of MBP-ToxT at other virulence promoters. In all radiographs, binding reactions in lanes 1-7 are with DMSO only, while lanes 8-14 are in the presence of 32μM linoleic acid. (A), (C), (E) are autoradiographs showing MBP-ToxT binding at P_{ctx}, P_{tagA}, and P_{aldA} respectively. (B), (D), (F) are the binding curves corresponding to each autograph: P_{ctx}, P_{tagA}, and P_{aldA} respectively. All autoradiographs and binding curves are representative of at least three separate experiments. K_d for each condition is inset and significant difference between the best-fit values of each data set is denoted by * ($p < 0.0001$).

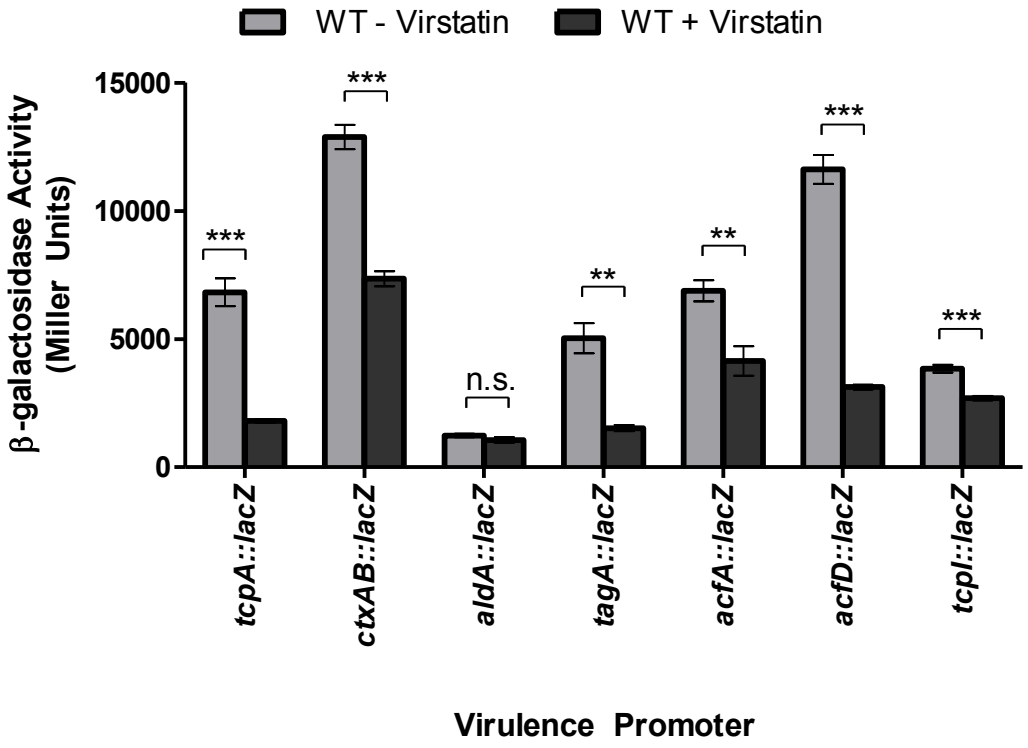
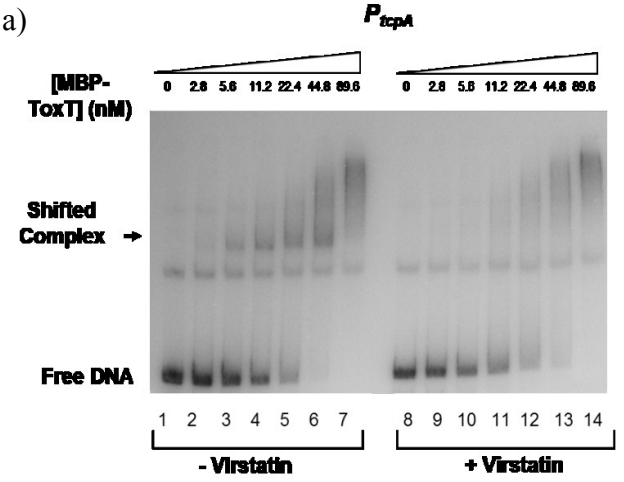
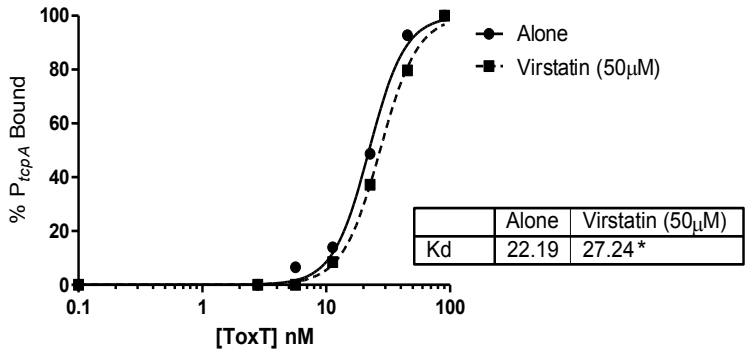


FIGURE 8. The effect of virstatin on ToxT-regulated virulence gene promoters. Cultures were grown under virulence-inducing conditions with and without virstatin. Promoter activity with and without 100μM was compared, and at all promoters except for *aldA*, virstatin caused a significant decrease in promoter activity. Statistical significance determined using Student's *t*-test (**, p<.005; ***, p<.0005).

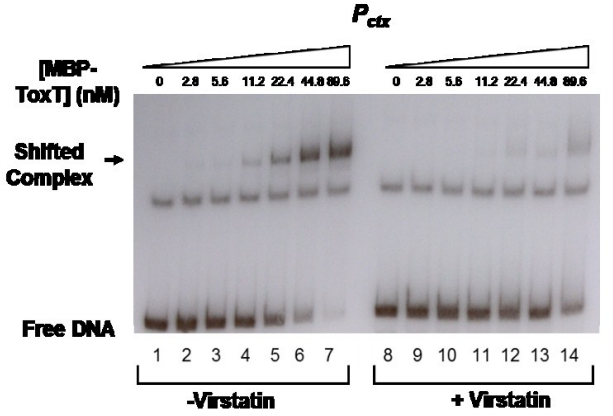
a)


P_{tcpA}
[MBP-ToxT] (nM)
0 2.8 5.6 11.2 22.4 44.8 89.6
0 2.8 5.6 11.2 22.4 44.8 89.6
Shifted Complex
Free DNA
1 2 3 4 5 6 7
8 9 10 11 12 13 14
- Virstatin
+ Virstatin

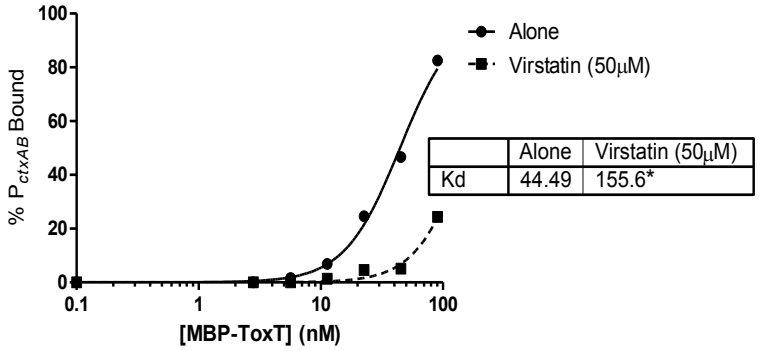
b)


% P_{tcpA} Bound
100
80
60
40
20
0
0.1
1
10
100
[ToxT] nM
Alone
Virstatin (50μM)
Alone | Virstatin (50μM)
Kd | 22.19 | 27.24*

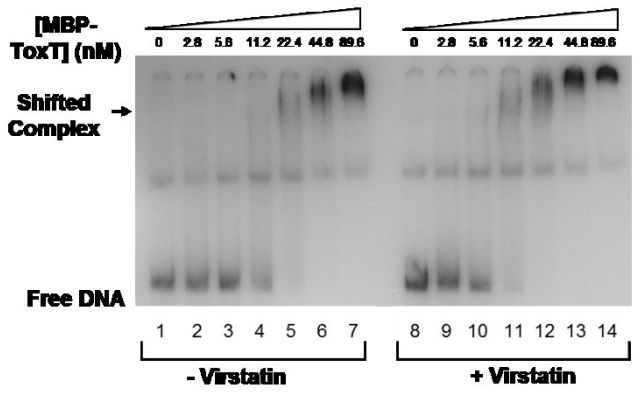
c)


P_{ctx}
[MBP-ToxT] (nM)
0 2.8 5.6 11.2 22.4 44.8 89.6
0 2.8 5.6 11.2 22.4 44.8 89.6
Shifted Complex
Free DNA
1 2 3 4 5 6 7
8 9 10 11 12 13 14
-Virstatin
+ Virstatin

d)


% P_{ctxAB} Bound
100
80
60
40
20
0
0.1
1
10
100
[MBP-ToxT] (nM)
Alone
Virstatin (50μM)
Alone | Virstatin (50μM)
Kd | 44.49 | 155.6*

e)


P_{aldA}
[MBP-ToxT] (nM)
0 2.8 5.6 11.2 22.4 44.8 89.6
0 2.8 5.6 11.2 22.4 44.8 89.6
Shifted Complex
Free DNA
1 2 3 4 5 6 7
8 9 10 11 12 13 14
- Virstatin
+ Virstatin

f)

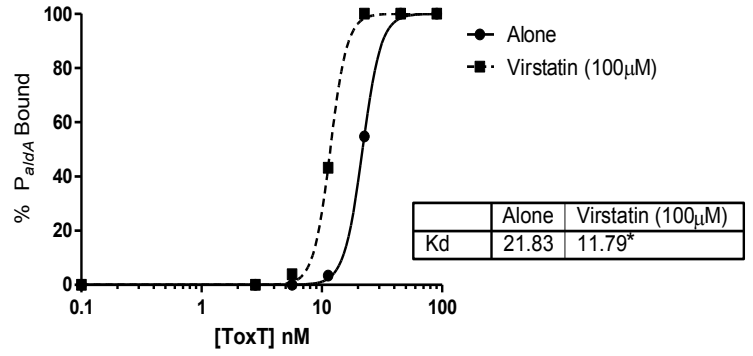

% P_{aldA} Bound
100
80
60
40
20
0
0.1
1
10
100
[ToxT] nM
Alone
Virstatin (100μM)
Alone | Virstatin (100μM)
Kd | 21.83 | 11.79*

FIGURE 9. Virstatin decreases binding affinity of MBP –ToxT at P_{tcpA} and P_{ctx}, but not at P_{aldA}. In all radiographs, binding reactions in lanes 1-7 are with DMSO only, while lanes 8-14 are in the presence of virstatin. 50µM virstatin was used for (A) and (C) and 100µM for (E). (A), (C), (E) are autoradiographs showing MBP-ToxT binding at P_{tcpA}, P_{ctx}, and P_{aldA} respectively. (B), (D), (F) are the binding curves corresponding to each autograph: P_{tcpA}, P_{ctx}, and P_{aldA} respectively. All autoradiographs and binding curves are representative of at least three separate experiments. K_d for each condition is inset and significant difference between the best-fit values of each data set is denoted by * ($p < 0.0005$).

CHAPTER TWO

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

ABSTRACT

V. cholerae is the causative agent of the severe diarrheal disease cholera. A complex network of transcriptional and post-transcriptional regulators controls production of the virulence factors that are required for human disease. 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 of bile and its unsaturated fatty acid (UFA) components on ToxT sensitivity. This analysis identified specific mutations within this unstructured region that limit the inhibition of virulence gene activation by bile and unsaturated fatty acids. These results suggest that a small unstructured region in the ToxT N-terminal domain is involved in virulence gene regulation and response to human host signals.

INTRODUCTION

V. 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 (44). 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) (100, 101, 198). CT is an ADP-ribosylating toxin composed of five binding B subunits and one enzymatic A subunit (216). 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 (8). TCP is a type IV bundle-forming pilus responsible for bacteria-bacteria interactions that result in microcolony formation during intestinal colonization (99, 101, 217).

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 (104, 133). 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 (109, 110, 113, 114, 133, 134, 183, 196). ToxT is a 276 amino acid protein that is part of the AraC/XylS family of transcription regulators (180). 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) (182). The CTD comprises the DNA-binding domain, consisting of two helix-turn-helix motifs, which has sequence similarity with the AraC/XylS family (181). The NTD shares no significant sequence similarity with any other protein, but shares secondary structural similarity with AraC (182) despite having only 14% identity at the amino acid level.

Transcription of *toxT* is initially activated by both TcpP/H and ToxR/S (104, 135-137). 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* (134, 136). Proteolysis of ToxT is required to break this auto-regulatory loop and completely shut off virulence gene expression prior to escape from the host (218). A region of the ToxT NTD between amino acids 100-109 was found to be required for proteolysis of ToxT (218). This region was not resolved in the ToxT crystal structure, indicating the absence of a fixed structure, at least in ToxT crystals (182).

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 (204, 205). The ToxT crystal structure contained a buried 16-carbon fatty acid, palmitoleic acid, which was shown to decrease binding of ToxT to the *tcpA* promoter when added exogenously (182). Another inhibitor of ToxT, virstatin, decreases ToxT activation of *ctxAB* and *tcpA* by presumably inhibiting ToxT dimerization (192, 193). On the other hand, ToxT activity is enhanced by bicarbonate, which is abundant within the upper small intestine where *V. cholerae* colonizes (202). 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 context of the crystal. This

unstructured region was shown to contain the site critical for proteolysis (218), and, together with the surrounding amino acids, has also been implicated in responding to bile, unsaturated fatty acids (UFAs), and virstatin (189, 190, 210). 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 and its UFA components from affecting ToxT activity. These 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 and 32 µM linoleic acid (Acros Organics). Cultures were grown shaking for 3 hours at 30°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).

Plasmid and strain construction. Construction of site-directed ToxT mutants was done using splicing by overlap extension PCR (219). For cloning C-terminal His-tagged *toxT* into

pBAD33, outside primers BP22 and BP195 (218) 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 (218) were paired with inside primers and PCR products were inserted into the pMAL-c2e vector using restriction enzymes *KpnI* and *PstI*. 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 (202).

β-galactosidase assays. Cells were grown under the appropriate conditions and β-galactosidase activity was measured and expressed in Miller units as previously described (202, 212, 218, 220).

Protein purification and electrophoretic mobility shift assays (EMSA). Protein purification and EMSAs were performed as previously described (214). 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 of 32 µM linoleic acid for 30 minutes at 37°C, and run through a 6% polyacrylamide 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 submitted for publication). 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 ToxT residues involved in responding to natural negative effectors, bile and linoleic acid. The crystal structure of ToxT revealed a small, unstructured region in the NTD of the protein (182). This region, as well as the surrounding amino acids, has been implicated in ToxT proteolysis, resistance to negative effectors of ToxT activity, and dimerization (190-193, 215, 218). To further characterize this region in regard to these roles, we performed site-directed alanine mutagenesis of ToxT amino acids 100-110, shown in Figure 10. The unstructured region of ToxT has previously been shown to have residues that are required for normal response of ToxT to bile and UFAs (189, 191). Bile and UFAs decrease the ability of ToxT to activate transcription of virulence genes (204, 205). Two ToxT mutations, M103A and N106A, have previously been described as insensitive to bile and UFAs (189). 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 (205). 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.

Figure 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* (190). The L107A mutation completely eliminated *tcpA::lacZ* expression. Prior reports have suggested that this mutation inhibits ToxT dimerization and therefore inhibits ToxT activity (189, 190). Additionally, ToxT

R105A had higher overall activity compared to WT, as has been previously reported (190). The fold decrease in activation of *tcpA::lacZ* upon addition of bile was calculated for each mutant (Figure 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 (189). However, a mutation at amino acid 106, which had been previously shown to decrease sensitivity to bile when measuring CT and TCP production (189) 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 (Figure 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 (189). 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 (189), 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. Interestingly, this mutant also showed decreased sensitivity for addition of bile as well as bicarbonate, a positive effector of ToxT, (data not shown). These results suggest that the

same ToxT residues within the unstructured region may be involved in both positive and negative effectors.

Alternate substitutions in the ToxT unstructured region affect response bile and UFAs. The results described above show that alanine mutagenesis within the unstructured region of ToxT alters the protein's sensitivity to bile and unsaturated fatty acids. Because amino acids mutated to alanine in the unstructured region showed reduced sensitivity to both bile/UFA and bicarbonate, we generated alternate substitutions of amino acids 105 (as it showed less sensitivity to bicarbonate, data not shown) 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 (191). 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 (189, 193). 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 (Figures 12A,B). 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 (Figure 12A). On the other hand, ToxT R105K exhibited

increased sensitivity to linoleic acid (Figure 12B). 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 (191), 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 (Figure 12A). The N106S mutation caused an increase in response to linoleic acid, but the double R105A/N106A mutant displayed reduced sensitivity to linoleic acid (Figure 12B). L114A was insensitive to bile and UFAs as previously described (189). 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.

Change in binding affinity of ToxT N106F is reduced in presence of the linoleic acid. Previous work has suggested that palmitoleic acid decreases the ability of ToxT to bind to P_{tcpA} (182). Additionally, we have shown that linoleic acid decreases the binding affinity of ToxT to virulence gene promoters regardless of toxbox orientation (Chapter One). To assess the ability of linoleic acid 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) is shown in Figure 13A. 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 (Figure 13B). The K_d of WT ToxT for P_{tcpA} 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 (Figures 13C,D). 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 (Figures 12,13D), providing a direct link between changes in DNA binding and transcriptional activation.

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 (189-193). Also, this region was required for ToxT proteolysis (218). Interestingly, this region was not visible in the solved ToxT structure due to the absence of a fixed structure in ToxT crystals (182). We discovered amino acids important for the response of ToxT to bile and linoleic acid including N106A, R105A/N106A, N106F, and L114A, which all showed decreased

sensitivity to the effector when mutated. These same amino acid substitutions were also important in the response to the positive effector bicarbonate (data not shown). Most mutations to amino acid 105 had no effect on reducing the response to bile and linoleic acid, but have been shown to have reduced sensitivity to bicarbonate (data not shown). Additionally, effector-insensitive ToxT N106F demonstrated no change in binding affinity to P_{tcpA} in response to UFA and 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 (189, 192). We hypothesize that negative effector binding forces ToxT into an inactive conformation that could be due to decreased interaction between ToxT monomers through the unstructured region, similar to has been previously proposed based on the ToxT crystal structure (182). 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 (182) 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 (221), which could also be the case with ToxT. It is unlikely that UFAs directly bind the unstructured region of ToxT, as mutations in the region do not reverse the proteolytic blockade by linoleic acid, but it is hypothesized that the positive effector bicarbonate could bind in this region. Previous findings in our lab revealed that the addition of linoleic acid blocked proteolysis of ToxT, but the UFA-insensitive ToxT N106F did not reverse the effect of linoleic acid on ToxT proteolysis (data not shown). Therefore, we conclude that amino acid N106 is not part of a binding site for UFAs, agreeing with results from (182). The unstructured region is likely to be important for the

change in ToxT structure, mediated by linoleic acid binding, which results in lower DNA binding affinity.

We have previously 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 (202). This model posits that ToxT is in an inactive form in the lumen of the intestine due to a lower local concentration of bicarbonate. The model was expanded in (222), 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 bind in the same region. 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.

In this model, shown in Figure 14, we propose that when ToxT is produced under 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 (182), and we add that this conformation impedes access to the unstructured region by the ToxT protease. This conformation could also inhibit ToxT dimerization. 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 that leads to a high degree of proteolysis.

In summary, we determined that an unstructured region in the NTD mediates the response of ToxT to negative effectors present in the intestine. 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

Work performed in the chapter is all my own; however, most of the text is from a manuscript with Joshua Thomson.

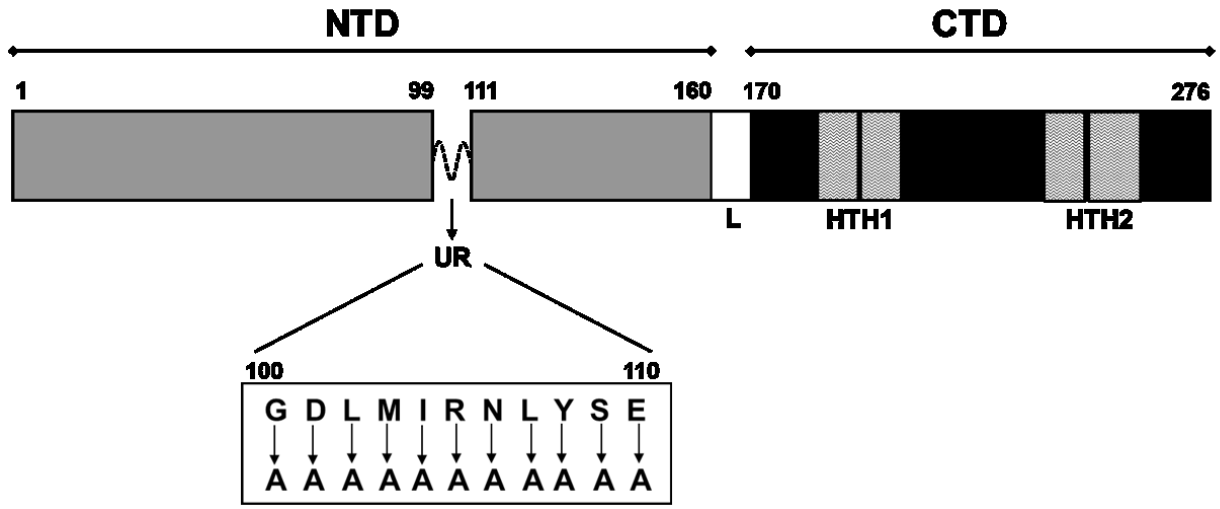


FIGURE 10. Linear representation of ToxT domain orientation and mutagenesis sites. Amino acids located in the unstructured region (UR) of ToxT between amino acids 100-110 were mutated to alanine for initial analysis of region. N-terminal domain (NTD) located between 100-160; Linker (L) located between 160-169; C-terminal domain (CTD) between 170-276. Helix-turn-helix domains 1 and 2 (HTH1,HTH2) located within the CTD.

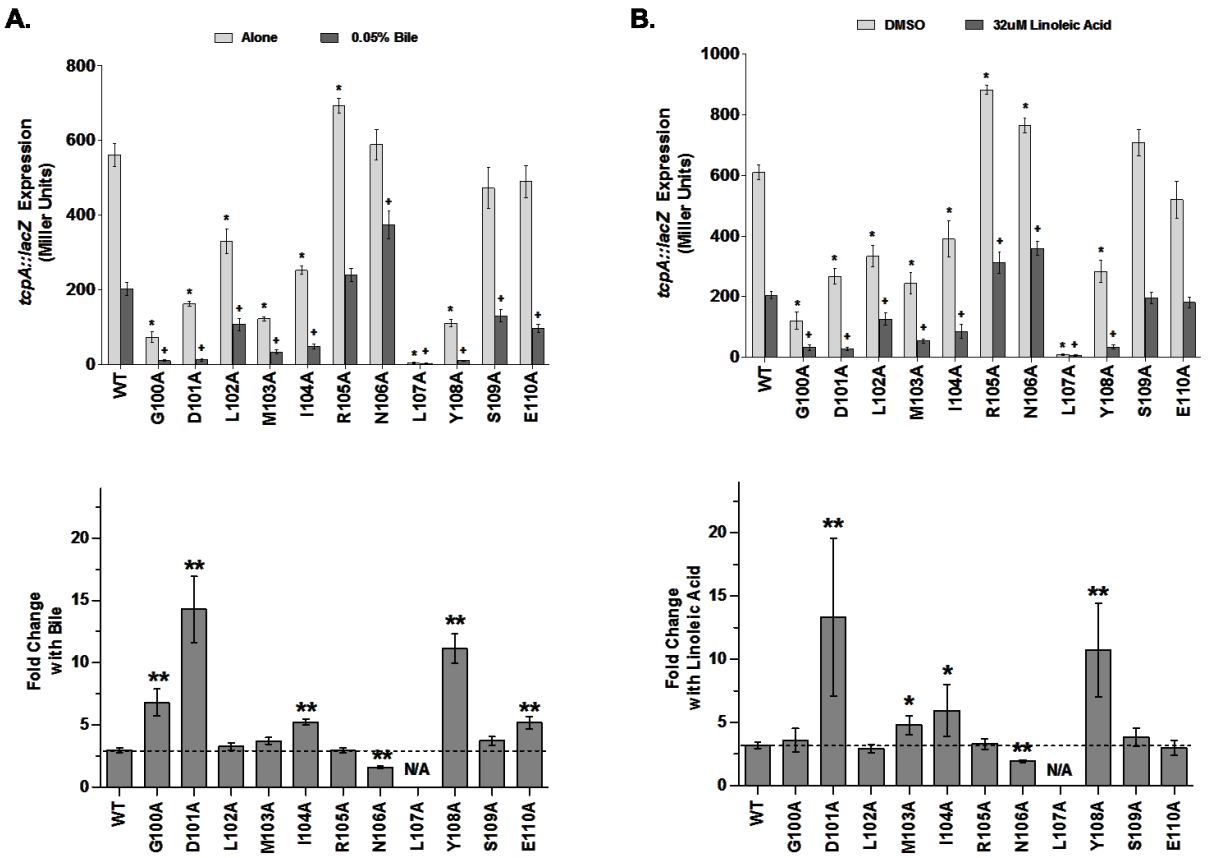


FIGURE 11. Effect of MBP-ToxT alanine mutagenesis in response to negative effectors, bile and linoleic acid. *V. cholerae* O395 Δ*toxT* mutant with plasmid-borne WT or MBP-ToxT mutants were grown under virulence-inducing conditions with or without the addition of negative effectors for 3 hours. (Top) 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) 32μM 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).

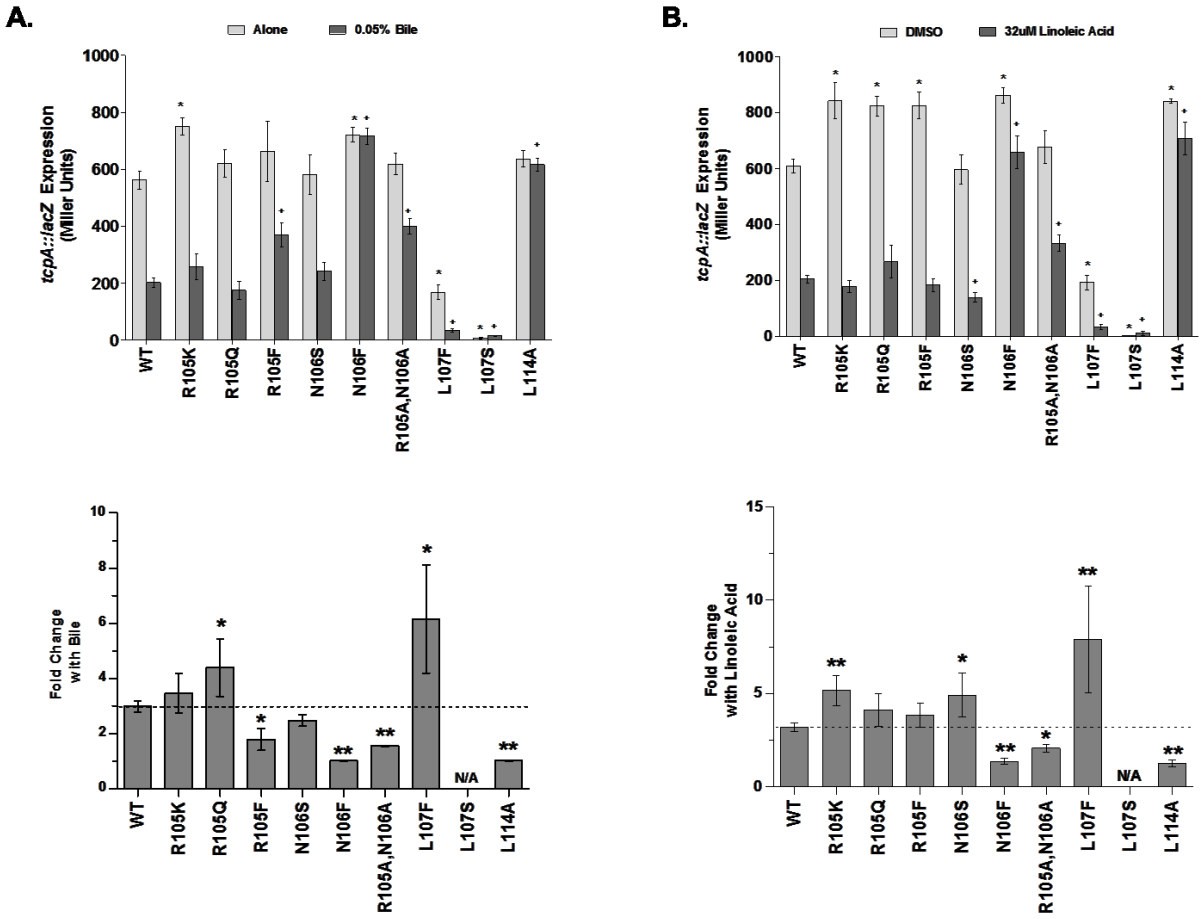


FIGURE 12. Alternate substitutions of unstructured region of ToxT affect response to negative effectors. *V. cholerae* O395 Δ*toxT* mutant with plasmid-borne WT or mutant MBP-ToxT were grown in the absence and presence of negative effectors. (Top) (A) β-galactosidase produced in absence and presence 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).

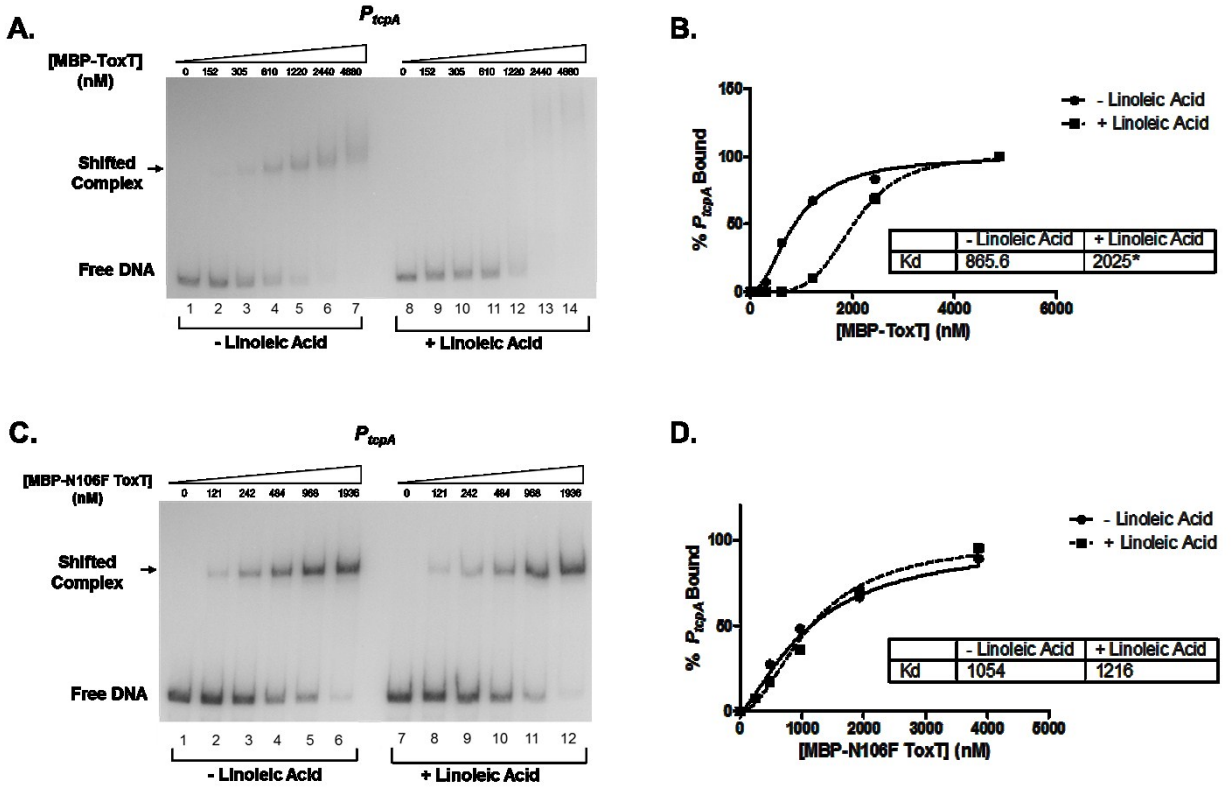


FIGURE 13. MBP-ToxT N106F has no change in binding affinity to P_{tcpA} after addition of linoleic acid. MBP-ToxT WT and N106F binding to P_{tcpA} was analyzed using EMSA. Autoradiographs of EMSAs presented are representative of three or more independent experiments. (Left) Binding reactions between (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 * ($P < 0.00025$).

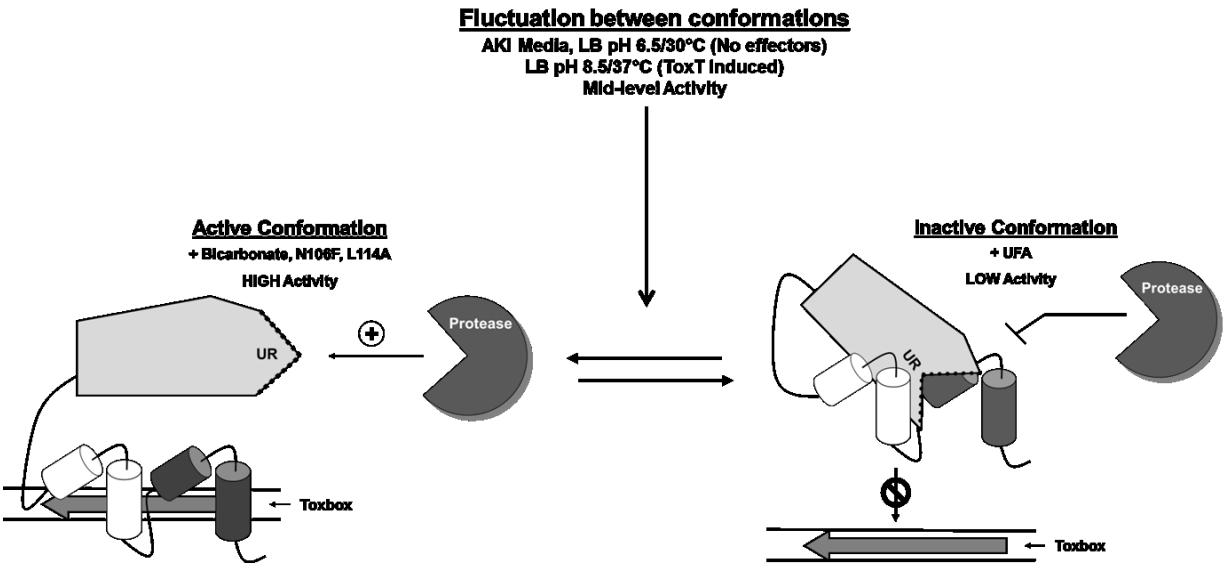


FIGURE 14. Model for effector control of ToxT activity. In the absence of effector molecules, there is mid-level transcriptional activation of ToxT and it is in fluctuation between inactive and active conformations. When bicarbonate is present, ToxT is at high-level transcriptional activation. The ToxT conformation has increased binding affinity to toxboxes but also leaves the unstructured region (UR) (proposed to contain a proteolytic cleavage site) accessible by protease. This conformation resembles ToxT mutants N106F and L114A. Conversely, when negative effectors are present, ToxT is in an inactive conformation where it has low-level transcriptional activation. The UR is inaccessible in this conformation, so the site is inaccessible to protease.

CHAPTER THREE

The use of conjugated linoleic acid as a potential cholera therapeutic

ABSTRACT

The severe diarrheal disease cholera is currently endemic in over 50 countries. Although much work has been done to develop effective vaccines against the causative agent, *V. cholerae*, these vaccines are currently only effective for very limited amounts of time and do not offer complete protection. Linoleic acid has been shown to be an effective negative regulator of *V. cholerae* virulence, not by killing the bacteria as an antibiotic would, but instead by acting on the major virulence transcriptional regulator protein, ToxT, to inhibit virulence gene expression. ToxT activates transcription of the two major virulence factors required for disease, cholera toxin (CT) and toxin co-regulated pilus (TCP). A conjugated form of linoleic acid (CLA) is currently sold on the market as a dietary supplement to aid in weight loss, is considered safe by the US Food and Drug Administration, and is relatively inexpensive. This study examines using CLA as a new potential prophylactic or as a therapy to reduce CT and TCP production, which, in turn, would decrease disease duration and intensity.

INTRODUCTION

Cholera is a devastating diarrheal disease that affects between 1.4-4.3 million people each year causing between 28,000-142,000 deaths (14, 15). The disease is characterized by severe dehydration that leads to organ shutdown and eventual death if not treated rapidly. It is caused by the gram-negative curved rod *V. cholerae* when a person ingests large quantities of contaminated water or raw shellfish colonized with *V. cholerae*. Current treatment is oral rehydration solution as specified by the World Health Organization, containing various salts and

glucose to rehydrate as well as replenish electrolytes (3). Without treatment, survival is approximately 50%, but rehydration with ORS bring survival to greater than 99% (8). Antibiotics are a treatment option; however their use is limited. By the onset of cholera symptoms, the bacteria are already being shed back into the environment, but antibiotics can act to slightly shorten the duration of disease symptoms. As only 25% of people show symptoms when infected with *V. cholerae*, the other 75% are still able to shed bacteria into the environment, potentially for another person to ingest (6, 7).

V. cholerae is classified based on the O antigen of its lipopolysaccharide, and though there are over 200 serogroups in the environment only one serogroup, O1, has been associated with pandemics (1, 8, 16). The O1 serogroup can be divided into two biotypes: classical and El Tor. *V. cholerae* has caused seven pandemics, and of these, the classical biotype was the causative agent of the first six, while the El Tor biotype caused the most recent, ongoing seventh pandemic (8, 16). The classical biotype also causes a more severe disease, but El Tor is thought to survive better in the environment, although the reasons for this are not understood (16, 17). O139, another serogroup, emerged in the early 1990s as being involved in outbreaks (18-21). As such, some refer to these outbreaks as an eighth pandemic (18).

Much work has been done on the development of effective vaccines. There are two oral vaccines currently available, each with questionable efficacy (28, 29). Neither vaccine is licensed in the United States due to their lack of complete and long-term protection. The first vaccine, Dukoral, consists of killed whole *V. cholerae* cells of both biotypes of serogroup O1 and the B subunit of CT. It has had very different success in field trials, including a trial in Peru involving over 17,000 people where the vaccine only offered 15% protection in persons aged six and up

after one year of surveillance (223). The now preferred vaccine is called Shanchol and it is composed of both O1 and O139 serogroups. Disappointingly, this vaccine also showed only 45% protection in all age groups after one year (224, 225). As over half the deaths due to cholera are in children under five (226), a vaccine that effective in small children is essential. Dukoral, actually offered negative protection in a field trial in Peru during the first year of surveillance, and Shanchol only offered 17% protection (223-225, 227). Clearly, more effective vaccines are necessary.

In order to initiate disease, production of two virulence factors is necessary: CT and TCP. CT is an A-B_5 toxin responsible for the profuse watery diarrhea, while TCP is necessary for host colonization. The genes encoding both of these virulence factors are both under control of the major transcriptional activator, ToxT (104, 132-134). *V. cholerae* encounters high concentrations of bile and its unsaturated fatty acid components, such as linoleic acid, in the small intestine before entering the mucosal layer where it is eventually able to colonize (42-44). In the presence of these negative ToxT effectors, *V. cholerae* expresses its motility genes, but its virulence inducing genes are not expressed, as ToxT is not active (40, 41).

To activate the transcription of CT and TCP, ToxT binds to their respective promoters on binding sites known as toxboxes (183). Linoleic acid can decrease virulence gene expression by directly reducing the ability of ToxT to activate these promoters (Chapter 1). Currently, CLA is sold over the counter as a weight loss supplement aimed at inhibiting fat absorption. As it is relatively cheap and easily accessible, we propose that CLA could potentially be used as either a cholera prophylactic or as a therapy used in conjunction with oral rehydration to reduce disease length and intensity by reducing virulence gene production. As antibiotic resistance is becoming

a more and more concerning problem, using a therapy that can inhibit pathogenesis, but not bacterial survival, is becoming much more attractive. Here, we show that CLA is able to downregulate virulence gene expression by acting on ToxT. We also show the effect of CLA in two animal models: the infant mouse model, which measures the ability of *V. cholerae* to colonize and the rabbit ileal loop model, which is used to measure disease by looking at CT-dependent fluid accumulation in a rabbit intestinal loop.

MATERIALS AND METHODS

***V. cholerae* strains and growth conditions.** All *V. cholerae* strains used in the study are derived from classical biotype strain O395. Strains were maintained in Luria Broth (LB) containing 20% glycerol and stored at -70°C. Overnight cultures were grown overnight at 37°C in LB and then subcultured 1:40 into LB pH 6.5 at 30°C for three hours for virulence inducing conditions in the presence or absence of 32 or 128μM CLA dissolved in DMSO, or in later experiments, 640μM of ME-CLA, or 32 μM of one of the three common isoforms of CLA: 9E,11E-; 9Z,11E-; or 10E,12Z- (Sigma-Aldrich). *V. cholerae* strains were grown with antibiotic concentration of streptomycin at 100μg/mL.

β-Galactosidase and CT assays. β-galactosidase activity was measured using the basic procedure of Miller (212). CT was detected in the culture supernatant by a GM1 enzyme-linked immunosorbent assay (ELISA) (228), using polyclonal anti-CT antibody (Sigma). Briefly, for both assays, bacteria were grown with or without CLA, CLA-ME or one the CLA isoforms for three hours under virulence-inducing conditions and then analyzed. DMSO was used as a solvent

control. A positive control assay for quantification of the level of CT in the samples was performed using purified CT (List Biological Laboratories).

Electrophoretic mobility shift assays (EMSA) and binding curve analysis. EMSAs were performed as previously described (214). Purified MBP-ToxT (described previously), was incubated with DNA probes made from the promoter sequence of interest that had previously been inserted into the plasmid pTL61T and labeled with γ-^{32}P (Perkin-Elmer) by T4 polynucleotide kinase (New England Biolabs). Binding reactions contained various amounts of MBP-ToxT with constant 10 μg/mL salmon sperm DNA, 10mM Tris-acetate (pH 7.4), 1mM Potassium EDTA (pH 7.0), 100mM KCl, 1mM dithiothreitol (DTT), 0.3mg/mL bovine serum albumin (BSA) and 10% glycerol in a volume of 30μL. To each reaction, a constant concentration of the labeled DNA probe was added. In reactions containing CLA, the final concentration was 32μM for each reaction. All other reactions contained 3.33% (1μL in 30μL) of DMSO as a solvent control. Binding reactions were incubated for 30 minutes at 37°C and then loaded into a 6% polyacrylamide gel to be run at 4°C. Gels were dried for 1 hour and then analyzed by autoradiography.

Binding curve analysis. Autoradiographs were analyzed using ImageJ software (NIH) as previously described. K_d values were determined for each binding curve and then the K_d for each condition were compared to each other using the extra sum of squares F test to determine if the two values were statistically different.

Infant mouse colonization assays. 5 day old CD1 mouse pups were inoculated with approximately 10^6 *V. cholerae* strain C6706 (El Tor) by oral gavage in a total volume of 50 μl.

CLA, if given, was administered at the time of initial gavage and boosted twice at hours 2 and 4 post-infection. 20 hours post infection the pups were sacrificed, their intestines dissected and homogenized using a bead beater, and serial dilution of the intestinal homogenate were plated to enumerate *V. cholerae*.

Infant mouse colonization assays. Infant mouse assays were performed as previously described (114). Briefly, 5 days old CD1 mouse pups were inoculated with ~10^6 *V. cholerae* classical strain O395 in 50 μl total volume, containing 0.5% Evans Blue as a tracer, by oral gavage. Mice treated with CLA were given either 5% or 10% CLA by volume in 10% Kolliphor RH40 or 10% Kollidon 25 as a carrier at the time of inoculation. These mice were also boosted with the indicated concentration of CLA at 3 hr and 6 hr post inoculation. At 20 hr post-infection the mice were sacrificed, dissected, and intestines homogenized using a bead beater. Intestinal homogenates were serially diluted and plated on LB agar containing 100 μg/ml streptomycin for the enumeration of colonizing *V. cholerae*.

Rabbit ileal loop assays. Fluid accumulation in rabbit ileal loops was performed as previously described (229). Briefly, 10 cm loops of small intestine were injected with 1 ml total volume containing approximately 10^6 *V. cholerae* strain C6706 (El Tor) and various amounts of CLA. A negative control loop was injected with 1 ml PBS. After 16 hours, the rabbits were sacrificed and their intestines dissected out. Fluid volume from each loop was measured and CT ELISA was performed to assess the mount of CT produced under each condition.

RESULTS

CLA acts as a negative ToxT effector for activation of both TCP and CT. Our previous work showed that linoleic acid decreased ToxT activity, leading to a decrease in virulence gene expression (Chapter One). Here, we wanted to assess whether CLA would also exert negative activity on ToxT, as measured by P_{tcpA} expression as well as CT production. This would confirm that CLA affects both major virulence factors in the classical biotype *V. cholerae* strain O395. To begin, we used the highest concentration of CLA that did not affect bacterial growth, 128 µM. In the presence of CLA, there is a significant reduction in P_{tcpA} activity when *V. cholerae* is grown under virulence-inducing conditions as compared to cultures grown in the same conditions, but in the absence of CLA (Figure 15A). We then wanted to confirm that CLA had a similar effect on the production of CT (Figure 15B). After growing the bacteria for three hours under virulence-inducing conditions, we looked at the supernatant to quantify the amount of secreted CT. CT production was also significantly reduced in the presence of CLA. Thus these results indicate that CLA works as expected to attenuate *V. cholerae* virulence gene expression in vitro

The CLA used in the previous experiment is a mixture of cis- and trans-9,11- and -10,12-linoleic acid. To look at the different effects of each isoform, we used the lowest concentration of the multi-isoform CLA that still had an effect on P_{tcpA} expression, 32 µM. By using this concentration, it allowed us to more easily compare effects of each isoform to one another as more or less effective at decreasing P_{tcpA} activity. To begin, we looked at CLA-ME. This is made by the transesterification of CLA with methanol. This compound had no effect on promoter expression even at a concentration 20 times the amount we used of the other CLA forms,

indicating it is the fatty acid and not only its aliphatic chain that is involved in downregulation of ToxT activity, and thus virulence gene expression (Figure 16). Next, we looked at the three most common isoforms of CLA and their effects on promoter expression (9E,11E-, 9Z,11E-, or 10E,12Z-CLA). Although all three isoforms caused a significant decrease in virulence gene expression, each did not do so to the same extent. Of the three isoforms, 9Z,11E-CLA was the most effective at reducing expression and slightly more effective than the CLA mixture but this was not statistically significant. 9E,11E-CLA affected promoter expression the least. Therefore, the different isoforms of CLA do not all affect the P_{tcpA} to the same extent.

ToxT binding activity is reduced in the presence of CLA. Previously we showed that linoleic acid was able to directly affect ToxT-binding to DNA, by using EMSA (Chapter One). Here, we used this same technique to determine if CLA also affected ToxT-binding to DNA. We also used this information to get the ToxT binding curve and the equilibrium constant, K_d. We again used the P_{tcpA} as our DNA probe labeled with ^{32}P while adding increasing amounts of purified MBP-ToxT to binding reactions. To each reaction we added DMSO to act as our solvent control (Figure 17A, lanes 1-7) or 32 μM CLA dissolved in DMSO (Figure 17A, lanes 8-14) and brought the binding reactions to equilibrium for 30 minutes. These reactions were then run on a 6% polyacrylamide gel, followed by autoradiography and analysis using ImageJ software. Densitometry analysis was used to calculate the percent bound MBP-ToxT of each concentration and then put into GraphPad to get a binding curve as previously described (Chapter one). The K_d with no CLA was 5.724nM and the K_d with CLA was 32.07nM. These values are significantly different from each other (Figure 17B).

Assessment of in vivo effects of CLA on virulence factor production. The results described so far indicate that CLA has a strong negative effect on ToxT activity in vitro, as assessed by both gene expression (CT production and P_{tcpA} activity) and DNA binding (EMSA). The next step was to determine whether administration of CLA in animal models for cholera would reduce virulence factor production, leading to decreased colonization and/or fluid accumulation induced by CT. The two most common animal models for cholera are the 3-5 day old infant mouse model and the adult rabbit ileal loop model. The former is an excellent model for assessing colonization, but does not produce a disease state in the mice. The latter is an excellent model for assessing CT production, which is exhibited by fluid accumulation in the ileal loops. Both models were used to determine whether CLA could reduce *V. cholerae* colonization and/or CT production.

First, *V. cholerae* colonization was assessed using the infant mouse model. Because infant mouse colonization requires TCP production, this would generally assess whether CLA inhibits TCP production in vivo. Mice were infected with classical biotype *V. cholerae* strain O395 or an isogenic *toxT* deletion strain as a negative control. Groups of mice infected with wild-type O395 were either given PBS as a control or CLA at the time of infection, then boosted with the appropriate reagent at 3 hours and 6 hours post-infection.

The results of these experiments are shown in Figure 18. CLA did not cause a significant decrease in colonization levels in infant mice. However, there were numerous problems with using this model. First, the inoculum was limited to 50 μl, due to the size of the mouse pups. CLA is also very viscous and difficult to pipet, which required that it be dissolved in a non-aqueous carrier that could then be emulsified in PBS for administration. This severely limited

the amount of CLA that could be administered to the mouse pups. Therefore, although this is a negative result, it may not be a good indicator of whether CLA would actually work in vivo in the human gut.

Second, CT production was assessed using the rabbit ileal loop model. In this case, a larger inoculum of 1 ml was used in each loop, which made administration of CLA much easier. CLA was either dissolved in ethanol and then suspended in PBS or dissolved in sterile corn oil, which is used in vaccine preparations. Preliminary results indicated that ethanol alone inhibited CT production, probably due to its toxic effects on the bacteria, so this was excluded from later experiments. El Tor strain C6707 was used in these experiments due to its stronger similarity to currently circulating disease strains. Figure 19 show the results of a representative ileal loop experiments. The administration of CLA, by separate injection, at the time of bacterial inoculation resulted approximately three-fold lower fluid volumes that when wild-type C6706 was injected without CLA (Figure 19A). CT ELISA indicated that the CT levels were also reduced when CLA was administered (Figure 19B). By combining the data from these two observations, the overall outcome of these experiments was that the total amount of CT produced per loop was approximately six-fold lower when CLA was administered (Figure 19C). While this was not as strong of an effect as having *toxT* deleted (Figure 19A), it did strongly suggest that administration of CLA in vivo could significantly reduce CT production, which could have an effect on the duration and intensity of disease.

DISCUSSION

Because of our findings that linoleic acid has such a strong inhibitory effect of ToxT activity, using the related compound conjugated linoleic acid (CLA) as a prophylactic or

therapeutic after exposure to *V. cholerae* became a possibility. There is a strong negative effect of CLA on virulence gene expression as shown by both P_{tcpA} activity and CT expression, as well as a decrease in ToxT binding to its toxboxes sites on the P_{tcpA} (EMSA). Different isoforms of CLA showed some minor differences in effect but no major differences, making CLA production easier as currently available mixtures could be used in over-the-counter therapies.

Although the infant mouse model showed no reduction in colonization, the model was slightly flawed as potentially we could not add a high enough CLA concentration to reduce virulence gene expression. This also only looked at O395 classical biotype, and it is possible that with El Tor, the more common biotype in the environment, would give different results. The duration of colonization could not be determined, and it is possible CLA could reduce its length. These are preliminary data, and more troubleshooting could lead to better and more complete results. Potentially even using a better solvent for CLA would help. The rabbit ileal loop model, showed great promise, however, as CT production was greatly reduced, showing that although CLA does not reduce colonization, it may reduce symptoms. Although these results are preliminary, the use of CLA with *V. cholerae* shows promise in reduction of disease. Future research will determine whether this will be a feasible and effective addition to current rehydration therapy.

All *in vitro* experiments were performed by me, but the *in vivo* work was done by our collaborators in India through NICED with Hemanta Koley, Ph.D.

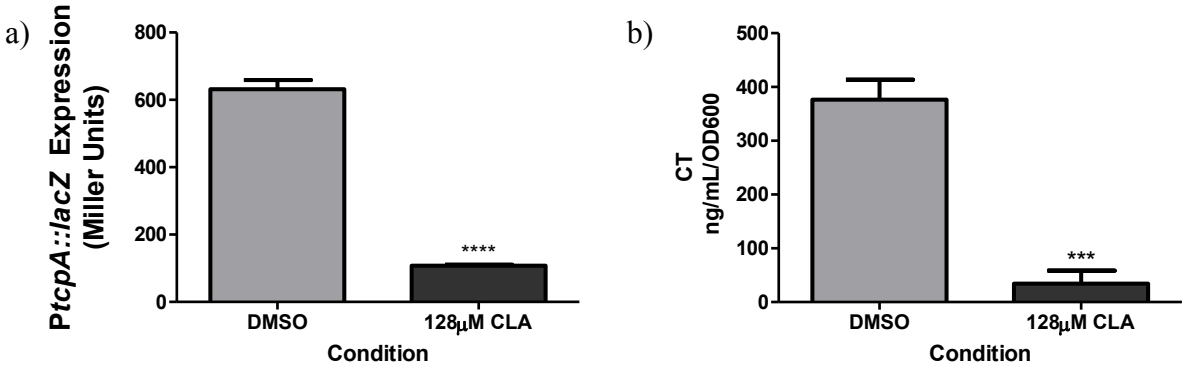


FIGURE 15. Cultures grown under inducing-conditions in the presence of CLA have downregulated virulence gene expression. (A) Looking at *tcpA::lacZ* activation by ToxT by β-galactosidase assay showed significant downregulation of promoter activity upon the addition of 128μM CLA. (B) CT expression was downregulated in the presence of CLA. Statistical significance determined using Student's *t*-test, *(p<.0001).

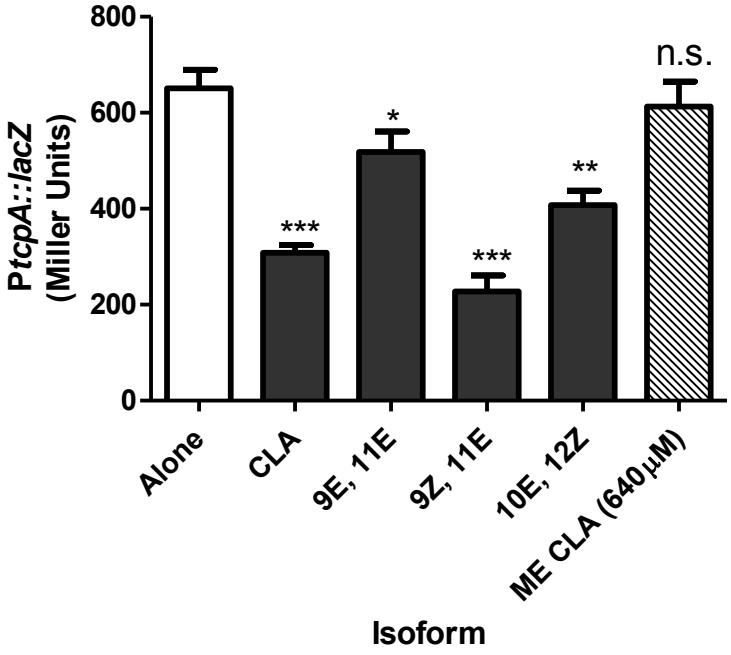


FIGURE 16. The different isoforms of CLA do not all affect P_{tcpA} to the same extent. *V. cholerae* was grown under virulence-inducing conditions with different isoforms of CLA present and β-galactosidase assays performed to look at promoter activity. Statistical significance determined using Student's *t*-test compared to alone (DMSO only) *,p<.05; **,p<.005; ***,p<.0005.

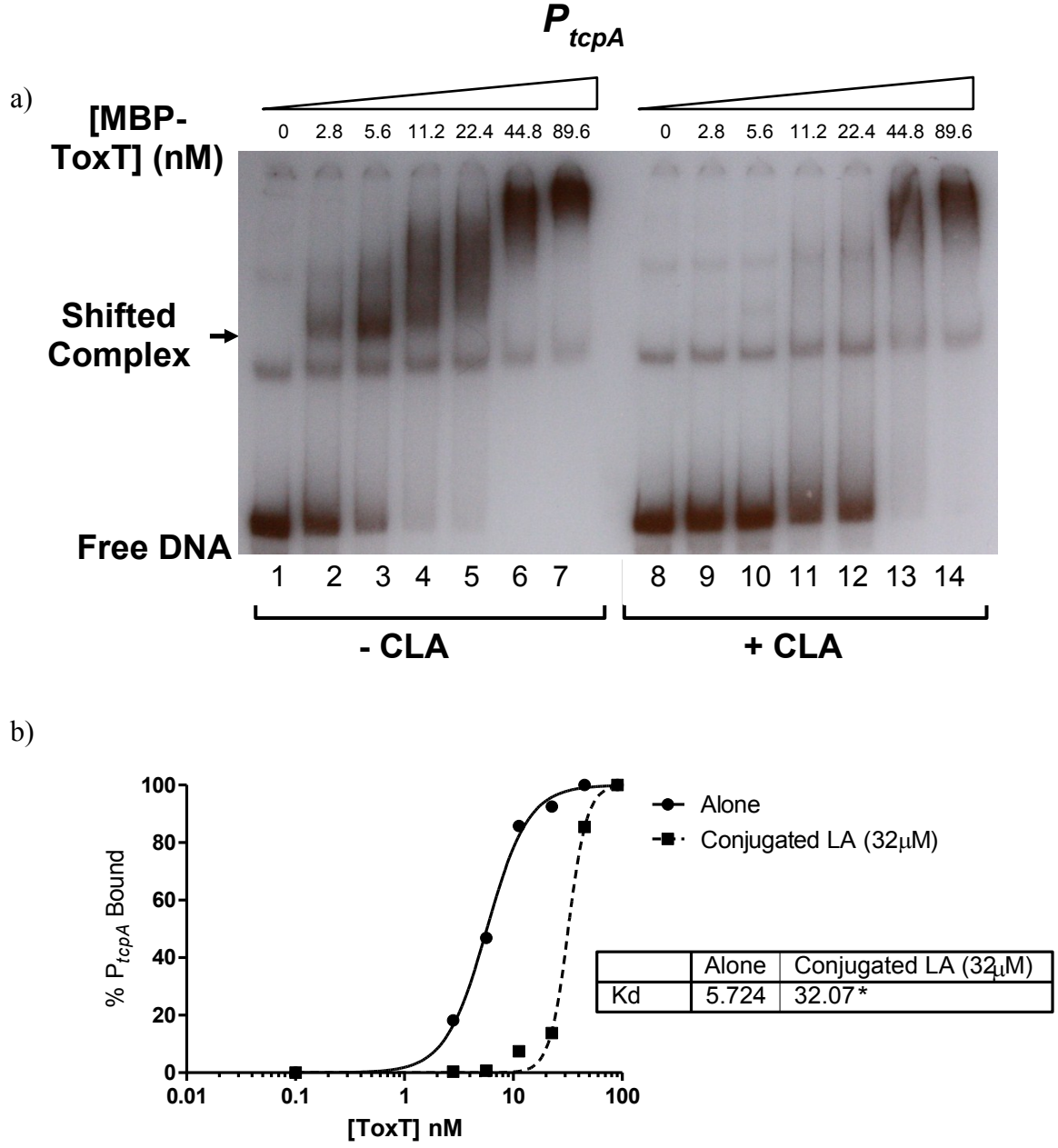


FIGURE 17. MBP-ToxT binding to P_{tcpA} DNA was negatively affected in the presence of CLA. (A) Autoradiograph of MBP-ToxT binding reactions with P_{tcpA} DNA. Lanes 1-7 have DMSO and lanes 8-14 have 32μM CLA. (B) Binding curve using densitometry of (A) and GraphPad software show significant reduction in the ability of ToxT to bind DNA. Autoradiographs of EMSAs presented are representative of three or more independent experiments. The K_d is inset with significant difference between the best-fit values denoted by * ($p < 0.0001$).

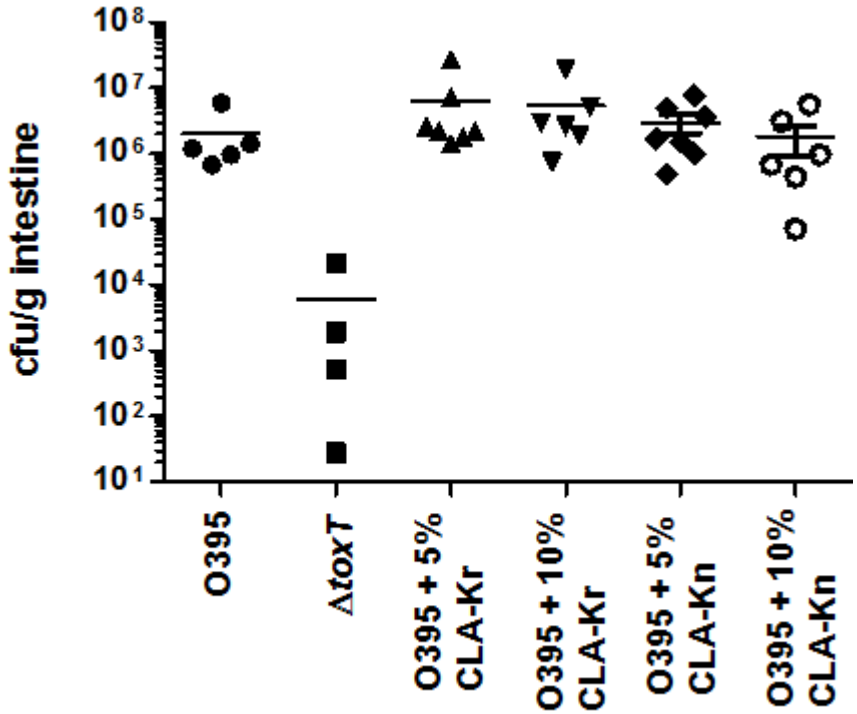


FIGURE 18. Infant mouse colonization assays. Each symbol represents the data from one mouse. CLA was administered using either Kolliphor RH-40 (Kr) or Kollidon 25 (Kn) as a carrier.

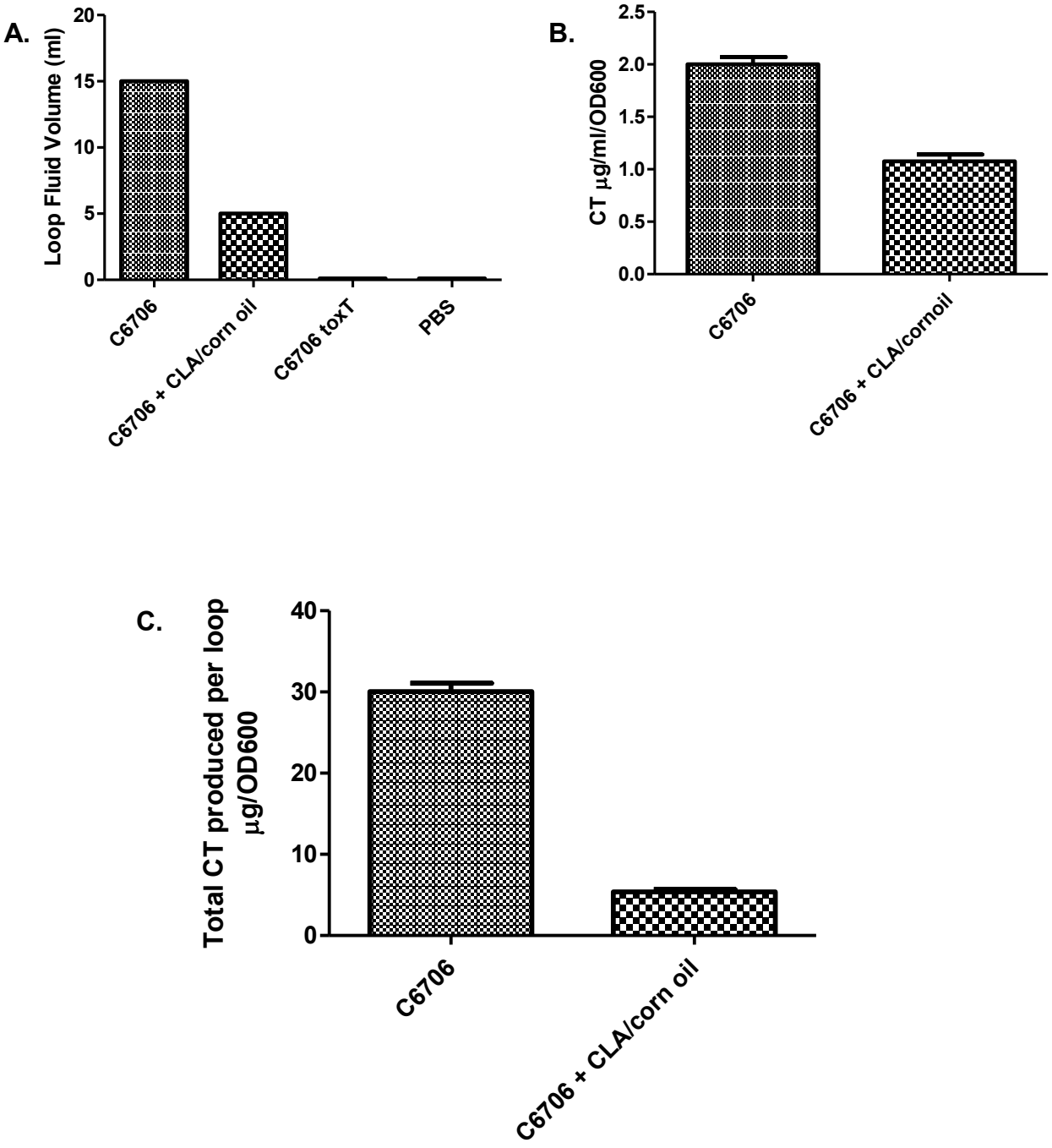


FIGURE 19. Rabbit ileal loop assays to assess the effect of CLA on fluid accumulation and CT production. (A) Ileal loop fluid volumes 16 hours after injection with the indicated mixture. (B) CT ELISA indicated CT levels in ileal loop fluid. C) Total CT production in loops containing the indicated inoculum.

CHAPTER FOUR

Identifying genes involved in the response to bile in *Vibrio cholerae* using fluorescence-activated cell sorting

Abstract

Vibrio cholerae causes the severe diarrheal illness cholera. Previous studies have shown that bile and its unsaturated fatty acid (UFA) components reduce virulence gene expression. The mechanism for the bile-mediated reduction of toxin co-regulated pilus and cholera toxin expression through the inhibition of ToxT activity has been described in earlier chapters of this dissertation, but it is thought that there are also other genes involved in the direct response to bile. To further characterize the mechanisms for bile inhibition of virulence gene expression, we used transposon mutagenesis to make a *V. cholerae* mutant library containing gene knockouts throughout the genome. Using fluorescence activated cell sorting methods, we screened and sorted the mutants using a virulence gene promoter (*tcpA)* fused to the gene encoding green fluorescent protein (*gfp*) to look for mutants that no longer caused a downregulation of *tcpA* promoter expression in the presence of bile or UFA. This method has resulted in identification of multiple mutants with insertions in *pckA*, which causes lowered sensitivity to bile when mutated or deleted. PckA is known to be involved in the citric acid cycle but potentially has another role in virulence. Identifying and characterizing the genes involved in the response to bile/UFAs will give us a clearer understanding of the regulatory networks controlling virulence gene expression.

INTRODUCTION

V. cholerae is the causative agent of the severe diarrheal disease cholera. Cholera is characterized by voluminous diarrhea. In order to initiate disease, production of two virulence factors is necessary: cholera toxin (CT) and toxin co-regulated pilus (TCP) both dependent on the major transcriptional protein activator ToxT. In the environment, *V. cholerae* expresses motility genes which encode a single, polar flagellum that is used to penetrate the mucus layer of the small intestine where *V. cholerae* then colonizes on the surface of epithelial cells (40). Upon finding a site for colonization, the bacteria then downregulate motility gene expression and upregulate virulence gene expression (40, 41). At the site for colonization, many host chemicals are present, but two, bile and bicarbonate, have been identified as a negative and positive effectors of ToxT, respectively (160, 202-205).

Bile is secreted by the gall bladder and is a heterogeneous mixture of many different components including bile salts, saturated and unsaturated fatty acids, cholesterol, proteins, and phospholipids. Bile normally acts as a fat emulsifier and bactericide, but *V. cholerae* uses it as a chemical signal. When added *in vitro,* bile is shown to cause decreased production of TCP and CT, and increased motility, biofilm formation, and outer membrane protein quantities (203-205).

To identify genes important for the response to bile, we made a transposon mutant library to look for mutants that were insensitive to the effect of bile. This was done by using the promoter of *tcpA* fused to *gfp*, such that when virulence gene expression is induced by ToxT, GFP is produced and can then be used as the marker for virulence gene induction. With GFP as our marker, we used fluorescence activated cell sorting (FACS), which has been previously

established as a method to isolate bacteria of interest from liquid cultures (230). Using this technology we looked for mutants grown in liquid culture that no longer downregulated virulence gene expression in response to bile and therefore were expressing high levels of GFP. By using FACS, we were able to collect these mutants for further identification and characterization. In doing so, we found several genes whose disruption was correlated with a decrease in sensitivity to bile, but a gene involved in the citric acid cycle, *pckA*, was the most common. Isolating mutants of *V. cholerae* that are no longer sensitive to bile allows us to identify genes that are involved in the bile response and gives us greater insight into how environmental signals affect virulence.

MATERIALS AND METHODS

***V. cholerae* strains and growth conditions.** All *V. cholerae* strains used in the study are from classical biotype O395 and this strain was also used to construct the transposon mutant library. Strains and transposon mutants were maintained in Luria Broth (LB) containing 20% glycerol and stored at -70°C. A P_{tcpA}::*gfp* fusion used for flow cytometry was made on pTL61T and P_{tcpA}::*lacZ* used for β-galactosidase assays was inserted into the chromosome using pKAS32 and conjugation. Overnight cultures were grown overnight at 37°C in LB and then diluted 1:40 into LB pH 6.5 at 30°C for virulence inducing conditions in the presence or absence of freshly prepared .05% bile (sodium choleate) or 160µM linoleic acid. *V. cholerae* strains were grown with antibiotic concentrations: streptomycin at 100µg/mL, ampicillin at 100µg/mL, and kanamycin at 50µg/mL. A deletion of the discovered bile insensitive mutant in *pckA* was made as previously described in a P_{tcpA}::*lacZ* chromosomal fusion in *V. cholerae* O395 classical to

characterize (220). This strain containing *pckA* in pBAD33 was also made as a complement to express in *trans*. Deletions of phosphoenolpyruvate carboxylase and isocitrate dehydrogenase were also made and put into *V. cholerae* with a P_{tcpA}::*lacZ* chromosomal fusion.

Transposon mutagenesis. Previously a transposon library had been made using the suicide plasmid pFD1 in our lab (230). Here we followed the same procedure. Briefly, *V. cholerae* was co-incubated with SM10 (λpir) in *E. coli* on nitrocellulose filter paper for three hours at 37°C. Cells were added to media with streptomycin, ampicillin and 2 mM IPTG to induce transposase expression. Bacteria were grown overnight and then transposon mutants were selected on LB plates with streptomycin, ampicillin and kanamycin. Plates were incubated for 7 hours at 37° C to allow for sufficient colony formation. Colonies were then collected and cultured in LB under inducing conditions with 0.5% sodium choleate (bile) or 160 µM linoleic acid. Bacteria alone were also cultured as a control. Collected mutants were directly sequenced using genomic DNA and primers that bind near the ends of the transposon: BP30 (5'-ATGCATTTAATACTAGCGACGCC-3') or BP 31 (5'-CGCTCTTGAAGGGAACTATGTTG-3').

Flow cytometry and FACS. As previously described (230), *V. cholerae* classical strain O395 and an otherwise isogenic strain containing a P_{tcpA}::*gfp* chromosomal fusion were grown under virulence-inducing conditions for 3 hours in the presence or absence of bile or linoleic acid. *V. cholerae* lacking the *gfp* fusion was grown in the absence of effector to set the parameter channels of the flow cytometer and the cell sorter in the three parameters; forward scatter (FSC), sideward scatter (SSC) and green fluorescence (515–545 nm), using a logarithmic scale. For flow cytometry analysis, the expression of *gfp* was measured by using a FACSscan flow cytometer (Becton Dickinson) and the data were further analyzed with FlowJo analysis

software. For bacterial sorting, high GFP-expressing mutants were sorted using a FACS Diva (Becton Dickinson) into LB media and plated to save individual colonies on LB plates with ampicillin, streptomycin and kanamycin.

β-galactosidase assays and CT ELISA. β-galactosidase activity was measured using the basic procedure of Miller to confirm mutants were insensitive to bile (212). CT was detected in the culture supernatant by a GM1 enzyme-linked immunosorbent assay (ELISA) (228), using polyclonal anti-CT antibody (Sigma). All strains and mutants tested were grown under virulence-inducing conditions for three hours with and without bile and compared.

RESULTS AND DISCUSSION

P_{tcpA}*::gfp* shows decreased expression in the presence of bile or linoleic acid. To look at the effect of bile on virulence gene expression, we used a previously constructed *gfp* plasmid fusion that contains the promoter of *tcpA.* TCP is one of the major virulence factors necessary for host colonization and therefore is a useful indicator of virulence gene expression. As an experiment for proof-of-concept, we began by looking at the effect of bile or one of its unsaturated fatty acid components, linoleic acid, on P_{tcpA} expression using flow cytometry (Figures 20A,B). After growing the bacteria under virulence-inducing conditions either alone or in the presence of one of the effectors, we measured GFP expression. Without bile or linoleic acid, there were high levels of GFP expressed, but upon the addition of either of the negative effectors, GFP levels were low. Figure 20B also shows that, at a concentration as low as 16 μM, linoleic acid still has an effect on *tcpA,* but at a concentration lower than that, virulence expression is unaffected. These data indicate that GFP levels are high under virulence-inducing

conditions, and low upon the addition of negative effectors, indicating this will be a useful screening system for use with FACS collection and analysis.

Collection of *V. cholerae* mutants potentially insensitive to bile by FACS. The general strategy to collect bile insensitive mutants is shown in Figure 21A. We began by creating a *mariner*-based transposon mutant library in *V. cholerae* O395 that carried chromosomal P_{tcpA}*::gfp.* To identify mutants that had lower sensitivity to bile, and therefore had high GFP expression in the presence of bile (Figure 21C), we grew the mutant pool in the presence of bile and selected for the highest expressing GFP producers (Figure 21D). We then took this pool, grew the bacteria overnight in LB alone to allow recovery, and then subjected the pooled mutants to bile treatment again under virulence-inducing conditions, hoping to enrich for the mutants that were the least sensitive to effects of bile and thus had the highest GFP levels (Figure 21E). On the second day of sorting, there were more mutants expressing high levels of GFP, and these mutants were collected for further characterization.

***pckA* was identified as the most common mutant in the screen.** After FACS analysis, mutants were characterized by β-galactosidase assay to ensure that the transposon insertion was not present in the P_{tcpA}*::gfp* construct to somehow cause constitutive activation of GFP. Each mutant was tested individually to look at bile sensitivity. The most common disrupted gene found in the screen was *pckA,* encoding phosphoenolpyruvate carboxykinase, an enzyme involved in central metabolism that converts oxaloacetate into phosphoenolpyruvate. Another gene involved in cell envelope biosynthesis was identified as well, D-alanyl-D-alanine carboxypeptidase, but was not studied further.

Although the high number of individual *pckA* mutations recovered from the screen was initially promising and suggested a key role for *pckA* in virulence, Δ*pckA* strains quickly reverted back to wild-type levels of virulence activity, making it very difficult to characterize them further. Compared to wild-type, Δ*pckA* showed only a slight decrease in bile sensitivity in both β-galactosidase assays and cholera toxin ELISAs (Figures 22A,B,C). In fact, in the absence of bile, Δ*pckA* actually made more cholera toxin when grown under inducing conditions. It is certainly possible, if not probable, that the Δ*pckA* strain acquired a secondary mutation that compensated for the deletion and increased bacterial fitness, as we suspect was rapidly happening with the *pckA* transposon insertion mutants. In spite of this, we examined other genes involved in the citric acid cycle to determine whether central metabolism may play a role in virulence gene expression levels (Figure 22C). Phosphoenolpyruvate carboxylase, which converts phosphoenolpyruvate into oxaloacetate, is an enzyme that works in the opposite direction of PckA, and, when deleted, showed no difference in virulence gene expression compared to wild-type (Figures 22D,E). Isocitrate dehydrogenase, which works at the rate-limiting step of the citric acid cycle to convert isocitrate into α-ketoglutarate, also showed no effect on virulence expression when deleted (Figures 22D,E). That said, more recent evidence suggests that metabolic proteins may have multiple functions and therefore act as "moonlighting proteins" (231). These proteins are generally highly conserved (232, 233). Although *pckA* has not yet been discovered to have multiple roles aside from metabolism, the possibility is there. The fact that the Δ*pckA* strain very quickly reverted back to wild-type virulence gene expression levels demonstrates the importance of this gene to bacterial fitness.

Even though the insensitivity of bile was a modest effect, at only a 10% difference from wild-type (Figure 3B), the possibility is there that *pckA* may have a small role in virulence regulation.

FACS is a useful technique that could be used in other liquid-based screens to identify genes involved in other types of effectors or conditions. As only the highest expressing GFP mutants were collected, it may be more useful in future work to collect other mutants that were expressing GFP levels more comparable to bacteria grown without bile.

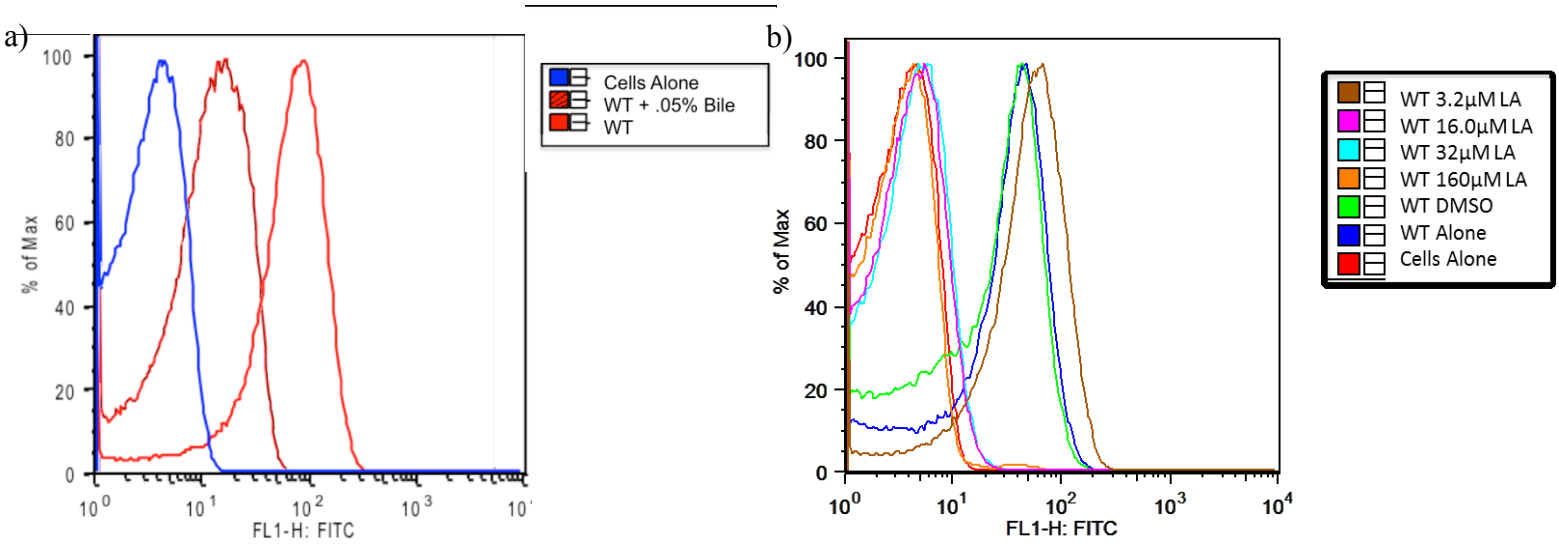


FIGURE 20. Flow cytometry of negative effectors on virulence expression. Classical biotype O395 *V. cholerae* was grown under virulence-inducing conditions. All histograms show the level of *gfp* fluorescence intensity with a *tcpA::gfp* fusion plasmid. Cells alone have no *gfp* reporter plasmid and are used to gate proper cell size and as a negative control (A) *V. cholerae* grown with or without the presence of .05% bile. (B) *V. cholerae* (B) *V. cholerae* grown with various dilutions of linoleic acid.

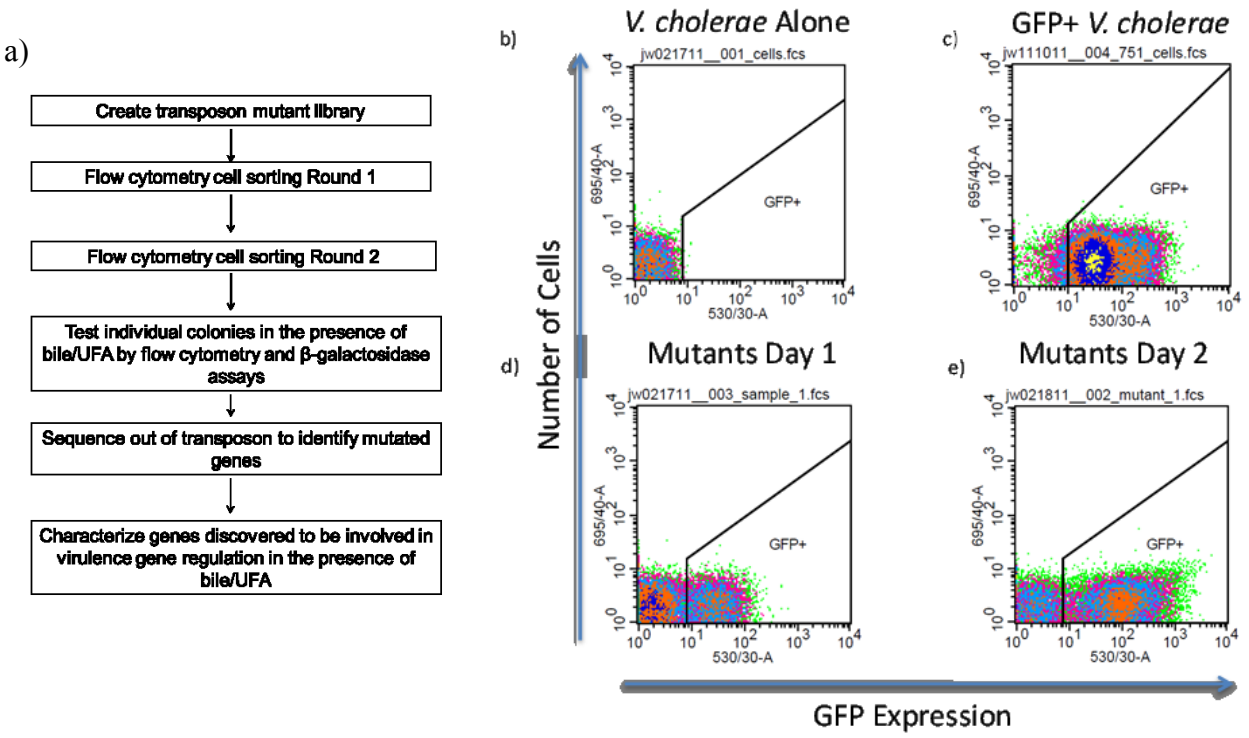


FIGURE 21. Collecting bile insensitive mutants using FACS (A) A general strategy for developing a transposon mutant library for identifying genes involved in response to bile. (B)-(D) FACS analysis of *V. cholerae* (B)*V. cholerae* grown without *gfp* as a size control (C) *V. cholerae* grown under virulence-inducing conditions without bile with high expression of P_{tcpA}::*gfp*. (D) A range of *gfp* expressing mutants was produced when grown under inducing conditions. Most of the mutants produce low fluorescence while some produced high *gfp*. (E) After overnight enrichment of high *gfp* expressing mutants, most mutants express high levels of *gfp*

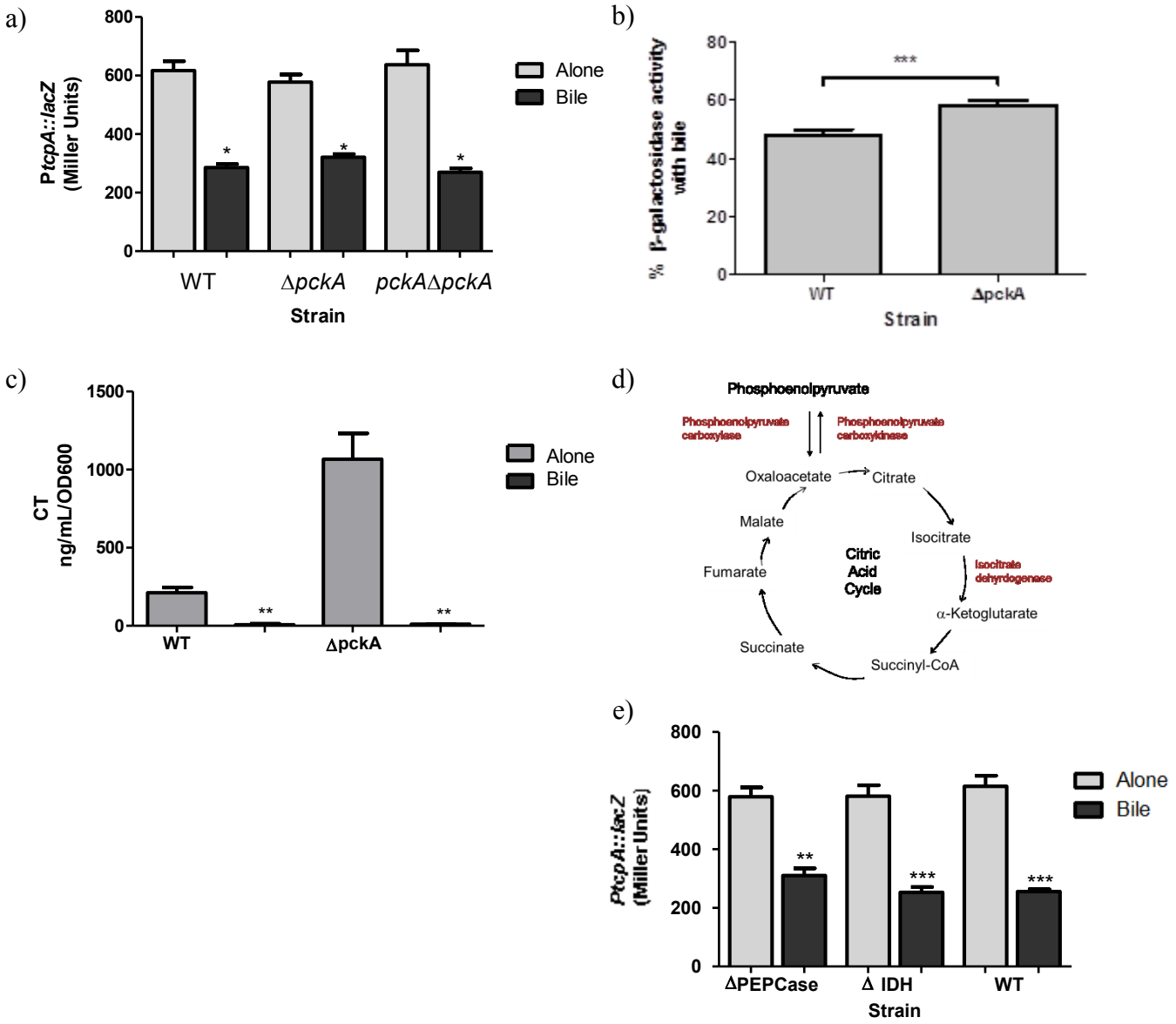


FIGURE 22. Characterization of *pckA* involvement in virulence regulation. (A) *V. cholerae* was grown under virulence-inducing conditions. β-galactosidase assay with and without .05% bile. WT – wild-type, *pckA*Δ*pckA* is deletion strain with a *pckA* complement in pBAD33 (B) Data expressed as a percent of activity between bile compared to no bile for WT and Δ*pckA* (C) CT expression with and without .05% bile from the supernatant of *V. cholerae* grown under virulence-inducing conditions (D) Citric acid cycle and location of deleted genes (E) Other metabolism genes deleted and grown under virulence-inducing conditions with and without .05% bile. PEPCase – phosphoenolpyruvate carboxylase, IDH – isocitrate dehydrogenase. Statistical significance determined using Student's *t*-test (*, p<.05; **, p<.005; ***,p<.0005).

CONCLUSIONS

Vibrio cholerae virulence gene expression is controlled by a complex regulatory cascade with its master gene regulator ToxT. ToxT directly activates the promoters of the two major virulence factors, cholera toxin (CT) and toxin-coregulated pilus (TCP). When *V. cholerae* colonizes the small intestine, it is able to use environmental signals produced by the host to regulate its gene expression. These environmental effectors include the positive effector, bicarbonate, as well as the negative effector bile, partially composed of unsaturated fatty acids (UFAs) that are thought to be directly responsible. This study has been aimed at elucidating the mechanism by which bile and its unsaturated fatty acid components act to downregulate virulence.

Here, we show that bile and UFAs decrease virulence gene expression. Linoleic acid, specifically, is able to enter the cell, where it can then act on ToxT in the cytoplasm. These negative effectors act directly on ToxT, causing decreased binding affinity of ToxT for the promoters of virulence genes including *acfA, acfD, aldA, tagA, tcpA,* and *tcpI*. As most promoters have two ToxT binding sites, it has been hypothesized that ToxT acts as a dimer and UFAs decrease the ability of ToxT to dimerize. However, *aldA* only has one ToxT binding site, and the observations that *aldA* gene expression was downregulated and the ability of ToxT to bind to the *aldA* promoter was inhibited by linoleic acid suggest a mechanism by which UFAs affect monomeric ToxT. As the data showed, *aldA* activity was substantially lower than the two ToxT-binding site promoters, suggesting that ToxT dimers activate better, but ToxT monomers can still activate gene expression. Interestingly, in RNAseq experiments, *aldA* was the gene

whose expression was most highly upregulated in the presence of bicarbonate, the positive ToxT effector (data not shown).

The unstructured region of ToxT, found when ToxT was crystalized, was mutated to look at the effect of bile and UFAs on ToxT itself. We found mutations that were involved in response to bile and UFAs. The activity of one mutant in particular, N106F, was not affected by the addition of bile or UFAs in terms of virulence gene expression as well as ToxT EMSAs. We hypothesize that the large phenylalanine residue may mimic the conformation of ToxT when bicarbonate is bound, and therefore, bile and UFAs no longer have an effect.

Conjugated linoleic acid (CLA) was studied for a potential use as a prophylactic or therapeutic. *In vitro* CLA downregulates virulence gene expression substantially. Using the infant mouse model, CLA had no effect on *V. cholerae* colonization. However, there were significant problems with using this particular animal model for these experiments. Rabbit ileal loop assays indicated that both fluid volume and CT concentration were lowered in the presence of CLA, suggesting that CLA may be effective as a therapeutic to reduce disease symptoms. Further work will be necessary to explore this possibility.

Last, a transposon mutant library for use in fluorescence activated cell sorting (FACS) genetic screening identified an insertion in *pckA*, a gene important in metabolism. The *pckA* mutant showed higher expression levels of P_{tcpA}-*gfp* in the presence of bile. Further studies showed that a *pckA* deletion mutant was only slightly less sensitive to bile, but this does suggest that genes important in metabolism can have effects on virulence gene expression.

Future Directions

Although much work has been done to determine the mechanism by which UFAs downregulate virulence, there is still much work that can be done. To begin, it would be useful to use the two-hybrid bacterial system to look at linoleic acid's effect on dimerization. Although our data suggest it is not dimerization by which linoleic acid decreases ToxT DNA binding affinity, this would add further confirmation. Although not discussed in this dissertation, surface plasmon resonance (SPR) was explored to identify binding changes upon addition of linoleic acid. Further exploration of this technique could give real-time data of the binding patterns of ToxT in response to linoleic acid, and potentially, the heterogeneous mixture of bile. This could include using the ToxT mutations in the unstructured region and comparing binding patterns as well. Also, the interplay of linoleic acid and the positive effector bicarbonate is something that could be further explored either through more EMSAs or SPR. Adding an additional toxbox to the *aldA* promoter or removing one from a promoter with two toxboxes may give some more useful information on the regulation of the effectors. Also, we could use human epithelial cells and add exogenous bile and/or bicarbonate to study surface colonization that could give greater understanding to how these effectors may act in the host.

The CLA aspect of this project shows a substantial negative effect of CLA *in vitro*. Experiments with rabbit ileal loops also indicated that CLA reduced CT production in vivo. The use of CLA as a prophylactic or therapeutic could be useful, but much work still needs to be done including identifying the right concentrations of CLA to use.

Characterization of the other genes identified in the FACS screen would also be useful. Potentially there is a transport mechanism that imports UFAs into the cell, and discovery of

genes responsible for this would further our knowledge of a location in which other negative effectors (therapeutics) could target to reduce disease in humans.

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ABSTRACT

THE MECHANISM FOR INHIBITION OF *VIBRIO CHOLERAE* VIRULENCE GENE EXPRESSION BY BILE AND ITS FATTY ACID COMPONENTS

by

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Vibrio cholerae is responsible for the diarrheal disease cholera by producing two major virulence factors: cholera toxin (CT) and toxin-coregulated pilus (TCP). During infection, *V. cholerae* downregulates its motility genes, which are actively expressed in the environment, and upregulates its virulence genes, which are inactive in the environment. Virulence gene transcription is regulated by the major transcriptional activator, ToxT. ToxT is regulated, in part, by host signals, which in turn, make ToxT active or inactive in respect to virulence gene expression. Host signals include temperature and pH as well as the chemical molecules bicarbonate and bile, which are positive and negative effectors of virulence, respectively. Bile is a heterogeneous mixture that includes unsaturated fatty acids (UFAs) that have been directly implicated in virulence gene downregulation. Another small molecule, virstatin, has also been shown to cause virulence gene downregulation. This dissertation focuses on the mechanism by which bile and its UFA components, specifically linoleic acid, downregulate virulence gene expression.

The first chapter focuses on the decreased binding affinity of ToxT for various virulence gene promoters at the ToxT binding sites, termed toxboxes. These toxboxes come in different configurations and orientations, and while most promoters have two toxboxes, *aldA* only has one. I showed that linoleic acid can enter the cell where it can go into the cytoplasm and then interact with ToxT. It has been hypothesized that UFAs cause decreased dimerization; however at P_{aldA}, linoleic acid still had an effect. Virstatin, which has been shown directly to decrease dimerization of ToxT, did not have an effect on ToxT DNA binding at P_{aldA}. This suggests a mechanism in which UFAs do not affect dimerization but can affect monomeric ToxT binding to DNA.

In chapter two, we characterize an unstructured region of the N-terminal domain of ToxT in response to bile and UFAs. By mutating each of the 10 amino acids in the region, we discovered a mutant, N106F, which had decreased response to bile and UFAs. It also showed decreased response to the positive regulator bicarbonate.

Chapter three discusses the possibility of using conjugated linoleic acid (CLA) as a therapeutic or prophylactic upon exposure to *V. cholerae*. The *in vitro* work showed a strong decrease of ToxT activity in response to CLA on both gene expression and DNA binding. In the infant mouse model, CLA did not inhibit colonization, but in the rabbit ileal loop model CT production was significantly decreased, suggesting that CLA may work to reduce cholera symptoms.

In the last chapter, we looked at the possibility of using fluorescence-activated cell screening (FACS) in addition to transposon mutagenesis as a method to collect mutants that were insensitive to bile. Use of this technique would allow us to discover genes that were

directly responsible for the effects of bile. Several genes were identified, including *pckA,* which is involved in metabolism, but could have a role in virulence. When deleted, however, the bacteria were only slightly less sensitive to bile than wild-type. FACS genetic screening is still a useful technique that, if further explored, could elucidate other genes involved in responding to effectors of virulence.

AUTOBIOGRAPHICAL STATEMENT

I was born in Bolingbrook, Illinois 1986 and moved to Rochester, Michigan in 1989. I lived there until I graduated from Stoney Creek High School in 2004 and went on to pursue a B.S. in biochemistry. It was during this time that I found my love of research. A microbiology professor of mine, Dr. Sonia Tequia, asked if I would go with her to Oak Ridge National Laboratory for a summer research project on discovering ethanol producing bacteria using only cellulose as their carbon source. It was here that I fell in love with research. Once I came back to Michigan, I immediately applied to Wayne State University to their Immunology and Microbiology program. I then joined Jeff Withey's lab to work on bile regulation of *Vibrio cholerae* virulence gene expression. Here, I have had the pleasure of working with many great scientists and have been so lucky to be able to also be able to travel to present my research. This entire experience has really been an amazing experience that will shape me into the best scientist that I can become.