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Post-transcriptional regulation of vibrio cholerae virulence activator toxt

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**POST-TRANSCRIPTIONAL REGULATION OF *VIBRIO CHOLERAE*
VIRULENCE ACTIVATOR TOXT**

by

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DISSERTATION

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of Wayne State University,

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GENERAL INTRODUCTION

Cholera is a human illness that is characterized by massive watery diarrhea, leading to severe dehydration and hypovolemic shock if left untreated. The characteristic symptom of the disease is the painless shedding of a voluminous stool that resembles rice-water. Individuals can purge up to one liter per hour, which is equivalent to 50lb water loss within one day. Cholera is endemic in Southeast Asia, Africa and Latin America where outbreaks are associated with poverty and poor sanitation. Although this illness can be treated simply by the replacement of fluid and electrolytes, it is associated with a 20-50% mortality rate without medical treatment (149).

Cholera, which has a long history associated with humans, has had a significant impact on science. There have been seven cholera pandemics since 1817, when the disease first spread from the Bay of Bengal along trade routes. In earlier times, it was thought that cholera was spread like a fog, or “miasma” coming from the river. However, during the third pandemic in 1854, which reached the British Isles, the classic epidemiologic study of John Snow showed that the disease was associated with ingestion of contaminated water (165). This study was the fundamental basis for the field of epidemiology. Around this same time, Filippo Pacini identified small comma-shaped particles, which he described as *Vibrios*, associated with the intestinal epithelium of deceased cholera patients in Florence, Italy (12). Pacini published this work in 1854 but it was almost completely ignored by the scientific community, and not until 85 years later was

he recognized as the discoverer of *Vibrio cholerae*, the causative organism of cholera. During the fifth pandemic in 1883, Robert Koch isolated *Vibrio cholerae* from a cholera patient in Calcutta, India, and was able to obtain a pure culture of the bacteria (71). Although this bacterium failed to adhere to Koch's postulate that it causes disease in animals, Koch correctly interpreted the reason for this as being the disease was human specific. Koch's work on cholera was essential in showing that a microorganism can cause disease, in addition to being a huge step in establishing the field of microbiology.

Currently cholera outbreaks are still causing health disasters in many different parts of the world. It has been estimated that there are 3–5 million cholera cases and 100,000-120,000 deaths per year worldwide (34, 201). A major outbreak occurred in 1994 in Goma, Zaire, which resulted in 70,000 cases and 12,000 deaths (158). A more recent epidemic occurred in Zimbabwe. By July 2009 it was reported that there were more than 98,000 cholera cases and 4,000 deaths (1). It is important to realize that these figures might worsen in the near future because cholera outbreaks are heightening. The rising sea levels and an increase in water temperature are likely to dramatically increase cholera outbreaks since coastal aquatic environments are a natural reservoir for *V. cholerae* (44), the etiological agent of the disease. Thus studying and understanding the pathogenesis and ecology of this organism should help in developing strategies to reduce the severity and spreading of the disease.

V. cholerae is a Gram-negative curved bacillus, and is highly motile having a single polar sheathed flagellum. It is classified into serogroups based on its lipopolysaccharide (LPS) O antigen, and more than 200 serogroups have been identified to date (44, 149). Only serogroups O1 and O139 are responsible for epidemic and pandemic cholera (14, 40, 131). Serogroup O1 can be further divided into two biotypes, classical and El Tor, and this biotype classification is based on differing biochemical properties and susceptibility to bacteriophages (44). Each biotype is further subdivided into Ogawa, Inaba, and Hikojima serotypes based on antigenic profile. Ogawa strains produce antigen A, B and a small amount of C. Inaba strains produce antigen A and C, and Hikojima strains produce small amount of all the three antigens (149). Although *V. cholerae* isolates are available only from the 6th and 7th pandemics, it is thought that the classical biotype was responsible for the first six pandemics while El Tor is responsible for the current, seventh pandemic which began in Indonesia in 1961 (44, 149). Non-O1/ non O139 serogroups have also been associated with diarrheal illness. However, these serogroups are not associated with endemic or pandemic cholera and they cause illness that is self-limiting and is rarely lethal (15).

V. cholerae is a natural inhabitant of coastal areas as it associates with crustaceans such as copepods and shellfish, insect egg masses, and vertebrate fish (17, 29, 60, 78, 153). Humans are the only known natural host in which *V. cholerae* causes disease and an infection is acquired orally by ingesting

contaminated food or water (44, 149). Once the bacteria enter the body, they swim to the small intestine where they adhere to the epithelial cells and cause diarrhea. By inducing diarrhea, *V. cholerae* are shed back into the environment in the form of rice-water stool. The exact stimuli that induce virulence during infection have not been identified. Once the bacteria reach the duodenum of the small intestine, motility gene expression is down-regulated, virulence gene expression is up-regulated and the disease is initiated. Later in infection, virulence gene expression is down-regulated, motility gene expression is up-regulated, and the bacteria escape the host. This “mucosal escape response (133)” also requires expression of other genes. For example, proteases are induced to help the bacteria detach from the epithelial surface while motility and chemotaxis genes allow the bacteria to swim away, exiting the host and entering back into the environment where *V. cholerae* completes its life cycle.

In aquatic environments, *V. cholerae* persist between epidemics and this persistence is thought to be facilitated by biofilm formation and entering into a viable but non-culturable state (VNC). *V. cholerae* are found free swimming in planktonic form, or associated with marine organisms such as plants, green algae, copepods, chironomid insects, and fish (17, 29, 60, 78, 80, 153). It has been shown that *V. cholerae* form biofilms in the aquatic environment (4). Bacteria within the biofilm are more resistant to several kinds of environmental stress including antibiotics (156). When environmental conditions change, *V. cholerae* sense these changes and modulate gene expression to adapt to these

new conditions. Nutrient starvation and other stress responses that are found in the aquatic environment are postulated to induce a VNC state form of *V. cholerae*. These VNC *V. cholerae* cannot be recovered by standard culture technique and so they are difficult to characterize. However, these seemingly “dead” bacteria can cause infection and revert back to culturable bacteria when grown with eukaryotic cells (5, 154).

There are two distinct acquired genetic elements that distinguish toxigenic *V. cholerae*, i.e. strains that can cause cholera, from non-toxigenic strains: the filamentous CTX bacteriophage (CTX Φ) (186), which encodes the cholera toxin genes, and the *Vibrio* pathogenicity island (VPI) (91), which encodes the genes that are required for production of toxin-coregulated structural pilus, accessory colonization factor genes and several other genes having unknown roles in pathogenesis. Non-toxigenic strains of *V. cholerae* can be converted into toxigenic strains under appropriate conditions (186). Toxigenic strains can be induced to produce extracellular CTX Φ particles. These phages can be propagated into recipient non-toxigenic strains and integrate into their chromosome to form stable lysogens. However, the propagation requires the production of the toxin-coregulated pilus (TCP) by recipient cells as the phage utilizes it as an entry receptor (186). Because TCP is produced during and absolutely required for an infection, it seems that the human intestine is not the only site that the bacteria exploit for infection, but also a site that augments the conversion of non-toxigenic strains into toxigenic ones.

Regulation of *V. cholerae* pathogenesis is complex and involves numerous regulatory factors; the virulence regulon is commonly known as the ToxR regulon due to the identification of ToxR as the first positive regulator of virulence (126). The ToxR regulon includes the cholera toxin genes, the toxin-coregulated pilus structural and assembly genes, the accessory colonization factor genes, and other genes with undefined functions (43, 72, 90, 137, 177). However, ToxR is not the direct activator of expression of most, if not all, of these virulence genes. Instead, ToxR is required for production of ToxT (68), the direct virulence activator, which will be discussed in more detail below.

For *V. cholerae* to cause cholera, two major virulence factors are absolutely required: Cholera toxin (CT), which causes the diarrhea (45, 119), and TCP, which is required for colonization of the human intestine (66). CT is the most important *V. cholerae* virulence factor and is directly responsible for the watery diarrhea that is the hallmark of cholera. CT is encoded by the *ctxAB* genes on the filamentous lysogenic CTX Φ bacteriophage (186) and is a classical AB₅ toxin composed of pentameric B subunits and one enzymatic A subunit (52, 110). The B subunits recognize GM1 ganglioside receptor on epithelial cells and facilitate translocation of the A subunit in the cells (70, 185). Activity of the A subunit requires processing to release the catalytic A₁ component. The catalytic A₁ subunit modifies adenylate cyclase by adding an ADP-ribosyl group to keep it locked in an active state and results in a greater than 100-fold increase in cellular cAMP (53, 149). As a consequence, an alteration in ion transport is manifested

by increasing chloride secretion and inhibition of sodium chloride absorption. Osmotic balance results in massive amounts of water being secreted along with the chloride ions, and the net result is a massive outpouring of fluid into the small intestine causing the watery diarrhea (45). In addition, CT inhibits water absorption by the colon, which also increases the severity of the disease (166). The secreted fluid is characterized by high amounts of sodium, chloride, potassium, bicarbonate and more importantly infectious *V. cholerae* (127). These secreted fluids could then get released back into the environment where water sources become contaminated. To make matters worse, the *V. cholerae* in rice-water stool are hyperinfectious; greater than 10^6 bacteria are required for an initial infection, but once the *V. cholerae* have passed through a host, the infectious dose is lowered to under 100 bacteria (21, 132, 199). The end result is a quick spreading of *V. cholerae* within the population and an epidemic is initiated.

The TCP type IV pilus is the other virulence factor that is absolutely required to be expressed by *V. cholerae* in order to colonize the human intestine and cause disease (66). It is termed “toxin co-regulated pilus” because its expression occurs under the same conditions as those of the cholera toxin. The TCP is composed of 5-6nm filaments containing a bundle of the TcpA pilin subunit (59). Production of the pilus by *V. cholerae* leads to agglutination of the cells. This auto-agglutination phenotype also provides a simple way to determine whether the TCP is produced in bacteria grown in the laboratory. The major

function of the TCP is to aid the overall colonization of the intestinal epithelial lining. A *tcpA* mutant strain of *V. cholerae* neither colonizes the intestine of human volunteers nor does it colonize the intestine of the infant mouse model (66, 179). The mechanism by which the TCP promotes intestinal colonization is not clear. However, *in vitro* and *in vivo* data suggest that it occurs through the formation of microcolonies via pilus-pilus contacts between individual bacteria to enhance overall colonization (89).

Although the pilus is a polymer of repeating subunits of one protein (TcpA), several other genes are involved in the biogenesis of the pilus structure. The *tcpB*, *tcpQ*, *tcpC*, *tcpR*, *tcpD*, *tcpS*, *tcpT*, *tcpE*, *tcpF* and the *tcpJ* genes are all encoded within one operon termed the *tcp* operon (44) (Fig. 1). These genes are located downstream of and in the same orientation as *tcpA* (79). They are transcribed along with *tcpA* as a long transcript to promote the assembly of the TCP structure on the surface of the bacteria. Transposon insertions that disrupt *tcpB*, *tcpC*, *tcpD*, *tcpE*, or *tcpF* prevent assembly of a functional pilus on the resulting bacterial surfaces, despite the fact that the TcpA pilin is still expressed (16). The functions of these pilus biogenesis genes are poorly understood. TcpB has a pilin-like prepeptide sequence and has been proposed to regulate pilus length and/or function as a pilus anchor (175). TcpT possesses a putative ATP-binding domain (135), which might function as an engine to drive the pilus translocation complex. TcpF is a soluble factor that is secreted by *V. cholerae* and is important for bacterial colonization of the intestinal epithelial cells (98,

128). TcpJ is the best-described protein, encodes a leader peptidase that is utilized to process the TcpA protein during secretion (87).

Expression of a collection of other genes carried on the VPI is also co-regulated with CT and TCP. These include the *acfABCD* genes, which encode the ACF, as well as *tagA*, *aldA*, and *tcpI* (39, 43, 61, 136, 137, 152, 190, 192). The functions of these genes and their requirements for inducing disease are not understood. However, deletion of any of the *acf* genes results in alteration of the swarm motility, a phenotype that is associated with altered chemotaxis (42, 74). In addition, based on protein alignments, AcfB and TcpI showed a significant relationship with other enteric methyl-accepting chemotaxis proteins (26, 42, 62). Collectively, *V. cholerae* may utilize these accessory genes to sense chemoattractants that are found in the small intestine milieu to guide the bacteria to the appropriate niche. *tagA* encodes a putative lipoprotein of unknown function (61), and *aldA* encodes an aldehyde dehydrogenase that has an unknown role in pathogenesis (136).

Aside from the virulence factors that are acquired by the two separated mobile genetic elements, motility and chemotaxis genes are found in all *V. cholerae* strains and play an important role in establishing an infection. *V. cholerae* is highly motile via a single polar flagellum. There are conflicting results regarding the requirement of motility for virulence (47-49, 146, 178, 194, 195). This is the case especially when comparing different animal models and also when comparing the classical versus the El Tor biotype. However, defined non-

motile mutants of the El Tor biotype are attenuated in the infant mouse model as several labs have demonstrated (20, 102, 159). The most convincing evidence to argue that motility is important in infection come from vaccine trials. Live-attenuated *V. cholerae* vaccine produces side effects such as nausea, cramps and diarrhea, while non-motile variants do not (31, 88, 120). These differences were further linked to the ability of *V. cholerae* to reach the epithelial surfaces. In addition, there is an inverse relationship between motility and virulence gene expression. Hyper-swarming motile bacterial strains produce lower levels of TCP and CT, while non-swarming motile bacteria produce a larger amount of TCP and CT when compared to the wild type parent strain (49, 63, 64). Thus the current favored model is that motility allows the bacteria to penetrate the mucus layer to reach the epithelial cells, which is important for colonization and inducing disease.

Chemotaxis genes have also been shown to be involved in the pathogenesis of *V. cholerae*. The genome of *V. cholerae* encodes three different general chemotaxis operons. However, only one of them is required for chemotaxis and the functions of the other two are unknown (54). Chemotaxis functions to direct movement of the bacteria in certain directions. This is achieved by changing the direction of flagellar rotation from the default counterclockwise (CCW) to clockwise (CW) direction. The CCW rotation propels the cells forward by smooth swimming in one direction while CW rotation causes the cells to tumble to change direction. CW-biased mutants have defects in net

movement and are attenuated for infection (20). However, mutants with a CCW-bias out-compete wild type *V. cholerae* during an infection (20). This competitive dominance correlates with increased infectivity. Additional analysis revealed that this CCW-biased mutant is able to colonize the entire mouse intestine, whereas wild type *V. cholerae* preferentially colonize the distal end of the infant mouse intestine (20). This may suggest that both motility and chemotaxis are tightly regulated to direct *V. cholerae* to a localized niche during an infection.

Regulation of *V. cholerae* virulence determinants requires a complex cascade of transcriptional events that involves numerous regulators. A schematic of virulence induction is shown in Fig. 2. ToxT is the major transcriptional activator that directly and positively induces expression of the vast majority of virulence genes including those that encode the major virulence factors CT and TCP (75, 190-192, 197, 198). Expression of *toxT* is induced by two membrane dimers, ToxR/ToxS and TcpP/TcpH (39, 65, 67, 100). While the *toxRS* operon is thought to be constitutively active, transcription of the *tcpPH* operon requires cooperation between two other transcription activators, AphA and AphB (94, 160). Other regulatory circuits that affect AphA induction include quorum sensing and small regulatory RNAs, which work through the master regulator quorum sensing regulator, HapR (97, 124).

ToxT is an AraC/XylS family protein (68) that directly activates transcription of the virulence genes of *V. cholerae*. ToxT positively activates transcription of several operons including *tcp*, *ctx*, and *acf*. It also activates

transcription of genes encoding a putative lipoprotein (*tagA*), aldehyde dehydrogenase (*aldA*) and methyl-accepting chemotaxis protein (*tcpl*) (75, 190-192, 197, 198). As previously mentioned, the functions of some of these genes in pathogenesis are well characterized while the functions of others are unknown. The 276 amino acid long ToxT protein is separated into two domains: amino acids 1-160 comprise the N-terminal domain (NTD), whereas amino acids 170-276 comprise the C-terminal domain (CTD). A linker (amino acids 161-169) further connects the two domains (111). The ToxT CTD shares the AraC/XylS family sequence homology and contains two helix-turn-helix motifs that are utilized for DNA binding. The NTD does not share significant sequence similarity with any protein in the database and its role is hypothesized to be for dimerization and/or for interacting with effectors that modulate ToxT activity. However, the crystal structures of ToxT protein and the AraC protein NTD indicate that the ToxT NTD and the AraC NTD share some structural similarity despite having only 11% amino acid identity (111). The AraC NTD is required for dimerization and binding to its effector, arabinose (151). It has been proposed that bile or the unsaturated fatty acids (UFA) present in bile are natural effectors of ToxT. Bile and UFA have been shown to inhibit ToxT activity and the overall growth of *V. cholerae* (57, 76, 152). Mutational analysis demonstrated that bile might interact with the N-terminal domain of ToxT, as a ToxT mutant somewhat resistant to bile was isolated that had a single amino acid change at residue 107 (141). Furthermore, the crystal structure of ToxT contained a buried 16-carbon

UFA, *cis*-palmitoleate, which inhibits the activity of ToxT and may be a minor component of crude bile (111). Virstatin, a synthetic compound that was found to reduce *ctxAB* expression, also likely interacts with ToxT in the same region as bile; a L114F mutation in ToxT makes ToxT resistant to virstatin (77, 155). This suggests that this region of the NTD is important for controlling ToxT activity.

Although the activities of ToxR/S and TcpP/H are required for ToxT production, evidence of ToxT auto-regulation also exists. The genetic organization of the *tcp* operon is shown in Fig. 1; the *toxT* gene is located within this *tcp* operon between *tcpF* and *tcpJ*. Transposon insertions in the region between *tcpA* and *tcpF* cause a polar effect on ToxT activity as determined by measuring CT production and expression of a *tcpA-lacZ* fusion (16). Further analysis of *toxT* transcripts using primer extension revealed the presence of transcripts that originated from two different promoters. Short transcripts correspond to product that depends on the *toxT* promoter and long transcripts correspond to *tcpA* promoter activity (67, 197). Transcription from the *tcpA* promoter depends on the activity of ToxT and the long *toxT* transcripts depend on the *tcpA* promoter, suggesting a model in which ToxT positively activates more of itself through an auto-regulatory loop that is initiated from the *tcpA* promoter.

ToxT activates the transcription of virulence gene promoters by binding to degenerate thirteen base-pair DNA sequences termed toxboxes (191) (Fig. 3). These toxboxes are organized differently within each promoter. For example,

within the *tcpA* promoter there are two toxboxes that are organized as a direct repeat, while between the divergently transcribed *acfA* and *acfD* genes the two toxboxes are organized as an inverted repeat. Furthermore, within the *aldA* promoter there is only a single toxbox, while the *ctxAB* promoter contains a series of half toxboxes that are oriented as direct repeats. Although the toxbox configuration and spacings are different within each promoter, all toxboxes are located upstream of the core -35 sequence. This is consistent with class I promoter architecture and suggests that ToxT directly interacts with RNA polymerase α subunits to induce transcription (19). Mutational analysis of toxbox spacings revealed that ToxT binds as a monomer to each toxbox and dimerization is not required for DNA binding (11, 190, 191). However, dimerization might be required to attract RNA polymerase and induce gene expression. It has been proposed that virstatin acts to inhibit ToxT activity by inhibiting dimerization; however, currently the evidence for this model is not compelling. The difference in the architecture of the toxboxes may suggest a unique interaction between ToxT and RNA polymerase at each promoter, which dictates the strength of that promoter.

The ToxR/ToxS and the TcpP/TcpH dimers act in conjunction to control the activation of *toxT* transcription (39, 65, 67, 100). The *toxR* gene is not linked to the two acquired genetic elements, CTX Φ and VPI. ToxR is found in all *V. cholerae* strains as well as other *Vibrio* species and was discovered based on its ability to activate expression of a *ctxAB::lacZ* fusion construct in *E. coli* (126).

ToxR is a 32kDa integral membrane protein that is localized to the inner membrane and contains a cytoplasmic DNA binding domain, a transmembrane domain, and a periplasmic domain. Its dimerization partner protein, ToxS, which is also an integral membrane protein, enhances ToxR activity (37, 41, 138). Current models suggest that ToxS functions to facilitate assembly and to stabilize ToxR. The ToxR/ToxS dimer also regulates the expression of other genes such as those encoding outer membrane porins (Omp). ToxR/S positively activates *ompU* transcription while repressing *ompT* transcription (33, 106). These outer membrane porins have been shown to protect the bacteria against bile that is secreted into the small intestine (142).

The TcpP/TcpH dimer is also required for activation of the *toxT* promoter (65, 100). Like ToxR, TcpP is an integral membrane protein that is localized to the inner membrane and contains a cytoplasmic DNA binding domain, a transmembrane domain, and a periplasmic domain. It also has a dimerization partner, TcpH that functions to stabilize TcpP and protect it from proteolytic degradation (8, 23). Utilizing the cytoplasmic DNA binding motifs, ToxR and TcpP bind to the *toxT* promoter and positively activate the transcription of *toxT* (65, 100). The binding sites for ToxR and TcpP proteins within the *toxT* promoters are located in close proximity; ToxR binding sites are located between -104 to -68 while the TcpP binding sites are located between -54 and -32 relative to the start of transcription. Interestingly, overproduction of TcpP/TcpH overcomes the ToxR/ToxS requirement and can alone activate the *toxT* promoter

(100). These data may suggest that the TcpP/TcpH dimer is essential to activate the *toxT* promoter and the ToxR/ToxS dimer functions to enhance this activation.

Additional regulators tightly regulate the *tcpPH* promoter, while the *toxRS* is constitutively active under several growth conditions. AphA and AphB cooperatively activate the *tcpPH* promoter (94, 160). AphA shares sequence homology with the PadR repressor, which regulates expression of genes involved in the detoxification of phenolic acids (6). AphB is a member of LysR family of transcriptional regulators that acts synergistically with AphA to activate transcription of the *tcpPH* promoter (94). The binding sites of AphA and AphB within the *tcpPH* promoter overlap. AphA binds to a region between -101 and -71 while AphB binds to a region between -78 and -43 relative to the start of transcription (96). The regulation of AphB is not yet defined. However, expression of AphA is controlled by the master quorum sensing regulator, HapR protein (97).

Besides controlling *tcpP* and *tcpH* induction, AphA and AphB have additional roles in *V. cholerae*. AphA represses expression of genes involved in the biosynthesis of acetoin from pyruvic acid (92). Shutting off this metabolic pathway increases the accumulation of organic acid when the bacteria are grown on excess glucose or carbohydrate. Because *V. cholerae* are sensitive to acidic pH, increasing organic acids in the culture medium will lower the pH and reduce cell viability. On the other hand, recent microarray analysis has identified five genes (*cadC*, VC0770, *clc*, *nhaB* and *cah*) besides *tcpP* and *tcpH* that are

activated by AphB (93). The *cadC* gene is the best characterized of these (181). CadC is a transcriptional activator that plays a role in acid tolerance. CadC protein activates the *cadBA* promoter, which encodes the lysine/cadaverine antiporter and lysine decarboxylase respectively. These genes function to induce the synthesis and excretion of cadaverine to buffer the external acidic environment. It seems that AphB counteracts the activity of AphA to allow the cells to tolerate any environmental acidification. AphB was also recently found to have increased activity in the presence of low pH and anaerobic growth conditions (93).

Expression of AphA is controlled by the quorum sensing master regulator HapR (97). HapR shuts off expression of AphA by binding to the *aphA* promoter and repressing transcription (97). As a result, the accumulated levels of AphA are reduced and become insufficient to activate the *tcpPH* promoter. In addition to virulence, HapR plays a central role in regulating other cellular processes including biofilm formation and protease production. It represses the *vps* operon, which prevents production of the exopolysaccharide involved in building a biofilm nest (200). HapR also induces expression of HapA, a secreted hemagglutinin and protease that functions in detaching the bacteria off epithelial cells during host escape (46, 85).

The action of HapR is controlled by several quorum sensing cascades and small regulatory RNA molecules. Quorum sensing is a process of cell-cell communication that allows the bacteria to respond as a group to chemical signals

and modulate their gene expression (123). *V. cholerae* has at least three systems of signaling to control the level of *hapR* (104) (Fig. 4). The first system involves the auto-inducer CAI-1 and the sensor kinase CqsS. The second system involves the auto-inducer AI-2 and the LuxPQ receptor complex. In the presence of low concentration of CAI-1 and AI-2 at low cell density, a phosphorelay mechanism is initiated that leads to transfer of a phosphate group to LuxO resulting in activation. LuxO in turn activates the production of four regulatory sRNAs termed Qrr1-4, and, together with the chaperone Hfq protein these RNAs destabilize *hapR* mRNA (105). The third quorum- sensing system, which involves the two components VarSA, activates expression of three sRNAs (CsrBCD) to inhibit the activity of the global posttranscriptional regulator CsrA. This also leads to activation of LuxO through an unknown mechanism (104).

Other regulators that affect virulence gene expression levels, which include the catabolic activator cAMP-CAP, the histone-like protein H-NS, and the EAL/GGDEF proteins that regulate cellular concentrations of the secondary messenger cyclic diguanylate, have been demonstrated to repress virulence induction. The catabolic activator cAMP-CAP represses *tcpPH* transcription (96). H-NS, which is a histone-like DNA binding protein that also acts as a major transcriptional repressor, represses several other *V. cholerae* virulence genes including *ctxAB* and *toxT* (134), and an increase in c-di-GMP concentrations lowers accumulation of *toxT* mRNA via a mechanism that is not yet clear (180).

cAMP-CRP negatively affects regulation of virulence gene expression by repressing the *tcpPH* promoter (96). cAMP-CRP is a catabolic activator that plays a major role in cellular metabolism by regulating the utilization of carbon sources. Deletion of the *crp* gene in both the classical and the El Tor biotypes causes virulence de-repression and increased levels of TCP and CT production under non-permissive growth conditions (161). Despite the presence of potential cAMP-CRP binding sites in the *tcpA* promoter, electrophoretic mobility shift analysis (EMSA) failed to prove that purified functional CRP could occupy the *tcpA*, *toxT*, or *ctxAB* promoters. Further analysis revealed that the *crp* deletion strain increased *tcpPH* promoter activity, a step before *toxT* activation in the regulatory cascade. In addition, cAMP-CRP binds to a region between -98 and -75 of the *tcpPH* promoter relative to the start of transcription (96). This region overlaps the sites where the two activators AphA and AphB recognize and bind to activate transcription. Thus it seems that cAMP-CRP competes with these activators to turn off further activation and affects overall virulence induction.

H-NS is a global repressor, which silences virulence gene expression of *V. cholerae* at multiple levels (134). H-NS has been implicated in transcriptional repression of foreign genes that are acquired by horizontal transfer and this is true for *V. cholerae* as well. It has been demonstrated that H-NS has the ability to repress transcription from the *tcpA*, *toxT*, and the *ctxAB* promoters (134). H-NS is a small protein that is encoded by the *vicH* gene on the large *V. cholerae* chromosome. A *vicH* deletion strain de-represses virulence and produces higher

levels of CT and TCP compared to wild type parent strains under non-permissive pH growth conditions (134). The *vicH* mutant also produces higher levels of β -galactosidase from *toxT-lacZ* and *ctx-lacZ* reporter constructs when grown under many different conditions (169).

Although H-NS silences the *tcpA*, *toxT* and the *ctxAB* promoters, the mechanism by which it does so is poorly understood. There are several mechanisms that describe how H-NS represses transcription. However, they all involve direct binding of H-NS to the promoter (169). The DNA binding sequences that are usually occupied by H-NS are rich in A + T nucleotides. The *toxT*, *tcpA*, and *ctxAB* promoter regions all possess A + T rich sequence, but the H-NS binding sites within these promoters have yet to be determined. Furthermore, there is conflicting data regarding *tcpA* repression by H-NS. If H-NS repression of *tcpA* occurs, it is less dramatic than that of *toxT* and the *ctxAB* (134). Repressing the *toxT* promoter would affect the level of ToxT protein and so it would indirectly affect the transcription of the *tcpA* and the *ctxAB* promoters.

Signaling by the cytoplasmic secondary messenger cyclic diguanylate (c-di-GMP) also modulates virulence gene expression. c-di-GMP is used by most bacteria to regulate numerous cellular processes including virulence (174). The intracellular concentration of c-di-GMP is controlled by its total synthesis and degradation. It is synthesized from two GTP molecules by enzymes that harbor GGDEF domains and is hydrolyzed by enzymes that contain EAL domains into linear pGpG, and then into two GMPs by enzymes that contain a HD-GYP

domain (174). The genome of *V. cholerae* encodes 31 GGDEF, 22 EAL, 9 HD-GYP, and 9 GGDEF-EAL proteins which indicate the complex regulation of c-di-GMP cytoplasmic concentration. However, in classical biotype *V. cholerae*, increasing and decreasing cytoplasmic c-di-GMP concentration is achieved by deletion or over-production, respectively, of the response regulator *vieA* (180). Increasing intracellular c-di-GMP causes a defect in colonizing the infant mouse gut due to decreased CT production. Further analysis shows that increasing cytoplasmic c-di-GMP concentration affects transcription of *toxT*, but does not affect the transcription of *tcpP* or *toxR* (180). These results suggest that increasing cytoplasmic c-di-GMP may affect the activity of TcpP and ToxR to activate the *toxT* promoter, or the ability of ToxT to amplify more of itself from the *tcpA* promoter.

In vivo environmental signals that are sensed by *V. cholerae* and induce its virulence determinants are not known. However, *in vitro* studies led to the discovery of a variety of physical and chemical environmental signals that the bacteria sense to modulate virulence regulation. Culturing the bacteria under specific pH, temperature, ion concentrations, carbon dioxide level and/or in the presence of certain amino acids (asparagine, glutamate, serine, and arginine) induces virulence (83, 125). This virulence induction is inhibited by the addition to growth media of bile or bile components, which are also present in the human small intestine where *V. cholerae* colonize (28, 57, 152). These *in vitro*

environmental signals might differ from those *in vivo*, but could mimic what occurs during the course of infection to regulate virulence expression.

Maximal virulence induction *in vitro* for the two biotypes of *V. cholerae* requires different sets of growth conditions. In the classical biotype, maximal virulence induction requires growing the bacteria in LB at pH 6.5 and 30° C. This was termed ToxR-inducing conditions due to the significant role of the ToxR protein in virulence induction. In contrast, minimal virulence induction results when the cells are cultured in LB at pH 8.5 and 37° C (ToxR-repressing conditions) (125). In the El Tor biotype, virulence induction occurs when growing the bacteria under biphasic conditions termed AKI (82, 83). During the first phase, the bacteria are cultured for several hours statically in AKI medium which contains 1.5% peptone, 0.3% yeast extract, 0.5% NaCl, and 0.3% sodium bicarbonate. During the second phase, an aliquot from the first phase is shifted to vigorous aeration for another several hours. Under virulence inducing conditions, both AphA and AphB induce TcpP/H expression and in conjunction with ToxR/S dimer, *toxT* transcription is activated. Once ToxT protein is expressed, it positively activates its own expression along with the *tcp* operon and other various virulence factors including CT (117).

The difference between the two biotypes in growth condition requirements that lead to virulence induction is due to differential transcription of the *tcpPH* operon. Both the classical and the El Tor strains of *V. cholerae* express their virulence factors under AKI conditions. However, the El Tor biotype does not

express these genes under classical ToxR-inducing conditions (low pH and temperature) (38, 129). Further analyses of virulence induction demonstrated that the *toxT* and *tcpPH* transcripts are absent in the El Tor biotype but are present in the classical biotype when culturing under classical ToxR-inducing conditions. In addition, overexpression of ToxT or TcpPH under low pH and temperature in the El Tor biotype results in restoration of virulence induction (129). Complementation studies showed that *toxT* and *tcpPH* from one biotype restored virulence expression in the corresponding alternate biotype deletion strain (38, 129). These results suggest that the difference in virulence induction between the two biotypes depends on activation of the *tcpPH* promoter, and this could be differentially regulated either by trans-acting elements, cis-acting elements, or both. AphA and AphB, which are the cis-acting activators of the *tcpPH* promoter, are expressed at the same level in both biotypes under different conditions (93, 160). Over-expression of AphA or AphB restores *tcpPH* induction by El Tor under low pH and temperature, which suggests the difference in amino acid sequence of these activators between the biotypes can't account for this non-induction phenotype. Instead, the *tcpPH* promoter DNA sequence slightly differs between the biotypes; this difference could affect the binding of either transcription activators or repressors that have been shown to bind to this region under certain growth conditions. One major change occurs in the binding site of AphB at the position of -65 relative to the start of transcription. Change from an A to a G (classical to El Tor) disrupts a dyad symmetry and reduces *tcpPH*

expression (95). Other changes are at the –87 and –86 positions within the binding sites of AphA and cAMP-CRP. At these positions, the classical biotype has a G and a C, while the El Tor has a T and an A, respectively. The significance of these changes to differences in virulence induction has not yet been characterized.

Alteration of pH, growth media, and temperature under ToxR inducing and repressing conditions for the classical biotype has also been characterized and acts to influence virulence induction at multiple levels. The expression and the activity of AphA and AphB are reduced when grown at high pH and temperature (93, 94, 160). This causes lower *tcpPH* expression and leads to lower *toxT* induction and lower virulence as a whole. However, constitutive expression of TcpPH or ToxT under ToxR-repressing conditions increases virulence expression, but the amount of virulence induction is still at a minimum when compared to ToxR-inducing conditions (129). These results suggest additional regulation occurs to control the activity of TcpP and ToxT posttranscriptionally. Although ToxT regulation at the posttranscriptional level has not been characterized (but it will be explored later in this dissertation), the level of TcpP is shown to be regulated by proteolysis (8, 23, 116). TcpP degradation occurs under ToxR-repressing conditions and is controlled by at least two sequential steps with different proteases (116). Initially, TcpP is cleaved by an unknown protease, and during the second step it is further degraded by YaeL protease.

Controlling the level of TcpP through expression and degradation processes could be an essential checkpoint to turn on and off the virulence cascade.

In vitro virulence growth conditions have been extensively studied and most of the regulators that are involved in the virulence cascade were discovered through these studies. However, *in vivo* studies have shown that virulence regulation during infection might differ from *in vitro* conditions (103). First, activation of *ctxA* *in vitro* requires both ToxR/ToxS and TcpP/TcpH dimers, whereas *in vivo* activation requires only the ToxR/ToxS dimer (not the TcpP/TcpH dimer) (103). Activation of *ctxA*, though, still occurs in a ToxT-dependent manner. This suggests that the discrepancy between *in vivo* and *in vitro* regulation occurs during the initial activation of the *toxT* promoter. Secondly, analysis of the temporal and spatial expression patterns of *tcpA* and *ctxA* revealed a difference in expression during infection versus during *in vitro* growth (103). During infection, *tcpA* transcription occurs in two separate peaks while *ctxA* occurs during the second peak of *tcpA* expression. In contrast, during *in vitro* growth, expression patterns of these genes occur simultaneously in a *toxT*-dependent manner (25, 36, 39, 197, 198). Lastly, *in vivo* TCP production is required to produce maximum *tcpA* and *ctxA* promoter activity while this requirement is absent during *in vitro* conditions. An in-frame *tcpA* deletion strain produces lower *tcpA* and *ctxA* activity *in vivo* when compared to *in vitro* conditions (103). Collectively, these results may suggest that the bacteria sense

additional signals during infection to promote maximum virulence induction and this requires production of TCP.

In summary, *V. cholerae* has evolved to utilize a complex array of gene regulation to induce a devastating pandemic disease. Understanding at the molecular level the mechanisms for how this pathogen induces virulence to cause illness could identify new therapeutic targets to disrupt this virulence regulation in cholera patients during outbreaks.

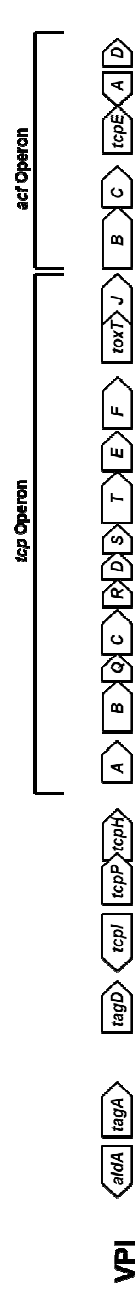


FIG. 1. Genetic map of *V. cholerae* pathogenicity island (VPI).

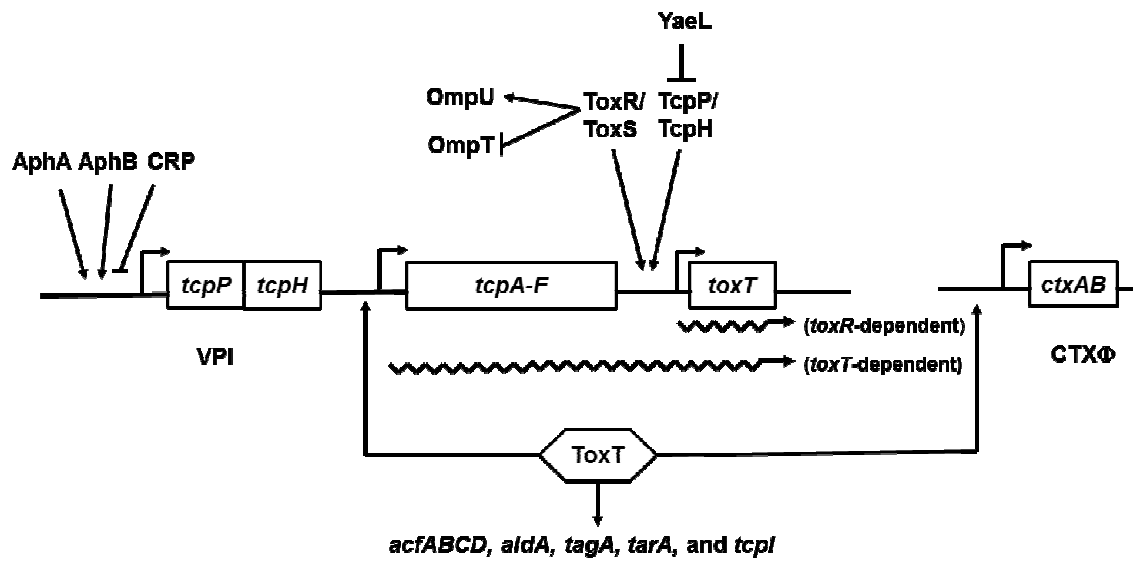


FIG. 2. The virulence regulatory network of *V. cholerae*.

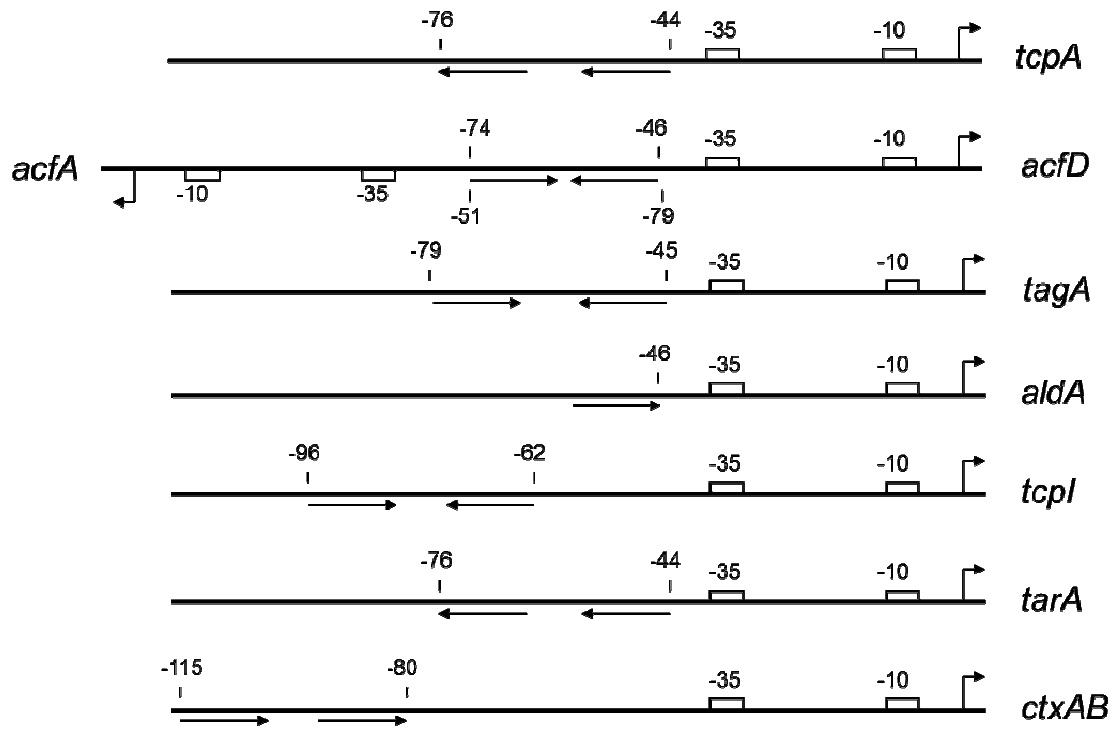


FIG. 3. The organization of toxboxes within ToxT activating gene promoters.

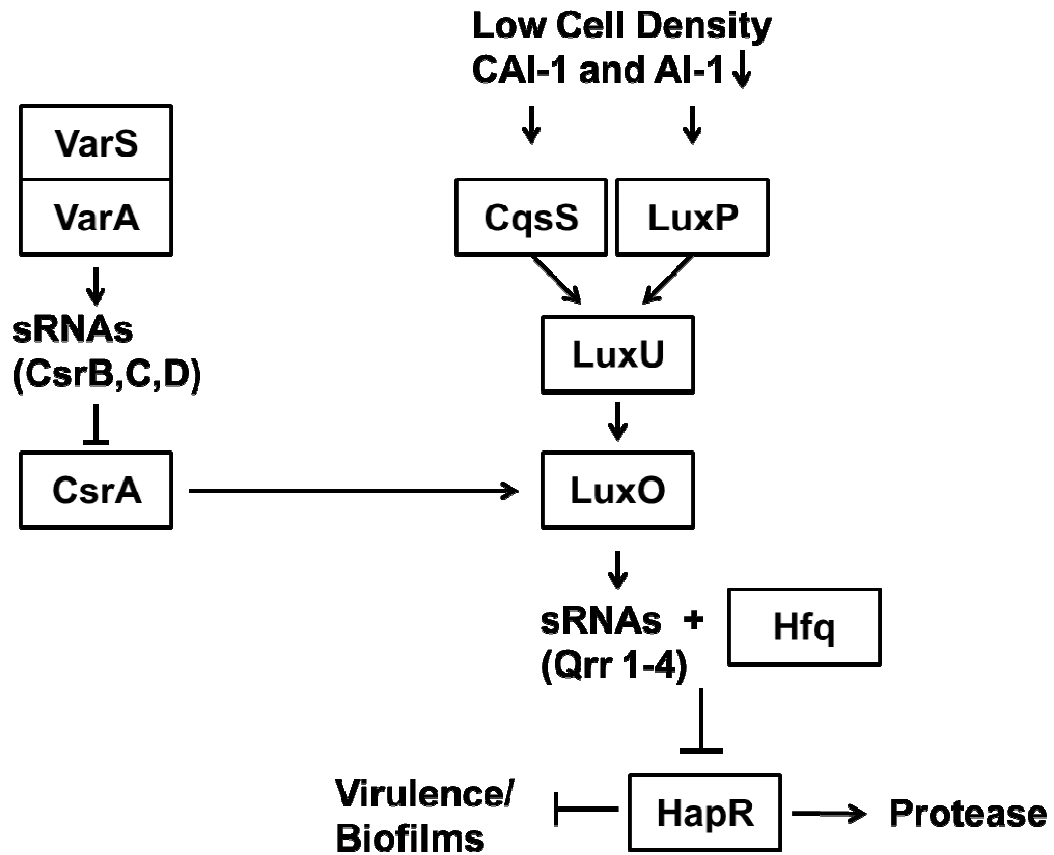


FIG. 4. Quorum sensing systems and the small RNA molecules of *V.cholerae* that regulate HapR expression.

CHAPTER ONE

Bicarbonate Induces *Vibrio cholerae* Virulence Gene Expression by Enhancing ToxT Activity

ABSTRACT

Vibrio cholerae is a gram-negative bacterium that is the causative agent of cholera, a severe diarrheal illness. The two biotypes of *V. cholerae* O1 capable of causing cholera, classical and El Tor, require different in vitro growth conditions for induction of virulence gene expression. Growth under the inducing conditions or infection of a host initiates a complex regulatory cascade that results in production of ToxT, a regulatory protein that directly activates transcription of the genes encoding cholera toxin (CT), toxin-coregulated pilus (TCP), and other virulence genes. Previous studies have shown that sodium bicarbonate induces CT expression in the *V. cholerae* El Tor biotype. However, the mechanism for bicarbonate-mediated CT induction has not been defined. In this study, we demonstrate that bicarbonate stimulates virulence gene expression by enhancing ToxT activity. Both the classical and El Tor biotypes produce inactive ToxT protein when they are cultured statically in the absence of bicarbonate. Addition of bicarbonate to the culture medium does not affect ToxT production but causes a significant increase in CT and TCP expression in both biotypes. Ethoxzolamide, a potent carbonic anhydrase inhibitor, inhibits bicarbonate-mediated virulence induction, suggesting that conversion of CO₂ into bicarbonate by carbonic anhydrase plays a role in virulence induction. Thus, bicarbonate is

the first positive effector for ToxT activity to be identified. Given that bicarbonate is present at high concentration in the upper small intestine where *V. cholerae* colonizes, bicarbonate is likely an important chemical stimulus that *V. cholerae* senses and that induces virulence during the natural course of infection.

INTRODUCTION

Cholera is a human disease that is characterized by massive loss of water and electrolytes, which leads to severe dehydration and hypovolemic shock if the condition is not treated. The causative agent of cholera is *Vibrio cholerae*, a highly motile, gram-negative, curved rod having a single polar flagellum. *V. cholerae* strains are classified into serogroups based on the lipopolysaccharide O antigen, and more than 200 serogroups have been identified to date. Only serogroups O1 and O139 are responsible for epidemic and pandemic cholera (145, 149). Serogroup O1 can be further divided into two biotypes, classical and El Tor, based on biochemical properties and susceptibility to bacteriophages (44, 149). Classical biotype *V. cholerae* strains are thought to have caused the first six cholera pandemics, beginning in 1817, whereas the El Tor biotype has been responsible for the seventh pandemic, which has been ongoing since 1961 (44, 149).

A major difference between the classical and El Tor biotypes is that they require different in vitro growth conditions for virulence gene induction. The classical biotype is cultured in LB medium at 30°C and pH 6.5 for maximal virulence gene expression and is cultured in LB medium at 37°C and pH 8.5 for

minimal virulence gene expression (125). The El Tor biotype is cultured under biphasic conditions termed AKI conditions for maximal virulence gene expression (82-84). During the first phase, the bacteria are cultured for several hours statically in a stationary tube in AKI medium, which contains 1.5% peptone, 0.3% yeast extract, and 0.5% NaCl (82). During the second phase, an aliquot is vigorously aerated and cultured for another several hours. Cholera toxin (CT) expression in the El Tor biotype was also observed when bacteria were grown in AKI media supplemented with 0.3% sodium bicarbonate under strictly static conditions (83). The mechanisms that induce virulence gene expression under either of these conditions are unknown.

Growth under virulence-inducing conditions results in production of the two major virulence factors, CT and toxin-coregulated pilus (TCP) (125, 176, 177), as well as an assortment of other gene products having functions that are poorly understood (43, 61, 62, 73, 74, 136). CT and TCP are absolutely required for *V. cholerae* to cause cholera. CT, a classical AB toxin composed of pentameric B subunits and one enzymatic A subunit (52, 110), is encoded by the *ctxAB* genes in the genome of a filamentous bacteriophage (CTX) (186). The CT A subunit ADP ribosylates a regulatory G protein in the intestinal epithelium, leading to constitutive adenylate cyclase activity and subsequent hypersecretion of water and electrolytes (149). This results in the voluminous watery diarrhea that is the hallmark of cholera. TCP is a type IV pilus that is encoded in the *tcpA* operon on the *Vibrio* pathogenicity island and aids in the formation of

microcolonies during colonization of the intestinal epithelial lining (89, 177, 179). TCP also acts as the receptor for CTX, which allows nontoxicogenic *V. cholerae* carrying the *Vibrio* pathogenicity island to acquire the CT genes (186).

The regulation of *V. cholerae* virulence gene expression is complex (117). The *V. cholerae* virulence genes have collectively been known as the ToxR regulon due to the central role that the ToxR protein plays in activating virulence gene expression. However, the major direct transcription activator of the virulence genes is the ToxT protein. ToxT is produced by the action of the transcription activators ToxR and TcpP (39, 65, 67, 100). Once produced, ToxT activates transcription of many virulence genes, including *tcpA* and *ctxAB*, leading to pathogenesis (75, 190-192, 197, 198). ToxR has also been shown to play a role in virulence gene expression independent of ToxT. Studies of classical biotype *V. cholerae* have shown that ToxR alone can induce CT production in the presence of bile (76). However, the amount of secreted toxin is very small compared to the amount of secreted toxin produced in a ToxT-dependent manner, and this ToxR-dependent expression has not been observed in El Tor strains or in vivo (103).

ToxT belongs to the large AraC/XylS family of transcription regulators (68). The carboxyl terminus of ToxT, corresponding to the conserved AraC family domain, contains two helix-turn-helix motifs that are utilized for DNA binding (114). The amino terminus does not share similarity with any protein in the databases and is hypothesized to be involved in dimerization and/or interaction

with an effector(s) that modulates ToxT activity (141). Bile and bile components have been shown to decrease ToxT activity, perhaps through direct binding by ToxT (28, 57, 152). However, no positive effector for ToxT activity has been identified.

While the *in vivo* signals that induce *V. cholerae* virulence gene expression have not been determined, *V. cholerae* has been shown to modulate the expression of its virulence genes *in vitro* in response to environmental factors and conditions, such as temperature, pH, osmolarity, chemotaxis toward certain amino acids, and bile salts (28, 57, 125, 152). Another potential inducer of virulence gene expression is sodium bicarbonate, which is included in some El Tor AKI media (83) and is present at a high concentration in the upper small intestine, which *V. cholerae* colonizes. Sodium bicarbonate protects the small intestine from the acidity of fluid arriving from the stomach and is secreted by the pancreatic duct epithelium at concentrations ranging from 70 to 140 mM (69). Data from human volunteers indicated that the infectious dose of *V. cholerae* decreased from 10^8 to 10^4 cells when volunteers were fed 2 g of sodium bicarbonate along with the inoculum (24). Although the interpretation of these data was that administration of bicarbonate enhanced survival of the bacteria in the acidic environment of the stomach, an alternative explanation is that bicarbonate could induce virulence gene expression, signaling to the bacteria that they are entering a human host. The bicarbonate ion has been shown to promote virulence gene expression in other bacteria, such as *Bacillus anthracis*

(189), *Streptococcus pyogenes* (22), enterohemorrhagic *Escherichia coli* (2), and the murine pathogen *Citrobacter rodentium* (196).

To determine whether bicarbonate alone could induce expression of the *V. cholerae* virulence genes, we monitored the effects of bicarbonate on expression of TCP and CT in both classical and El Tor biotype *V. cholerae*. The data strongly suggest that bicarbonate enhances virulence gene expression in both biotypes in a ToxT-dependent manner. ToxT protein was observed in bacteria grown with and without sodium bicarbonate in the growth medium, but virulence gene expression was observed only if bicarbonate was present. Addition of a carbonic anhydrase (CA) inhibitor caused a significant reduction in virulence gene expression. Thus, we propose that bicarbonate induces *V. cholerae* virulence gene expression by enhancing ToxT activity and that this may be the primary mechanism for virulence gene induction in vivo.

MATERIALS AND METHODS

Strains and growth conditions. *V. cholerae* classical biotype strain O395 and El Tor biotype strain E7946 and isogenic ΔtoxT (25) and ΔtoxR (100) mutants of these strains were maintained at -70°C in LB medium containing 20% glycerol. All strains were grown overnight in LB medium at 37°C and then subcultured in AKI medium (83) in the presence or absence of 0.3% bicarbonate. Sodium bicarbonate was freshly prepared and used on the day of the experiment. The classical strains were subcultured in the AKI medium from an overnight culture using a 1:100 dilution, while the El Tor strains were diluted

1:1,000.

Plasmid and strain construction. A chromosomal *tcpA-lacZ* fusion was constructed as follows. The *V. cholerae* O395 *tcpA* promoter region was amplified by PCR with 500-bp segments on either side corresponding to the DNA sequence surrounding the *V. cholerae lac* promoter. The product was cloned into suicide vector pKAS32, transformed into *E. coli* SM10(λ pir), and moved into *V. cholerae* strain O395 by conjugation as previously described (162). Thus, P_{tcpA} replaced P_{lac} as the promoter driving *lacZ* expression from the normal chromosomal locus. A *toxT::lacZ* fusion plasmid was constructed by PCR amplifying the *toxT* promoter from *V. cholerae* O395 genomic DNA and cloning it into pTL61T (108) using XbaI and HindIII restriction sites. The *tcpA::lacZ* fusion plasmid was constructed previously (191). An arabinose-inducible *toxT* plasmid (pBAD-*toxT*) was constructed by amplifying the *toxT* gene from *V. cholerae* O395 genomic DNA and cloning it into pBAD33 (58) using XbaI and PstI restriction sites.

RNA isolation and reverse transcription (RT)-PCR. Cell pellets harvested at different time points during static growth or AKI growth were collected by centrifugation. RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer's protocol. Total RNA was subjected to DNase I digestion for 1 h at 37°C to eliminate any DNA contamination. RNA was recovered by ethanol precipitation and resuspended in RNase-free water. The RNA concentration was adjusted to 5 μ g/ μ l based on A_{260} measurement. To

monitor the presence of *toxT* mRNA, an aliquot of each RNA sample was reverse transcribed using SuperScript III reverse transcriptase (Invitrogen). This was followed by PCR using *Taq* DNA polymerase (Denville Scientific) and an Eppendorf Mastercycler gradient thermal cycler. The PCR conditions were as follows: 60 s at 94°C, followed by 30 cycles of 30 s at 94°C for denaturation, 30 s at 55°C for annealing, and 60 s at 72°C for extension and then 5 min at 72°C. The upstream forward primer ATGATTGGGAAAAATCTTTTC was used (the underlined sequence is the start codon). The reverse primer sequence was TCAAGATCATCAGTAATAAATATAG (the underlined codon is complementary to the leucine codon at position 168 of ToxT relative to the start codon).

β-Galactosidase and CT assays. β-Galactosidase activity was measured using the basic procedure of Miller (122). CT was detected in the culture supernatant by a GM1 enzyme-linked immunosorbent assay (ELISA) (173), using polyclonal anti-CT antibody (Sigma). A positive control assay for quantification of the level of CT in the samples was performed using purified CT (List Biological Laboratories).

Immunodetection of ToxT. Aliquots of cells harvested at different time points during growth were normalized based on the optical density at 600 nm and resuspended in 10 µl water and 10 µl of 2X protein buffer (123 mM Tris-HCl, 4% sodium dodecyl sulfate, 1.4 M 2-mercaptoethanol, 20% glycerol, 0.2% bromophenol blue). The samples were boiled for 5 min and subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The gel was blotted

on nitrocellulose paper for 2 h using a semidry electroblotter apparatus (Fisher Scientific). Blots were incubated for 2 h in TBS buffer (20 mM Tris-HCl, 0.5 M NaCl, 0.025% Tween 20; pH 7.5) containing 5% milk to reduce nonspecific binding. After the blots were washed with TBS buffer, they were incubated overnight in TBS buffer containing 5% milk and a 1:3,000 dilution of rabbit polyclonal anti-ToxT serum. After three washes for a total of 15 min with TBS buffer, each blot was incubated for 2 h in TBS buffer containing 5% milk and a 1:5,000 dilution of goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase (Southern Biotech). After the blots were washed with TBS buffer, they were developed using 5 ml of immuno-BCIP (5-bromo-4-chloro-3-indolylphosphate)—nitroblue tetrazolium liquid substrate (Invitrogen).

RESULTS

Sodium bicarbonate stimulates CT and TCP production. To begin our investigation into the effects of bicarbonate on *V. cholerae* virulence gene expression, we assessed expression of the *tcpA* and *ctxAB* operons. Previous work indicated that CT was produced by El Tor *V. cholerae* cells when the bacteria were grown in a stationary tube (static conditions) in the presence of bicarbonate (83). CT was not detected when the cells were cultured under the same conditions in the absence of bicarbonate. However, adding a shaking phase after the static growth phase resulted in production of high levels of CT regardless of the presence of bicarbonate in the medium (82). We repeated these experiments using a CT ELISA to measure CT production and β -

galactosidase assays to measure *tcpA::lacZ* expression. CT and β -galactosidase levels were measured at 3, 4, 5, and 6 h after subculturing and again after 4 h of shaking for AKI conditions.

Our results indicate that bicarbonate does indeed induce expression of both CT and TCP under static growth conditions. As shown in Fig. 5A, CT was expressed at very low or undetectable levels when *V. cholerae* El Tor strain E7946 was grown statically in the absence of bicarbonate. High levels of CT were expressed when the E7946 strain was grown statically in the presence of 0.3% sodium bicarbonate. CT expression peaked at 4 h after subculturing and remained stable during the rest of the experiment (Fig. 5A). An isogenic *V. cholerae* strain in which *toxT*, which encodes the major activator of *ctxAB* and *tcpA* transcription, was deleted did not produce detectable levels of CT at any time point under any growth conditions, indicating that the observed CT production required ToxT protein activity.

Induction of *tcpA* transcription was also observed to be dependent on both bicarbonate and ToxT. β -Galactosidase production from a plasmid-borne *tcpA::lacZ* fusion in E7946 was measured at 3, 4, 5, and 6 h after subculturing in medium either containing or lacking 0.3% sodium bicarbonate (Fig. 5B). In contrast to the very low CT production that we observed when bacteria were grown under static conditions in media lacking bicarbonate, some *tcpA::lacZ* expression was observed over the time course in the absence of bicarbonate, starting at 4 h after subculturing. However, the β -galactosidase levels were much

higher when the bacteria were grown in the presence of 0.3% sodium bicarbonate. Once again, an otherwise isogenic $\Delta toxT$ strain did not produce significant β -galactosidase activity at any time point or under any growth conditions. These results demonstrate that bicarbonate stimulates the expression of *tcpA* and *ctxAB* and that this stimulation is *toxT* dependent.

Under AKI growth conditions, which include an additional shaking phase after the static growth phase, CT production was observed to be much higher if bicarbonate was present in the growth medium, but *tcpA::lacZ* expression was similar in the presence and in the absence of bicarbonate (Fig. 5A and B). Similar results were obtained using El Tor strains C6706 and N16961 (data not shown). An otherwise isogenic $\Delta toxT$ strain did not produce significant CT or β -galactosidase activity under AKI conditions, confirming that the induction of virulence gene expression under AKI conditions is *ToxT* mediated.

To determine whether bicarbonate also induces virulence gene expression in classical biotype *V. cholerae*, CT production and *tcpA-lacZ* expression in classical strain O395 were measured. Previously, it was found that some classical strains produced CT under AKI conditions, whereas other did not (82). Our results indicate that bicarbonate induces virulence gene expression in classical *V. cholerae* strain O395. Both CT levels (Fig. 5C) and β -galactosidase production from a chromosomal *tcpA-lacZ* fusion (Fig. 5D) were greatly increased by addition of 0.3% bicarbonate to the growth medium. AKI growth conditions with medium lacking bicarbonate resulted in some induction of CT and

tcpA-lacZ expression, but addition of bicarbonate under AKI conditions still caused a significant increase in CT and *tcpA-lacZ* expression. No significant CT or *tcpA-lacZ* expression was observed in an isogenic O395 Δ *toxT* strain under any conditions (data not shown).

The pH of AKI medium increased from 7.0 to 7.2 when 0.3% bicarbonate was added. To rule out the possibility that the induction of virulence was due to the pH difference, we measured the expression of *tcpA::lacZ* after the starting pH of AKI medium was increased to 7.2 with sodium hydroxide. No significant induction was observed for either El Tor strain E7946 or classical strain O395 (Fig. 6). The pH of the culture with added bicarbonate remained 7.2 during 4 h of static growth, whereas the pH of the culture whose pH was raised to 7.2 with NaOH dropped to 6.8 during 4 h of static growth, indicating that bicarbonate buffers the medium. Addition of morpholinepropanesulfonic acid (MOPS) buffer to AKI medium with a starting pH of 7.2 kept the pH at 7.2 during 4 h of static growth but had no effect on virulence gene expression unless bicarbonate was also added (data not shown). Overall, these results demonstrate that bicarbonate induces *tcpA* and *ctxAB* expression in El Tor and classical *V. cholerae* strains and that this induction is ToxT dependent.

***toxT* mRNA production is independent of bicarbonate.** *toxT* transcription is initially activated by the inner membrane protein pairs ToxR-ToxS and TcpP-TcpH (39, 65, 67, 100). Once ToxT protein is expressed, it can produce more of itself by activating transcription of the *tcpA* operon, in which the

toxT gene is located (197). One possible explanation for the minimal virulence gene expression observed when *V. cholerae* was cultured under static conditions in the absence of bicarbonate is that the initial activation of *toxT* did not occur. To examine this possibility, we analyzed the effects of bicarbonate on β -galactosidase production from a *toxT::lacZ* reporter plasmid. Because the reporter is plasmid based and not located downstream of *tcpA*, ToxT should have no effect on its expression. When *V. cholerae* was grown in the absence of bicarbonate, the amount of β -galactosidase activity per cell was larger than the amount when *V. cholerae* was grown in the presence of bicarbonate (Fig. 7A), strongly suggesting that bicarbonate is not required for and does not positively affect *toxT* transcription. Addition of bicarbonate to the growth medium caused a ~50% reduction in *toxT::lacZ* expression at every time point. Regardless of the presence of bicarbonate, *toxT::lacZ* expression peaked at 4 h after subculturing, consistent with the ToxT-dependent CT and *tcpA::lacZ* expression peaks shown in Fig. 5. As a control, β -galactosidase production by a Δ *toxR* strain that harbors the same *toxT::lacZ* fusion plasmid was also measured. The *toxT::lacZ* expression in the Δ *toxR* strain was low at all time points, and no effect of bicarbonate was observed, confirming that *toxT* transcription is ToxR dependent and bicarbonate independent.

To directly assess whether the *toxT* mRNA level or stability was affected by bicarbonate, RT-PCR experiments using primers specific for the *toxT* gene were performed (Fig. 7B). *toxT* transcripts were present under both static and

AKI growth conditions regardless of the presence of bicarbonate. These results indicate that the effect of bicarbonate on virulence gene expression is mediated downstream from *toxT* transcription.

ToxT protein is produced but inactive in the absence of bicarbonate. Because *toxT* mRNA production was not dependent on bicarbonate, we next investigated whether ToxT protein production was dependent on bicarbonate. Bicarbonate could possibly act at the translational level, affecting ToxT protein synthesis, or at the protein level, affecting ToxT activity. Using polyclonal antibodies specific for ToxT, we assessed by Western blotting whether ToxT protein was produced in *V. cholerae* grown in the presence or absence of bicarbonate. Our results paralleled the results that we obtained for *toxT* mRNA expression. ToxT protein was stably produced regardless of the presence of bicarbonate in the growth medium (Fig. 8). Using cell extracts harvested from El Tor strain E7946 after 4 h of static growth in medium either containing or lacking bicarbonate, a ToxT-specific band was visible in Western blots under both growth conditions (Fig. 8A, lanes 3 and 4). An isogenic Δ *toxT* strain did not produce this band (Fig. 8A, lane 2).

Similar results were obtained using cell extracts from classical strain O395. At the 4- and 6-h time points, a ToxT-specific band that corresponded to purified ToxT protein was observed (Fig. 8B, lanes 1, 3, 4, 6, and 7). An isogenic Δ *toxT* strain did not produce this band (Fig. 8B, lane 2). The ToxT-specific band was also observed for extracts of O395 grown under AKI conditions (Fig. 8B,

lanes 5 and 8). However, the ToxT levels in bacteria grown under AKI conditions with bicarbonate were significantly lower; the reasons for this are unclear. Paralleling the *toxT::lacZ* expression results indicating that *toxT* transcription was lower in the presence of bicarbonate, lower ToxT protein levels were observed in cells grown in the presence of bicarbonate in general, strongly suggesting that bicarbonate does not increase ToxT protein expression or stability.

Over-expression of ToxT can compensate for the absence of bicarbonate. The experimental results described above are consistent with a model in which bicarbonate positively affects ToxT activity rather than ToxT expression levels. Previous work indicated that ToxT expression from a plasmid in *V. cholerae* or *E. coli* resulted in activation of virulence factor transcription, even under virulence-repressing growth conditions (198). To investigate whether this over-expression of ToxT could compensate for the absence of bicarbonate as the inducing agent, we constructed a plasmid carrying *toxT* fused to P_{araBAD} (pBAD-*toxT*). ToxT expression can be induced from pBAD-*toxT* by addition of 0.2% arabinose to the growth medium. This plasmid was transformed into our E7946 $\Delta toxT$ derivative, and CT production and ToxT protein levels in the new strain were then measured.

Our results indicate that ToxT was indeed overproduced from the pBAD-*toxT* plasmid and that this resulted in increased CT production. In Western blot experiments (Fig. 8A, lanes 5 and 6) much higher levels of ToxT were present in extracts of the E7946 $\Delta toxT$ strain carrying pBAD-*toxT* than in extracts of wild-

type strain E7946 carrying the pBAD33 vector. CT production was approximately sixfold greater in the E7946 $\Delta toxT$ strain carrying pBAD-*toxT* than in wild-type strain E7946 carrying the pBAD33 vector when both strains were grown in the absence of bicarbonate (Fig. 9). The CT production by the E7946 $\Delta toxT$ strain carrying pBAD-*toxT* grown without bicarbonate was remarkably similar to the CT production by wild-type strain E7946 carrying the pBAD33 vector grown with bicarbonate. However, bicarbonate still increased CT expression in the E7946 $\Delta toxT$ strain carrying pBAD-*toxT* more than twofold, suggesting that ToxT activity could be enhanced by bicarbonate even when ToxT levels are far higher than normal.

A CA inhibitor, ethoxzolamide (EZA), inhibits the effect of bicarbonate on virulence induction. The results described above strongly suggest that bicarbonate induces ToxT-dependent *V. cholerae* virulence gene expression. In mammals, bicarbonate is secreted by the pancreas into the upper small intestine at a concentration of ~140 mM (69). *V. cholerae* colonizes the upper small intestine and therefore encounters high levels of bicarbonate during the course of infection. Bicarbonate could enter bacterial cells by at least two routes. First, bicarbonate and CO₂ are interconvertible in aqueous solution. CO₂ can enter the bacterial cell by passive diffusion and then be converted into bicarbonate by CA. Second, bicarbonate transporters can directly bind to bicarbonate and import it into the cell. Recent studies of other pathogens that utilize bicarbonate/CO₂ to induce virulence have produced different results

regarding the effects of CA inhibition. Inhibition of CA reduces virulence activation in *C. rodentium* (196) but has no effect on *B. anthracis* (189). These results suggest that *C. rodentium* utilizes CA to accumulate bicarbonate in the cell, while *B. anthracis* mainly utilizes direct bicarbonate transport.

To determine whether CA inhibition affects bicarbonate-mediated virulence induction in *V. cholerae*, we measured β -galactosidase production from *tcpA::lacZ* in the presence of EZA in both classical (O395) and El Tor (E7946) *V. cholerae* strains. Addition of 400 μ M EZA resulted in a >50% reduction in *tcpA::lacZ* expression in both E7946 and O395 grown with bicarbonate in the medium (Fig. 10). The effect of EZA was observed in bacteria grown under both static and AKI conditions. In the absence of bicarbonate, EZA had no effect when *V. cholerae* was cultured under static conditions but caused 50% inhibition of *tcpA::lacZ* expression when *V. cholerae* was cultured under AKI conditions, suggesting that the shaking phase of AKI conditions mimics the presence of bicarbonate in static culture. EZA had no detectable effect on *V. cholerae* growth rates. These data suggest that CA plays an important role in *V. cholerae* virulence induction by modulating intracellular bicarbonate levels.

DISCUSSION

Sodium bicarbonate has previously been found to induce CT production in the El Tor biotype of *V. cholerae* (82, 83), but the mechanism for this induction was unknown. Medrano et al. (118) found that ToxR-dependent *toxT* transcripts were produced when El Tor biotype *V. cholerae* was cultured under static

conditions in the absence of bicarbonate. However, ToxT activity in terms of increased *ctxAB* transcription was not detected. These results suggested that the ToxT protein was either present but inactive or not present when the cells were cultured statically in the absence of bicarbonate. Here we have shown that the initial activation of the *toxT* promoter by ToxR-ToxS and TcpP-TcpH occurs when the bacteria are cultured statically in the presence or absence of bicarbonate. These results are in agreement with previous data showing that transient expression of *toxT* occurs during the static phase under AKI conditions (118). Moreover, our data indicate that the ToxT protein was present but had minimal activity in both the classical and El Tor biotypes when bacteria were grown in the absence of bicarbonate. Growing the bacteria with bicarbonate in the medium enhances the activity of ToxT, and both CT production and *tcpA* promoter activation are maximally induced. This bicarbonate-mediated virulence induction is ToxT dependent, as the isogenic *toxT* deletion strains did not express virulence genes in the presence of bicarbonate.

The *V. cholerae* in vitro virulence-inducing conditions do not resemble the conditions that *V. cholerae* encounters in the small intestine. The classical biotype is cultured in LB medium at pH 6.5 and 30°C for maximum virulence gene induction (125). Neither low temperature nor low pH occurs in the upper small intestine that *V. cholerae* colonizes. The El Tor biotype is cultured in rich medium statically for several hours, followed by shaking for several more hours, for maximal virulence gene induction (82). Again, these conditions are not found

in the upper small intestine. The presence of bicarbonate is not required for CT production and *tcpA::lacZ* expression under AKI conditions, although bicarbonate does increase the amount of CT produced. However, static growth conditions with bicarbonate in the medium might more closely mimic what happens during the course of infection, as *V. cholerae* is grown at 37°C, with exposure to very low levels of oxygen, and in the presence of bicarbonate, all of which are similar to conditions in the small intestine. Based on the evidence that we obtained regarding the requirement of bicarbonate for maximal ToxT activity and knowing that bicarbonate is present at high concentrations in the small intestine, we propose that *V. cholerae* utilizes bicarbonate during infection as an effector molecule to induce virulence.

Bicarbonate could also be responsible for the temporal regulation of virulence that has been observed in vivo. Bicarbonate is produced by pancreatic cells and secreted into the lumen of the small intestine to neutralize the acid that comes from the stomach. Lee et al. (103) have observed that during infection *tcpA* expression is induced in two stages, while *ctxAB* expression is induced subsequent to the second stage of *tcpA* induction. On the basis of these results, these authors proposed a model for temporal regulation in which the primary pulse of *tcpA* expression allows the bacteria to colonize the epithelial lining and in response to a second signal *ctxAB* expression is induced. Studies have shown that there is a pH gradient in the mucus gel in the human duodenum (144), suggesting that the concentration of bicarbonate is higher close to the epithelial

surface, so bicarbonate could be the second signal that stimulates maximum virulence induction.

The following model describes a mechanism for bicarbonate-mediated virulence gene induction and its involvement in temporal regulation patterns (Fig. 11). As the bacteria enter the lumen of the small intestine, they encounter the primary signal, which remains unclear and which induces transcription of *toxT*. In the lumen, lower levels of pancreatic bicarbonate are present due to diffusion throughout the intestine. The low concentration of bicarbonate produces low levels of ToxT activity, resulting in a low level of *tcpA* expression. At later stages of infection, the bacteria enter the mucus layer, where they encounter a higher concentration of bicarbonate that is secreted by the epithelial cells. This leads to enhanced ToxT activity and maximal *tcpA* and CT expression.

Bicarbonate has the same effects on ToxT protein activity and virulence gene expression in both the classical and El Tor *V. cholerae* biotypes. This suggests that the mechanism of bicarbonate-mediated enhancement of ToxT activity is conserved. Bicarbonate could act as a positive effector molecule and directly modulate ToxT protein activity, or it could act indirectly to enhance ToxT function. Recent work on RegA of *C. rodentium*, which like ToxT shares sequence homology with the AraC/XylS protein family, has shown that bicarbonate stabilizes RegA binding to promoter regions. Addition of bicarbonate in vitro resulted in different migration patterns of RegA-DNA complexes in electrophoretic mobility shift assay (EMSA) experiments (196). ToxT binds to

promoter DNA sequences in vitro without bicarbonate (190-192, 198). We tested for a similar direct effect of bicarbonate on ToxT-DNA complexes using EMSA. However, no differences in the binding profiles were observed after addition of bicarbonate to the binding reaction mixtures (data not shown). Although we did not observe direct effects of bicarbonate on ToxT using the EMSA technique, the possibility that bicarbonate directly binds to the ToxT protein and modulates its activity cannot be ruled out. The possible mechanisms for modulation of ToxT activity by bicarbonate include enhancing the binding affinity of ToxT for toxboxes, enhancing the interaction between DNA-bound ToxT monomers, enhancing the interactions between ToxT and RNA polymerase, and some other direct mechanisms. Our data indicating that overexpression of ToxT can compensate for the absence of bicarbonate in the growth medium suggest that bicarbonate may enhance the binding affinity of ToxT for its DNA or protein partners. Thus, an increased ToxT concentration compensates for reduced ToxT binding affinity.

Indirect effects of bicarbonate on ToxT are also possible. Bicarbonate could induce or modulate gene products to enhance the activity of ToxT. Sträter et al. have found that bicarbonate ion activates *E. coli* aminopeptidase A (PepA) (172). The *pepA* gene product is a multifunctional protein. It has peptide proteolysis activity (27), acts as a repressor involved in regulation of the carboamoylphosphate synthetase operon (35), and plays a role in site-specific recombination at the ColE1 site, a mechanism that is involved in resolving

multimers of multicopy plasmids into monomers to allow stable heredity of the plasmids (168). The X-ray crystallographic structure of PepA indicated that a bicarbonate anion is bound to an arginine side chain (171). Interestingly, deletion of the *V. cholerae pepA* gene has also been shown to increase the levels of CT, *tcpA*, *toxT*, and *tcpP* when the cells are cultured under ToxR-repressing conditions (LB medium at pH 8.5 and 37°C) (10). One possible indirect effect of bicarbonate is that *pepA* or other gene products negatively regulate the activity of ToxT and addition of bicarbonate could modify such gene products to relieve this inhibition.

Bacteria can increase cytosolic bicarbonate levels through at least two routes. First, transporters can directly bind to and import bicarbonate. The *cmpABCD* gene cluster encodes the bicarbonate transport system of *Synechococcus elongatus* PCC 6301 (112). Proteins with sequence homology to proteins in this system have been shown to play a role in *B. anthracis* pathogenesis (189). However, BLAST searches of the *V. cholerae* genome have yielded no sequences having homology to this system. This suggests either that *V. cholerae* does not utilize transporters to accumulate cellular bicarbonate or that *V. cholerae* utilizes a different system of transporters to import bicarbonate. Second, both metabolic CO₂ and atmospheric CO₂ that enter the cell by simple diffusion are converted into bicarbonate by the action of CA. CAs are zinc metalloenzymes that catalyze the hydration of CO₂ into bicarbonate. CAs have been shown to be involved in many cellular processes, such as photosynthesis,

respiration, CO₂ transport, and cyanate metabolism in *E. coli* (164). CAs are classified into three classes, α , β , and γ , and they share little sequence homology with each other but catalyze the same reaction. *V. cholerae* encodes one putative CA homolog belonging to each class; the *cah* gene (VC0395_0957) product belongs to the α class; the putative gene VC0395_A 0118 product belongs to the β class; and the putative VC0395_A2463 product belongs to the γ class. Our data indicate that addition of the CA inhibitor EZA resulted in a >50% reduction in *tcpA* promoter activity. This suggests that one or more of the putative CAs and/or other CA-like molecules play a role in virulence induction in *V. cholerae*. Notably, EZA also caused a decrease in virulence gene expression under AKI conditions in the absence of bicarbonate. This finding suggests that the shaking phase of AKI conditions has an effect that is similar to addition of bicarbonate to a static culture and that the induction of virulence gene expression induced by both bicarbonate and AKI conditions is due to increased cytosolic bicarbonate levels mediated by one or more of the *V. cholerae* CAs.

Culturing *V. cholerae* under AKI conditions likely mimics the presence of bicarbonate in the medium by increasing the CO₂ concentration in the medium. CO₂ and HCO₃⁻ freely interconvert in solution, so an increase in the CO₂ concentration would result in an increase in the bicarbonate concentration. Production of CT has been observed when the El Tor biotype was cultured in the absence of bicarbonate. However, either a shaking period or culturing the bacteria with a low ratio of volume to surface area was required (82, 150). Under

these conditions, *V. cholerae* could produce enough CO₂ so that there was an increase in cellular bicarbonate levels. The increase in bicarbonate levels could occur through direct transport of bicarbonate by the bacteria and/or through CO₂ uptake and conversion into bicarbonate by CAs. Increasing the cellular bicarbonate level would enhance ToxT activity so that CT was maximally expressed. In the absence of bicarbonate, *V. cholerae* produces mainly CO₂ as a product of metabolism. When the bacteria are grown under static conditions with a small exposed surface area, the amount of CO₂ produced by the cells is relatively small as the growth rate is low. CO₂ diffuses out of the cells at a rate greater than the rate of conversion into cellular bicarbonate by CA. The low level of bicarbonate produced by CA under these conditions is not sufficient to induce virulence. In contrast, addition of a shaking period or culturing using a large exposed surface area increases the growth rate due to aerobic metabolism, and thus the cells produce more CO₂. The higher level of CO₂ could increase the cytoplasmic level of bicarbonate, and virulence gene expression would be induced. It has been reported that under anaerobic growth conditions in either classical virulence-inducing medium (classical biotype) (99, 113) or syncase medium (El Tor biotype) (113) TCP expression is observed but CT production is low or nonexistent. This is somewhat similar to the expression patterns that we observed under AKI conditions in the absence of bicarbonate, in which a high level of *tcpA* expression but a low level of CT production was observed (Fig. 5). Both of these growth conditions may mimic the early stages of infection shown in

Fig. 11, in which the bicarbonate concentration is relatively low. A possible mechanism for the observed differential expression of CT and TCP under low-bicarbonate conditions arises from the observation that H-NS is a major negative regulator of *ctx* transcription (51, 134), whereas H-NS has little or no effect on *tcpA* transcription (198). When bicarbonate levels are low, a small pool of “activated” ToxT may be blocked from binding to the *ctx* promoter by H-NS but may bind to the *tcpA* promoter unhindered and activate its transcription.

In summary, we have found that bicarbonate induces expression of the *V. cholerae* major virulence factors by enhancing the activity of the ToxT protein that is already present in the bacteria. This is the first example of a positive effector for ToxT activity, and bicarbonate is likely to be an important in vivo signal that induces *V. cholerae* virulence gene expression during infection.

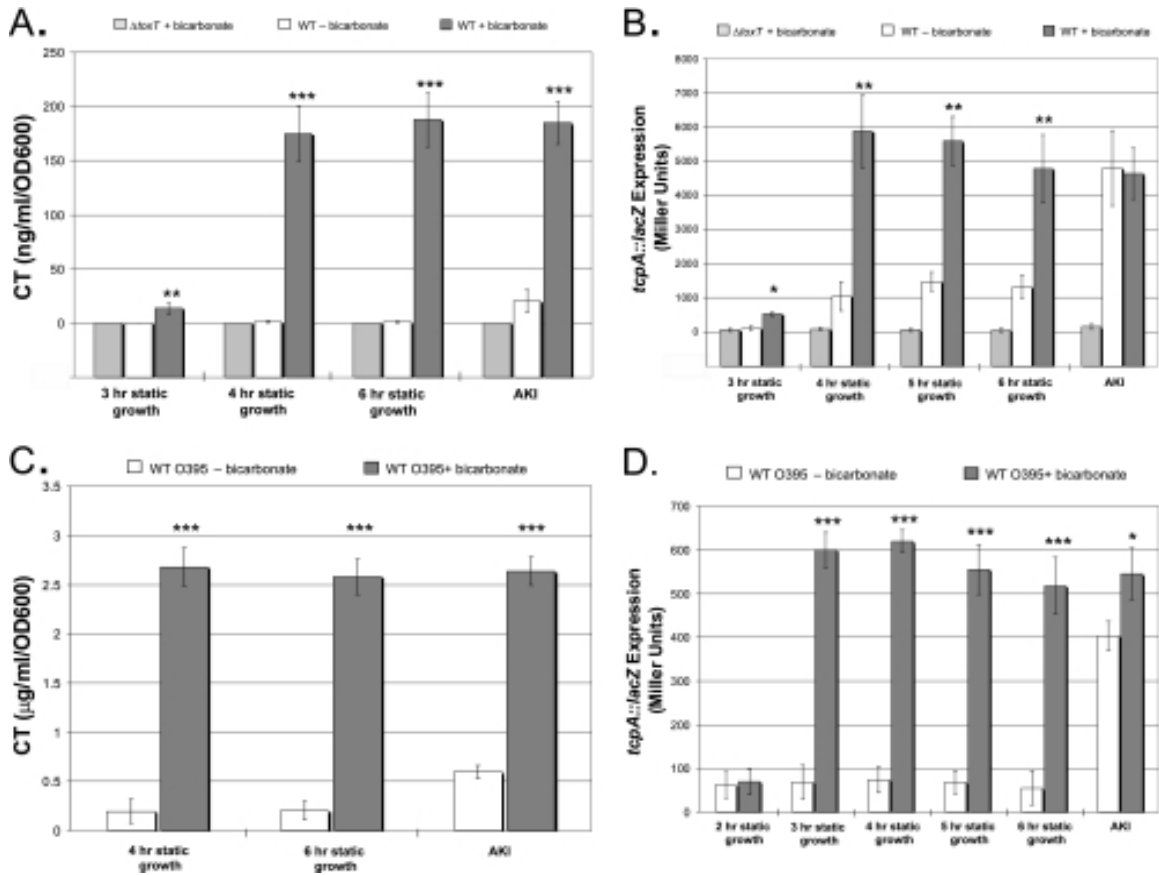


FIG. 5. Effect of bicarbonate on CT and *tcpA::lacZ* expression. Open bars, wild-type *V. cholerae* grown without bicarbonate; dark gray bars, wild-type *V. cholerae* grown with 0.3% bicarbonate; light gray bars, *V. cholerae* Δ toxT mutant grown with 0.3% bicarbonate. (A) CT production by El Tor strain E7946. (B) β -Galactosidase produced from plasmid-borne *tcpA::lacZ* in El Tor strain E7946. (C) CT production by classical strain O395. (D) β -Galactosidase produced from chromosomal *tcpA-lacZ* in classical strain O395. Statistical significance was determined by Student's *t* test (*, $P < 0.025$; **, $P < 0.005$; ***, $P < 0.0005$). OD600, optical density at 600 nm; WT, wild type.

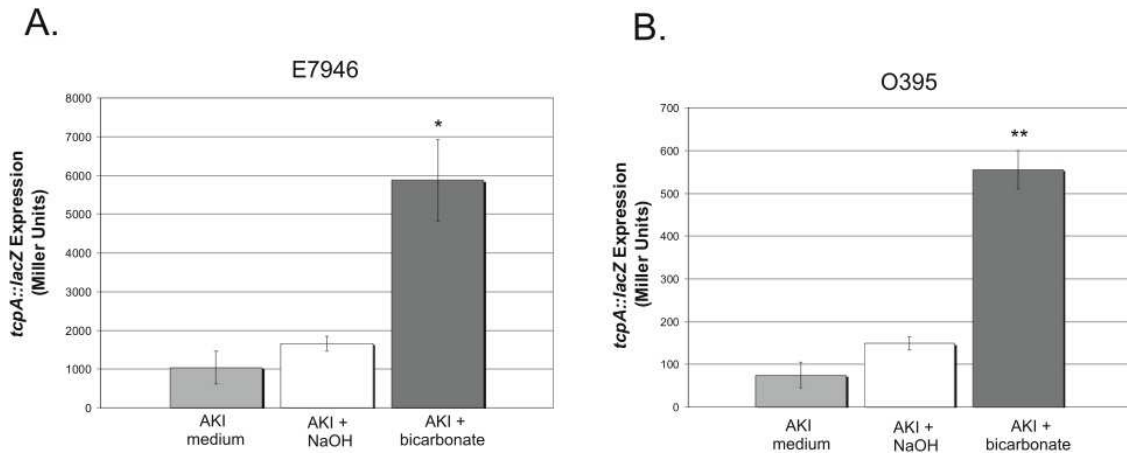


FIG. 6. Effect of pH on *tcpA* expression. *V. cholerae* were grown for 4 hours in a static tube in AKI medium. Light gray bars indicate neither NaOH nor bicarbonate were added to the medium, white bars indicate NaOH was added to adjust starting pH to 7.2, and dark gray bars indicate 0.3% bicarbonate was added, which changed pH to 7.2. A. β -galactosidase produced from plasmid-borne *tcpA::lacZ* in El Tor strain E7946. B. β -galactosidase produced from chromosomal *tcpA-lacZ* in classical strain O395. Statistical significance was determined by Student's T test with * indicating $P < 0.0025$ and ** indicating $P < 0.0001$.

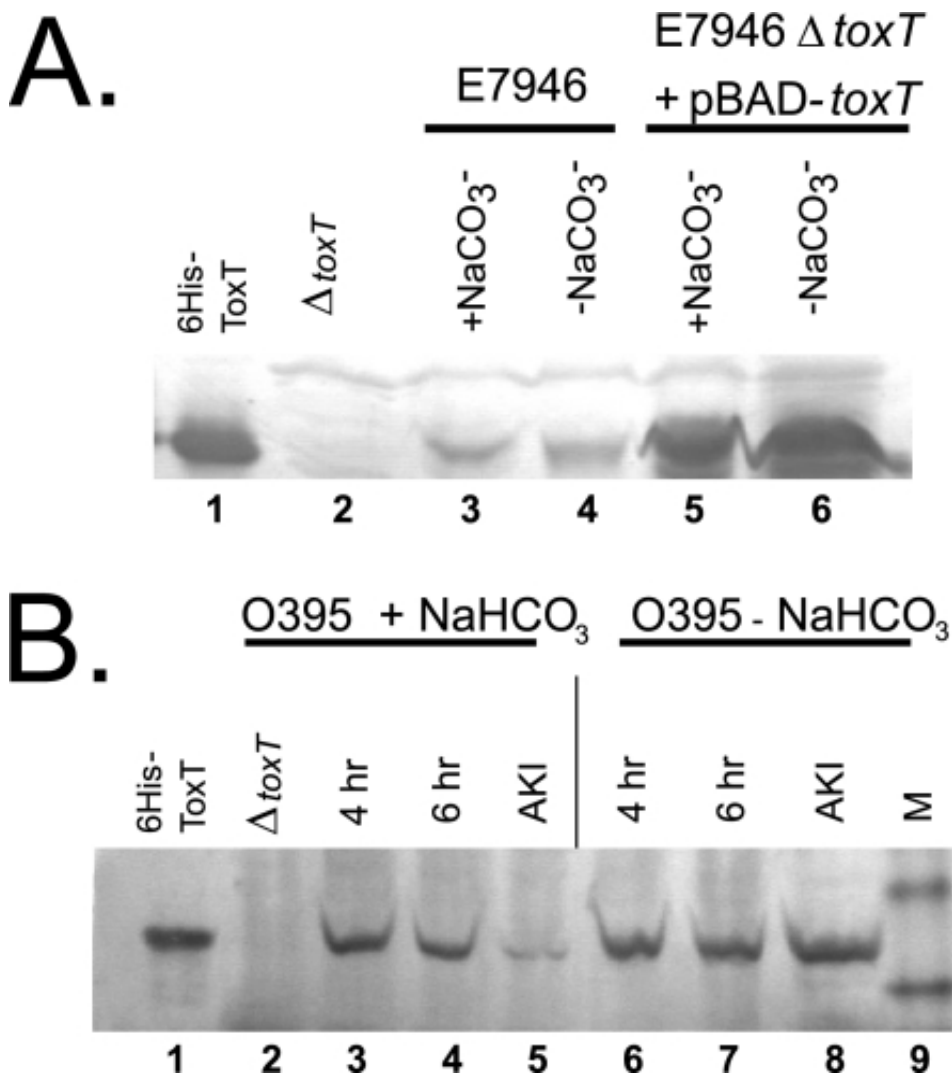


FIG. 8. Detection of ToxT protein in *V. cholerae* grown in the presence or absence of bicarbonate. ToxT protein was detected by Western blotting using polyclonal anti-ToxT antibodies. 6His-ToxT, purified His-tagged ToxT protein loaded as a control. (A) El Tor strain E7946. The presence or absence of bicarbonate in the growth medium is indicated above the lanes. E7946 $\Delta toxT$ + pBAD-*toxT* indicates that the $\Delta toxT$ strain was complemented in *trans* with pBAD-*toxT* and arabinose was included in the growth medium. (B) Classical strain O395. 4 hr and 6 hr indicate the time of growth in a stationary tube, and AKI indicates that a shaking phase of growth was added. Lane M contained protein molecular weight markers.

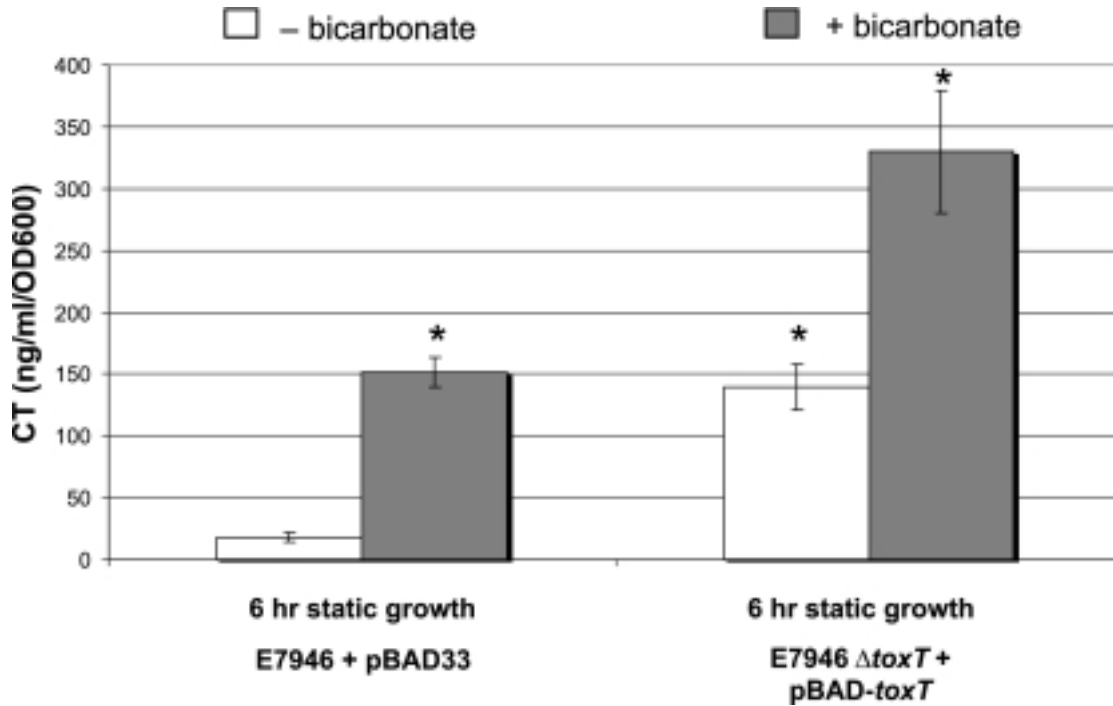


FIG. 9. Effect of ToxT overproduction from pBAD-*toxT* on CT production in *V. cholerae* El Tor strain E7946. Open bars, bacteria grown without bicarbonate; gray bars, bacteria grown with 0.3% bicarbonate. Both strains were grown statically for 6 h in the presence of arabinose before a CT ELISA was performed. E7946 Δ *toxT* + pBAD-*toxT* indicates that the Δ *toxT* E7946 strain was complemented in *trans* with pBAD-*toxT*, and E7946 + pBAD33 indicates wild-type *V. cholerae* carrying the empty pBAD33 vector. Statistical significance was determined by Student's *t* test (*, $P < 0.005$). OD600, optical density at 600 nm.

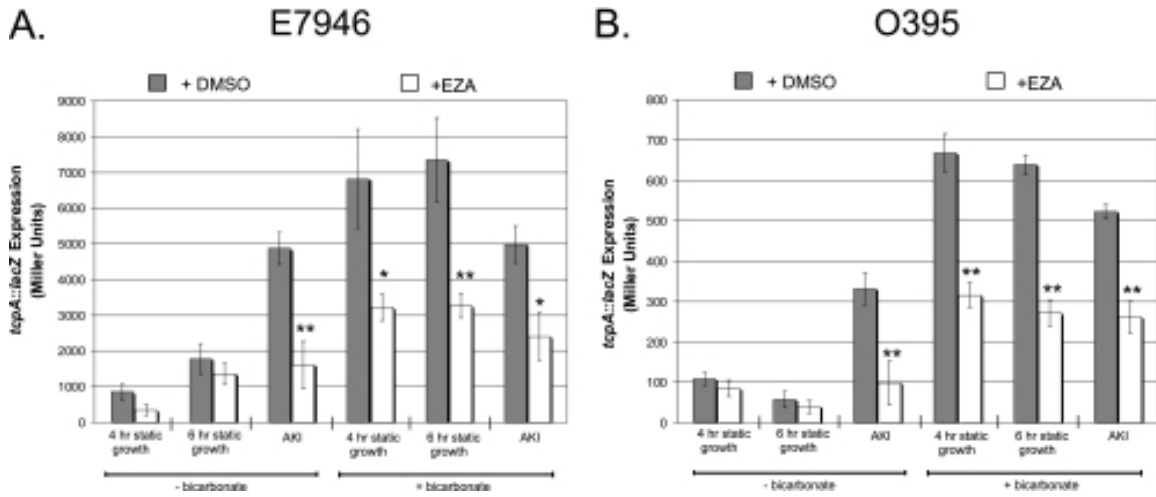


FIG. 10. Effects of the CA inhibitor EZA on *tcpA::lacZ* expression. Gray bars, dimethyl sulfoxide (DMSO) alone added to the growth medium; open bars, EZA dissolved in dimethyl sulfoxide added to the growth medium. (A) β -Galactosidase produced from plasmid-borne *tcpA::lacZ* in El Tor strain E7946. (B) β -Galactosidase produced from chromosomal *tcpA-lacZ* in classical strain O395. Statistical significance was determined by Student's *t* test (*, $P < 0.015$; **, $P < 0.005$).

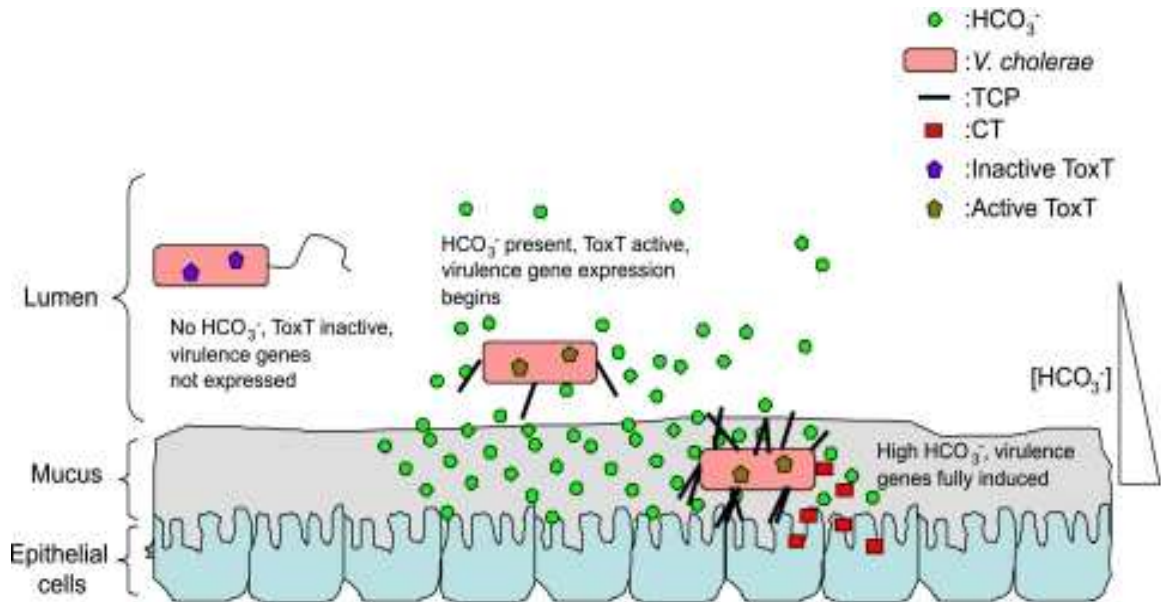


FIG. 11. Model for induction of virulence gene expression by bicarbonate *in vivo*. On the left, motile *V. cholerae* containing inactive ToxT protein enters the upper small intestine. In the center, *V. cholerae* in the intestinal lumen encounters bicarbonate, ToxT becomes active, and TCP production begins. On the right, bacteria entering the mucus layer encounter higher levels of bicarbonate, virulence genes are fully induced, and CT production begins. The gradient of increasing bicarbonate levels from the lumen to the mucosal surface is indicated by the triangle on the right.

CHAPTER TWO

Regulation of *Vibrio Cholerae* Virulence by Proteolysis of ToxT

ABSTRACT

Vibrio cholerae is a gram negative bacterium that is the causative agent of the severe diarrheal illness, cholera. While over 200 different *V. cholerae* serogroups have been identified, only serogroups O1 and O139 have exhibited the ability to cause epidemics. *V. cholerae* O1 is further divided into two biotypes, classical and El Tor. For the classical biotype, maximal virulence induction *in vitro* requires growing the bacteria at 30° C in LB at starting pH 6.5 (ToxR-inducing conditions). In contrast, minimal virulence induction results when the cells are cultured at 37° C in LB at starting pH 8.5 (ToxR-repressing conditions). Under virulence inducing conditions, both the ToxR/S and TcpP/H heterodimers initially activate *toxT* transcription. Once ToxT is expressed, it activates expression of the two major virulence factors: cholera toxin (*ctx* operon) and toxin co-regulated pilus (*tcp* operon). ToxT also produces more of itself via a positive feedback loop by activating transcription of the *tcp* operon, within which *toxT* is located. It is known that *V. cholerae* terminates virulence gene expression prior to escape from the host, but it is unknown how the ToxT positive feedback loop is broken, which is an essential step in terminating virulence gene expression. To understand better the regulation of ToxT protein, we monitored its accumulation and its activity under virulence inducing and repressing growth conditions. Our results suggest that ToxT protein undergoes proteolytic degradation under

virulence repressing conditions. The degradation of ToxT under repressing conditions suggests a model for terminating *V. cholerae* virulence gene expression during the late stage of infection, with both ToxT and TcpP undergoing proteolysis prior to escaping the host.

INTRODUCTION

Vibrio cholerae, the causative agent of severe dehydrating diarrhea, uses a complex array of gene regulation to control virulence gene expression during infection (117). ToxT is the direct transcriptional activator that induces expression of most virulence factors including the cholera toxin (CT) and the toxin co-regulated pilus (TCP) (75, 190-192, 197, 198). CT is responsible for fluid loss into the intestinal milieu that results from the constitutive production of cAMP by the intestinal epithelial cells (45, 53). CT is an AB₅ toxin composed of five B subunits that binds to the GM1 ganglioside receptor of the epithelial cells, and an enzymatic A subunit that upon activation modifies adenylyl cyclase by adding an ADP-ribosyl group and keeps it in its active state (52, 110). The TCP is a type IV pilus that is thought to be involved in bacteria-bacteria interaction to form microcolonies and enhance colonization over the epithelial cells that line the small intestine (89, 177, 179).

ToxT protein belongs to the AraC/XylS family of proteins (68) that directly binds to virulence gene promoters of *V. cholerae*, recruits RNA polymerase and positively activates these promoters (75, 191, 198). It is composed of 276 amino acids that are separated into two domains: The N-terminal domain (NTD)

comprises amino acids 1-160, whereas the C-terminal domain comprises amino acids 170-276. These two domains are connected by a short linker, amino acids 161-169 (111). The ToxT CTD is a conserved AraC/XylS family domain, having two helix-turn-helix motifs that are utilized for DNA binding. The NTD does not share significant sequence similarity with any protein in the database and its role is hypothesized to be dimerization and/or interacting with effectors that modulate ToxT activity. However, the crystal structures of ToxT protein and the AraC protein NTD indicate that the ToxT NTD and the AraC NTD share some structural similarity despite having only 11% amino acid identity (111). The AraC NTD is required for dimerization and binding to its effector, arabinose (114). It has been proposed that bile or the unsaturated fatty acids (UFA) present in bile are natural effectors of ToxT. Bile and UFA have been shown to inhibit ToxT activity and the overall growth of *V. cholerae* (28, 57, 141-143, 152, 188). Mutational analysis demonstrated that bile might interact with the N-terminal domain of ToxT, as a ToxT mutant somewhat resistant to bile was isolated that had a single amino acid change at residue 107 (141). Furthermore, the crystal structure of ToxT contains a buried 16-carbon UFA, *cis*-palmitoleate, which inhibits the activity of ToxT and may be a minor component of crude bile (111). A synthetic inhibitor of ToxT activity, virstatin, also apparently interacts with the ToxT NTD (155).

toxT expression is controlled by two promoters. Initially, activation of *toxT* transcription occurs via the activity of the two membrane localized heterodimers,

toxR/S and *tcpP/H* (39, 65, 67, 100). ToxR and TcpP have been shown to bind to the *toxT* proximal promoter to produce *toxT* transcripts. Deletion of *toxR* or *tcpP* inhibits activation of this promoter and abrogates *toxT* mRNA production. Once ToxT protein is present in the cells, a second, longer mRNA containing *toxT* is produced from the distal *tcpA* promoter. Deletion of the *tcpA* promoter or the ToxT helix-turn-helix motifs required to inactivate *tcpA* transcription decreases *toxT* mRNA level (197). Furthermore, transposon insertions in the region between *tcpA* and *tcpF* cause a polar effect on ToxT activity as determined by measuring CT production and expression of a *tcpA-lacZ* fusion (16). These results support a positive auto-regulatory mechanism that controls *toxT* expression and activity.

While virulence induction has been vigorously studied in the past decade and is characterized to some extent, repression of virulence once it has been established is not well understood. Down-regulation of virulence genes including those encoding TCP and CT during the late period of infection (in rice water stool) has been observed (101, 121, 132) and has been proposed to be important for environmental survival. In order for *V. cholerae* to shut down the virulence cascade, two things must occur: 1) activation of the *toxT* promoter must be curtailed, and 2) activation of the *tcpA* promoter auto-regulatory loop that produces ToxT must be curtailed. In the former case, studies have shown that the TcpP level is tightly controlled by expression and degradation processes, which could provide a check point to turn on and off the *toxT* promoter (8, 23, 93,

94, 116). *tcpP* expression requires two transcription activators, AphA and AphB, and *tcpP* transcription is inhibited by cAMP-CRP, which is responsive to the presence or absence of glucose as an available carbon source (96). Once TcpP protein has been produced, it is degraded by a sequential event involving YaeL and another unknown protease (116); the role of TcpH is to protect TcpP from this degradation. In the latter case, it is unknown how the *tcpA* promoter auto-regulatory loop that continues ToxT production is broken. In this report, we present data that suggest proteolysis of ToxT protein is the major factor responsible for breaking this auto-regulatory loop. ToxT proteolysis occurs when *V. cholerae* is grown *in vitro* at pH 8.5 and 37°C, a growth condition that is known to repress virulence production. These are the same growth conditions under which TcpP is proteolyzed. These results indicate that rapid shutdown of virulence prior to escape of *V. cholerae* from the human host back into the aquatic environment may be mediated by directed proteolysis of both TcpP and ToxT.

MATERIALS AND METHODS

Bacterial strains, plasmids, culture conditions and reagents. All bacterial strains and plasmids are listed in Table 1. Strains are maintained at -70°C in LB media containing 20% glycerol. The strains were grown overnight at 37°C in LB medium with a starting pH of 8.5 and then were sub-cultured using a 1:40 dilution into either LB at starting pH 6.5 and 30°C (virulence inducing conditions) or LB at starting pH 8.5 and 37°C (virulence repressing conditions).

For the experiments shifting from inducing to repressing growth conditions, cells were harvested by centrifugation after 3 hours growth under inducing conditions and cell pellets were re-suspended into the same volume of fresh LB at either pH 8.5 or pH 6.5 and continued to be cultured at the corresponding temperature (37°C or 30°C). Antibiotics were used at the following final concentrations unless otherwise stated: streptomycin 100 µg/ml; ampicillin 100 µg/ml; and chloramphenicol 5 µg/ml. To induce protein expression, a final concentration of 0.2% arabinose was used to induce pBAD33 vector derivatives while a final concentration of 1mM of isopropyl-β-D-thiogalactopyranoside (IPTG) was used to induce pMAL-c2e vector derivatives. To deplete cellular ATP, cells were treated with a combination of 10 mM of α-methylglucoside and 20 mM of sodium azide as previously described (86).

β-Galactosidase assay. After the cells were grown under the indicated time and growth conditions, β-galactosidase activity was measured and expressed in Miller units as previously described (122).

Plasmid and strain construction. The *tcpA* promoter deletion strains were constructed as previously described (197). Briefly, 500-bp segments on either side of the *tcpA* promoter were amplified by nested recombinant PCR method and cloned into suicide vector pKAS32 using *KpnI* and *SacI* restriction sites. The resulted plasmid was transformed into *E. coli* SM10(λpir), and moved into both *V. cholerae* strains O395 and JW 611 by filter conjugation. Arabinose-inducible *toxT* plasmids which has either N-terminal or C-terminal polyhistidine

tag was constructed by amplifying the *toxT* gene from *V. cholerae* O395 genomic DNA and cloning it into pBAD33 (58) using *XbaI* and *PstI* restriction sites For N-terminal His tagged ToxT, the forward primer BP 81 (5'-GATCTCTAGATTTAGG ATACATTTTTATGCATCATCATCATCATATGATTGGGAAAAAATCTTTTCA AACTAATG-3') and the reverse primer BP 193 (5'-GATCCTGCAGTTATTTTTCT GCAACTCCTGTCAAC-3) is used. For C-terminal His tagged, the forward primer BP 22 (5'-GCTCTAGATTTAGGATACATTTTTATGATTGGGAAAAAATCTTTTCA AAC-3') and the reverse prime BP 195 (5'-GATCCTGCAGTTAATGATGATGATG ATGATGTTTTTCTGCAACTCCTGTC-3') were used. IPTG inducible plasmids that contain translational fusion of MBP with ToxT that bears his tag at the C-terminal and its N-terminal truncation derivatives using the reverse primer BP 195 and the following forward primers; for full length of ToxT₁₋₂₇₆, BP 171 (5'- GA CAAGGTACCGATGATTGGGAAAAAATCTTTTCAAAC-3'), for ToxT₁₀₁₋₂₇₆, BP 174 (5'- GACAAGGTACCGATCTCATGATAAGGAATTTATATAG-3'), for ToxT₁₁₀₋₂₇₆, BP 185 (5'- GACAAGGTACCGGAAAATAAAGATCTATTACTTTGG-3'), for ToxT₁₁₅₋₂₇₆, BP 186 (5'- GACAAGGTACCGTTACTTTGGAATTGTGAACA TAATG-3'), for ToxT₁₂₀₋₂₇₆, BP 187 (5'-GACAAGGTACCGGAACATAATGATATA GCTGTCCTTTC-3'), for ToxT₁₃₀₋₂₇₆ BP 188 (5' GACAAGGTACCGGTGGTAAAT GGTTTCAGAG-3'), for ToxT₁₅₁₋₂₇₆, BP 175 (5'- GACAAGGTACCGTTCTTCTCG AAAGTAGAAAAAATATAAC-3'). The PCR products were cloned into pMAL-c2e using *KpnI* and *PstI* restriction sites.

RNA isolation and qRT-PCR. Cell pellets from different strains were harvested by centrifugation after 3 hours growth under virulence inducing conditions. RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer's protocol. Total RNA was subjected to DNase I digestion for 1 hr at 37°C to eliminate any DNA contamination. RNA was recovered by ethanol precipitation and re-suspended in RNase-free water. The RNA concentration was adjusted to 1 µg/µl based on A_{260} measurement. To monitor the level of *toxT* mRNA, 1 µg of total RNA was subjected to one-step SYBR Green quantitative RT-PCR using the manufacturer's protocol (Invitrogen). Specific primers for the *toxT* gene were used. The forward BP 105 primer is (5'-TGGGCAGATATTTGTG GTGA-3') and the reverse BP 106 primer is (5'- AAACGCTAGCAAACCCAGAC-3'). The level of *toxT* mRNA in each sample was normalized to the level of *rpoB* mRNA using the forward BP 100 primer (5'- ATCGAGCGTAACGTCGCGGTTGA C-3') and the reverse BP 101 primer (5'- AGTCAGGTTGTAGATGTGCGATACC-3'). The data represent an average of three independent experiments ± standard deviation (S.D.) Statistical significance of lower levels of *toxT* mRNA in the *tcpA* promoter deletion was determined by student's t-test with p value of 0.0101.

Protein analysis and purification. Aliquots of cells harvested at different time points during growth were normalized unless otherwise stated based on the optical density at 600 nm and re-suspended in 30 µl water and 10 µl of 4x protein buffer (246 mM Tris-HCl, 8% sodium dodecyl sulfate, 2.8 M 2-mercaptoethanol, 40% glycerol, 0.4% bromophenol blue). The samples were boiled for 5 min and

subjected to 12% or 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was blotted on nitrocellulose paper as previously described (3) and probed with a 1:3000 dilution of rabbit polyclonal anti-ToxT serum or 1:5000 dilution of mouse anti-His tag monoclonal antibody (Millipore). As an internal loading control, the primary mouse anti-elongation factor alpha 1 (anti-EF- α 1) was used at a dilution of 1:3000 (Millipore). Goat anti-rabbit or anti-mouse immunoglobulin antibodies conjugated to either alkaline phosphatase (AP) or Horseradish peroxidase (HRP) were used at a dilution of 1:5000 (Southern Biotech). After washing the blots, they were developed using the corresponding substrate. For AP, immuno-BCIP (5-bromo-4-chloro-3-indolylphosphate)—nitroblue tetrazolium was used (Invitrogen). For HRP, western lightning chemiluminescence substrate (PerkinElmer) was used and the blots were visualized using Autoradiography X-ray Film.

To purify ToxT fragments, C-terminal His tagged ToxT was overexpressed for 3 hours under virulence repressing conditions. Bacteria were collected by centrifugation and re-suspended into buffer A (50 mM sodium phosphate, 300 mM NaCl, 10 mM Imidazole, and 8M Urea at pH 8.0). The cells were lysed by French press at 1200 psi. Cell debris was removed by centrifugation and the clear cell extract was incubated overnight with nickel coated beads (Bio-Rad). The beads were washed 4 times for a total of 40 minutes with buffer A and collected after each wash by brief centrifugation. The His-tagged protein

fragments were eluted using 2 ml of elution buffer B (50 mM sodium phosphate, 300 mM NaCl, 250 mM Imidazole, and 8M Urea at pH 8.0).

RESULTS

Monitor the expression and activity of ToxT protein under different conditions. In classical biotype *V. cholerae*, maximal virulence induction *in vitro* is induced by growing the bacteria at 30° C in LB at starting pH 6.5 (125). These growth conditions were termed ToxR-inducing conditions due to the significant role of the ToxR in virulence induction; however, these conditions do not significantly affect ToxR activity. Instead, these conditions permit production of TcpP, which acts together with ToxR to activate *toxT* expression (94, 96, 100, 160), and we will refer to them as virulence inducing conditions. Minimal virulence induction results when the cells are cultured at 37° C in LB at beginning pH 8.5 (125), which we will refer to as virulence repressing conditions. Under virulence inducing conditions, both ToxR and TcpP initiate the activation of *toxT* from the *toxT* promoter. Once ToxT protein is made, it amplifies itself by activating transcription of the large *tcp* operon, which contains genes encoding TCP components and the *toxT* gene (197). Virulence induction is minimal under the high pH and temperature virulence repressing conditions, likely due to the absence of significant TcpP levels, and thus low ToxT levels, under these conditions. However, there is basal *toxT* transcription that should result in some ToxT expression, which could then ostensibly activate *tcpA* and produce more ToxT. Overexpression of *tcpPH* from a constitutive promoter in El Tor *V. cholerae*

under virulence repressing conditions results in an increase in CT expression but the amount of CT produced is still minimal when compared to CT production under inducing conditions (129), suggesting that ToxT activity is low under these conditions even when its production is induced by TcpP.

To better understand the regulation of ToxT, a time course experiment was conducted to monitor the accumulation of ToxT protein by western analysis and its activity in terms of *tcpA* activation under both ToxT-inducing and repressing conditions. *tcpA* activation was monitored using a chromosomal *tcpA-lacZ* fusion present at the endogenous *lacZ* locus. The data in Fig. 12A show that maximal ToxT activity occurs when culturing under virulence inducing conditions but minimal activity results when culturing under virulence repressing conditions. Consistent with the observed ToxT activity, ToxT protein (Fig. 12B) was detected in the bacteria as early as two hours and continually present in the cells up to at least four hours after subculture into inducing conditions. In contrast, ToxT protein was undetected at any time point after subculture into repressing conditions. As a positive control, lane 1 shows purified His₆-ToxT and as a negative control, lane 2 shows a ToxT deletion strain, which lacks the corresponding ToxT band.

Absence of any detected ToxT protein under virulence repressing conditions can be explained either by the fact that maximal activation of the *toxT* promoter is required to overcome the dilution of ToxT that results from bacterial replication, or ToxT can't amplify more of itself by activating the *tcpA*

autoregulatory loop under repressing conditions. To assess whether *toxT* transcription via the autoregulatory loop occurs under virulence repressing conditions, we utilized a derivative of classical *V. cholerae* strain O395 carrying a chromosomal *toxT-lacZ* fusion. This strain does not produce functional ToxT because the *lacZ* gene is inserted into *toxT* gene at the *HindIII* restriction endonuclease site (65), but transcription into *toxT* can be monitored by β -galactosidase assay.

First we assessed the contribution of the *tcpA* autoregulatory loop under virulence inducing conditions but in the absence of ToxT. Approximately 300 Miller units of β -galactosidase were produced; this total is the combined production from both the *toxT* and *tcpA* promoters. However, if functional ToxT was expressed *in-trans* from a pBAD-*toxT* plasmid, a three-fold increase in β -galactosidase production to about 800 Miller units was observed (Fig. 13A). These results suggest that this increase in β -galactosidase production is due to activation of the *tcpA* promoter autoregulatory loop by ToxT. Deletion of the *tcpA* promoter in this strain prevents the increase in β -galactosidase production induced by ToxT *in-trans*, which confirms that autoregulation occurs by activating the *tcpA* promoter.

When grown under virulence repressing conditions, the *V. cholerae toxT-lacZ* parent strain produced about 10 Miller units of β -galactosidase, but when ToxT is applied *in trans* there is more than a sixty-fold increase in *lacZ* production. These results suggest that ToxT is highly active under virulence

repressing conditions, at least when is it expressed at a high level *in trans*. The observed increase in β -galactosidase is a result of the autoregulatory loop of activation, as parallel experiments in the strain having a deletion of the *tcpA* promoters showed no increase in β -galactosidase production despite applying ToxT *in-trans*. To more directly measure how much the autoregulatory loop contributes to the level of *toxT* mRNA, we performed qRT-PCR to compare the levels of *toxT* mRNA in the wild type *V. cholerae* strain and the *tcpA* promoter deletion strain. Deleting the *tcpA* promoter causes a 2 fold reduction in *toxT* mRNA (Fig. 13B). Collectively, these results confirm that the ToxT positive autoregulatory loop functions through activation of the *tcpA* promoter under both virulence inducing and repressing conditions, and contributes to *toxT* expression.

The above results confirm that ToxT is active when over-produced under virulence repressing conditions and that ToxT is produced via activation of the *tcpA* promoter autoregulatory loop. The next question we asked was whether ToxT protein produced at natural levels could amplify itself as part of an autoregulatory loop under repressing conditions. To circumvent the problem that the initial activation of the *toxT* promoter by ToxR and TcpP is required for ToxT production, we cultured the bacteria first under virulence inducing conditions to induce ToxT production, and then the bacteria were harvested by centrifugation and resuspended in an equal volume of either fresh inducing medium or fresh repressing medium. This ensured that there was no dilution of the culture. A sample from each set of conditions was taken every hour and ToxT protein was

monitored by western analysis. Fig 14A shows that in primary culture under inducing conditions, ToxT is present in the cells (Lane 2). However, after shifting the bacteria into repressing conditions, ToxT protein disappeared after one hour (Lanes 6, 7, and 8). In contrast, when the cells were resuspended into inducing media, ToxT protein was continuously detected in the cells (Lane 3, 4 and 5). It is important to note that the dilution factor that arises from cell division is controlled by collecting the same volume of cultured media over time. This is apparent in the internal loading control as the amount of control protein increased over time. To determine an earlier time point for the fate of ToxT protein after shifting the culture from inducing to repressing conditions, samples were taken at 0, 15, 30, 60, and 90 minutes and western analysis was performed. The results in Fig. 14B show that the level of ToxT protein declined after 15 minutes and became completely undetectable after one hour of incubation. These results suggest that pre-existing ToxT protein is degraded under virulence-repressing conditions and this could prevent ToxT auto-amplification under these conditions.

Proteolysis of ToxT requires protein synthesis and ATP. Most of the cytoplasmic degradation in prokaryotes requires energy (55). *V. cholerae* possess at least five different ATP-dependent protease systems (ClpXP, ClpAP, Lon, HslUV, and HflB) and expression of some of these systems increases in response to high temperatures as their promoter regions contain a heat shock σ^{32} factor binding site (163). To test whether ToxT degradation under virulence-repressing conditions requires protein synthesis to induce a specific protease

and/or if proteolysis occurs through an ATP-dependent mechanism, two methods were utilized. We either stopped further protein synthesis by adding high amounts of chloramphenicol (200 $\mu\text{g}/\text{m}/$) to the bacteria, or we depleted the bacteria of ATP by adding a combination of sodium azide and non-metabolized glucose (α -methylglucoside) prior to shifting into repressing conditions; after both these treatments ToxT accumulation was monitored by western blot. This ATP depletion method was chosen because it has been shown to rapidly lower ATP concentration in the cells within ten minutes (86). Treatment of the cells with chloramphenicol or ATP depletion prior to shifting to repressing growth conditions prevents ToxT degradation (Fig. 15, lanes 5 and 6). However, depleting the cells of ATP causes a smaller ToxT band to appear (Fig. 15, lane 5). These results suggest that ToxT degradation might occur in a sequential manner with at least two different proteases. ToxT could be initially cleaved by a protease that is less sensitive to ATP depletion but is induced in response to temperature and pH, and then further ToxT degradation occurs in an ATP-dependent proteolysis.

ToxT degradation analysis. Because ToxT protein is not detected under normal growth conditions but produces a smaller protein band when ATP is depleted from the cells after shifting into repressing conditions, we hypothesized that overexpression of ToxT under repressing conditions could lead to the production of ToxT degradation fragments that can be detected by western analysis. To test this possibility, we overexpressed ToxT protein that carries a polyhistidine tag at either the N-terminal or the C-terminal end. When His-tagged

ToxT was over-expressed under virulence repressing conditions, a major smaller band was reproducibly detected in the cell lysate in addition to full length His-tagged ToxT (Fig. 16). Interestingly, the size of this smaller band differed between the N-terminally and C-terminally tagged ToxT constructs. N-terminally His-tagged ToxT produced a predominant band smaller than 17kDa. However, C-terminally His tagged ToxT construct produced a predominant band smaller than 23kDa but bigger than the 17kDa marker band. These data suggest that ToxT is initially cleaved at one primary site and then subjected to further degradation. To confirm that the smaller bands that were detected by western blotting are part of ToxT, we purified the predominant band that arises from the C-terminal His tagged ToxT on a nickel column, subjected it to SDS-PAGE, and then subjected a tryptic digest of the excised band to mass spectroscopy analysis. The peptide mass spectroscopy fingerprinting from the fragment band successfully identified multiple peaks corresponding to the C-terminal domain of ToxT protein. These data confirmed proteolytic cleavage of ToxT under virulence-repressing conditions.

We attempted to identify the protease that is responsible for ToxT degradation by both screening a transposon library for mutants that fail to degrade ToxT and by directly obtaining in-frame deletions of specific cytoplasmic proteases and assessing whether the deletion prevented ToxT proteolysis under virulence repressing conditions. However, these methods did not identify any

individual protease responsible for ToxT degradation. This could be due to the possibility that more than one protease is capable of degrading ToxT.

Next we determined whether the protease(s) that degrade ToxT are conserved between the classical and El Tor *V. cholerae* biotypes and/or other gram negative bacteria. We assessed whether ToxT degradation occurs in the El Tor biotype of *V. cholerae* and in *E. coli* when His-tagged ToxT is over-expressed from a plasmid. Overexpression of C-terminally His tagged ToxT protein both in the El Tor biotype of *V. cholerae* and in *E. coli* K12 under virulence repressing growth conditions resulted in detection of the same small fragments that arise from ToxT degradation in classical *V. cholerae* (Fig. 17A and B). Overexpression of C-terminally His tagged ToxT protein in classical *V. cholerae* and *E. coli* K12 under virulence inducing growth conditions resulted in minimal ToxT degradation (Fig. 18B) when compared to the amount of ToxT proteolysis under repressing conditions. However, some degradation fragments were detected in *E. coli* when cultured under inducing conditions. These results suggest that maximal ToxT degradation occurs under virulence repressing growth conditions and minimal degradation occurs under virulence inducing conditions in both *V. cholerae* biotypes and even in *E. coli*.

Identification of the cleavage site(s) within ToxT. To better understand the sequential degradation of ToxT, we attempted to identify the cleavage site(s) within ToxT. Several attempts were made to obtain N-terminal sequence information from the fragments that arise from the C-terminally His tagged ToxT

degradation. However, these fragments were blocked for Edman degradation chemistry. As an alternative we sought to map the domain within ToxT that is recognized by the protease and gives rise to the predominant band that is detected from the C-terminally His tagged ToxT construct. Translational fusions of maltose binding protein (MBP) with wild type ToxT-His₆ or various N-terminal ToxT-His₆ truncations were constructed, and these proteins were over-expressed under virulence repressing conditions such that the degradation intermediate could be detected. Truncation of the first 110, 115, 120,130, and 150 amino acids abolishes detection of the predominant band that migrates just below the 23kDa maker band (Fig. 18, lanes 5-9). However, this band was produced by full length ToxT or ToxT lacking the first 100 amino acids (Fig. 18, lanes 3 and 4). These results suggest that the region between amino acids 100 and 110 plays a role in ToxT proteolysis. It is significant to note that this region of ToxT has been shown to be important for its function. Alanine substitute mutations in amino acids 102, 103, 105, and 106 causes increase in ToxT activity (30). Increasing the activity of ToxT could be due to increase in stability of ToxT.

DISCUSSION

V. cholerae uses a coordinate regulatory cascade to induce its virulence determinants (117). While ToxT protein is the primary transcriptional activator that directly activates expression of major virulence factors including CT and TCP (75, 190-192, 198), ToxT expression is controlled by ToxR/S and TcpP/H as well as the positive auto-regulatory mechanism mediated through activation of *tcpA*

transcription (39, 65, 67, 100, 197). The current model for the virulence regulatory circuit is that ToxR and TcpP initiate *toxT* expression from the *toxT* promoter. Once ToxT is made, it maintains its expression through the auto-regulatory mechanism by activating the *tcpA* promoter. Evidence of virulence down-regulation during the late stage of infection, prior to escaping the host, has been observed (101, 121, 132). However the mechanism of this down-regulation is not understood. To turn off virulence expression, *toxT* expression must be stopped and this can be achieved by terminating activation of both the *toxT* and the *tcpA* promoters. Recent studies have shown that the level of TcpP is negatively controlled at multiple levels through promoter repression and protein degradation (8, 96, 116). This complex regulation might account for shutting off transcription from the *toxT* promoter. However, the mechanism for turning off the *tcpA* promoter is unknown, and here we present evidence for ToxT proteolysis when the bacteria are grown under conditions that do not favor virulence production. Maximal ToxT degradation occurs when the cells are grown under classical virulence-repressing conditions, whereas minimal or no degradation occurs when bacteria are grown under virulence-inducing conditions. Further analysis suggests that the unstructured motif located between ToxT amino acids 100-110 is required for this degradation. ToxT proteolysis likely accounts for the shutting down of the autoregulatory loop as the bacteria prepare to escape from the human host and resume their environmental lifestyle.

There are few examples in the literature of proteolysis controlling the level and activity of transcriptional regulators in bacteria. It is widely accepted that the concentrations of these regulators are controlled by their rate of synthesis and their concentration is decreased due to repeated cell division. However, it has been shown that lambda CII, lambda CI repressor and LexA repressor proteins are controlled by proteolytic degradation (56, 109, 147). The CII protein is a positive regulator for lysogen formation and is cleaved by the ATP-dependent Lon protease (56). Both lambda and lexA repressors that normally prevent gene transcription are also autocleaved in an ATP-dependent manner when targeted by RecA, and so their repression is relieved (109, 187). Further analysis revealed that the proteolytic degradation of these repressors results in production of two stable smaller fragments (109, 147). Interestingly, a similar circumstance occur in regard to ToxT degradation. Proteolytic cleavage of ToxT also produces smaller fragments with one predominant fragment that is approximately half the size of ToxT. Proteolytic cleavage of ToxT into fragments should inactivate pre-existing ToxT as it loses specific motifs that are required for its activity (30).

Depletion of cellular ATP prior to culturing the cells under high pH and temperature was able to rescue ToxT from degradation. However, a band smaller than full length ToxT was resolved on SDS-PAGE. This band could result from cleavage of ToxT protein to produce a slightly smaller polypeptide in a step that is less sensitive to ATP depletion, and further ToxT degradation is inhibited by ATP depletion as it occurs in ATP-dependent manner. *V. cholerae* encodes at

least three different cytoplasmic ATP-dependent proteases (ClpP, Lon, and HslV). Thus, we suspected that one of these systems is responsible for ToxT degradation. However, individual in-frame deletions of *clpP*, *lon*, or *hslV* did not prevent ToxT degradation. Additionally, inactivation of the *recA* gene, which prevents proteolysis of Lambda and LexA repressors (109, 187), did not prevent proteolysis of ToxT. The degradation fragments that resulted from ToxT degradation in wild type *V. cholerae* strain were still detected by western analysis in *recA*, *lexA*, *clpP*, *lon*, and *hslV* mutant strains of *V. cholerae* (data not shown). These results suggest that these proteases individually are not required for ToxT degradation and additional proteases might be able to cleave ToxT. It is worth noting that deletion of the *lon* gene from *V. cholerae* does not result in production of mucoid colony morphology as it is in *E. coli*. Searching the *V. cholerae* genome revealed additional proteases that are related to the Lon protease. These or other proteases could possibly substitute for any defective Lon function and prevent the mucoid phenotype and perhaps ToxT degradation.

Degradation of ToxT is enhanced by increasing temperature. Maximum ToxT proteolysis occurs when culturing the bacteria in LB with high pH and temperature. In contrast, minimal degradation occurs when culturing the bacteria in LB with a lower starting pH and temperature. Further analysis suggests that the temperature, rather than pH, is the major environmental factor that enhances ToxT degradation (data is not shown). In general, increased temperature leads to increased expression and activity of several cytoplasmic protease systems. *V.*

cholerae also responds to increased temperature with increased expression of several proteases and chaperones (163). Additionally, *clpB1* and *clpB2*, which function to dissociate inactive protein aggregates, are differentially induced *in vivo* (inside rabbit intestine) and *clpB1* expression increases in response to higher temperature and pH (130).

Treatment of *V. cholerae* with chloramphenicol to stop protein synthesis rescues ToxT degradation. These results suggest that the protease(s) that degrade ToxT require protein synthesis to function. We further tested whether any of the above proteases induced at higher temperature are responsible for ToxT degradation. However, none of the individual mutants prevented ToxT degradation. Again, a possible explanation is that more than one protease can degrade ToxT and inactivating one protease at the time might not be sufficient to prevent ToxT degradation.

The unstructured region of ToxT plays a role in controlling ToxT expression and activity. Protein fusions of MBP with different parts of ToxT suggested that the motif located between amino acids 100-110 is important for ToxT degradation as this region is required to produce the predominant degradation fragment of ToxT. The recently solved ToxT crystal structure revealed the 100-110 region of ToxT remained unstructured under the conditions that were used (111). Unstructured regions of proteins commonly serve as initiation sites for efficient degradation by the mammalian proteasome (140). In bacteria these unstructured regions of proteins are less common, but a recent

study showed that the unstructured motif of the bacterial Pub (prokaryotic ubiquitin-like) protein is required for its degradation (107). These results may suggest that bacteria could recognize these unstructured regions of protein to initiate their degradation. Furthermore, mutational analysis by alanine substitution in this region enhances ToxT function. Changing amino acids at positions 103, 105, or 106 causes more than a 300% increase in ToxT activity when compared to wild type ToxT when measured in terms of *ctxA-lacZ* expression (30). Amino acid substitutions in this region could result in a conformational change in this motif to stabilize and/or enhance the activity of ToxT.

In summary, we have demonstrated that the ToxT protein is regulated by proteolysis. The degradation of ToxT maximally occurs under conditions that do not favor virulence induction while it minimally occurs under conditions that do favor virulence induction. Furthermore, the disordered domain that extends between amino acids 100 and 110 appears to play a role in ToxT degradation. ToxT proteolysis may suggest a model for terminating *V. cholerae* virulence gene expression during the course of infection, with both ToxT and TcpP undergoing proteolysis prior to escape from the host.

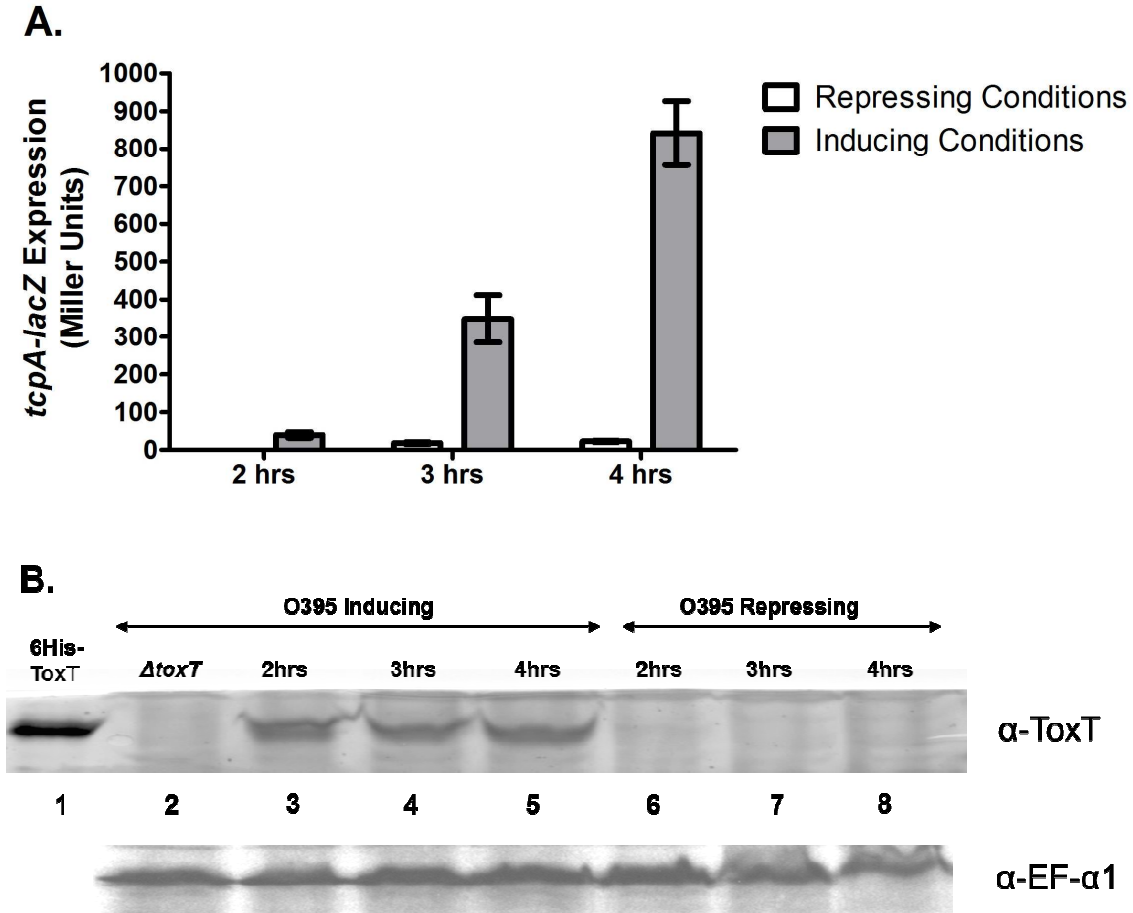


FIG. 12. ToxT protein levels and activity under virulence inducing and repressing conditions. (A) ToxT activity was measured by *tcpA-lacZ* fusion expression. Open bars, culturing under virulence repressing conditions. Grey bars, culturing under virulence inducing conditions. (B) Time course monitoring ToxT protein accumulation under inducing and repressing conditions. ToxT protein was detected using anti-ToxT antibody. For protein loading control; mouse anti-EF- α 1 (elongation factor- α 1) was used.

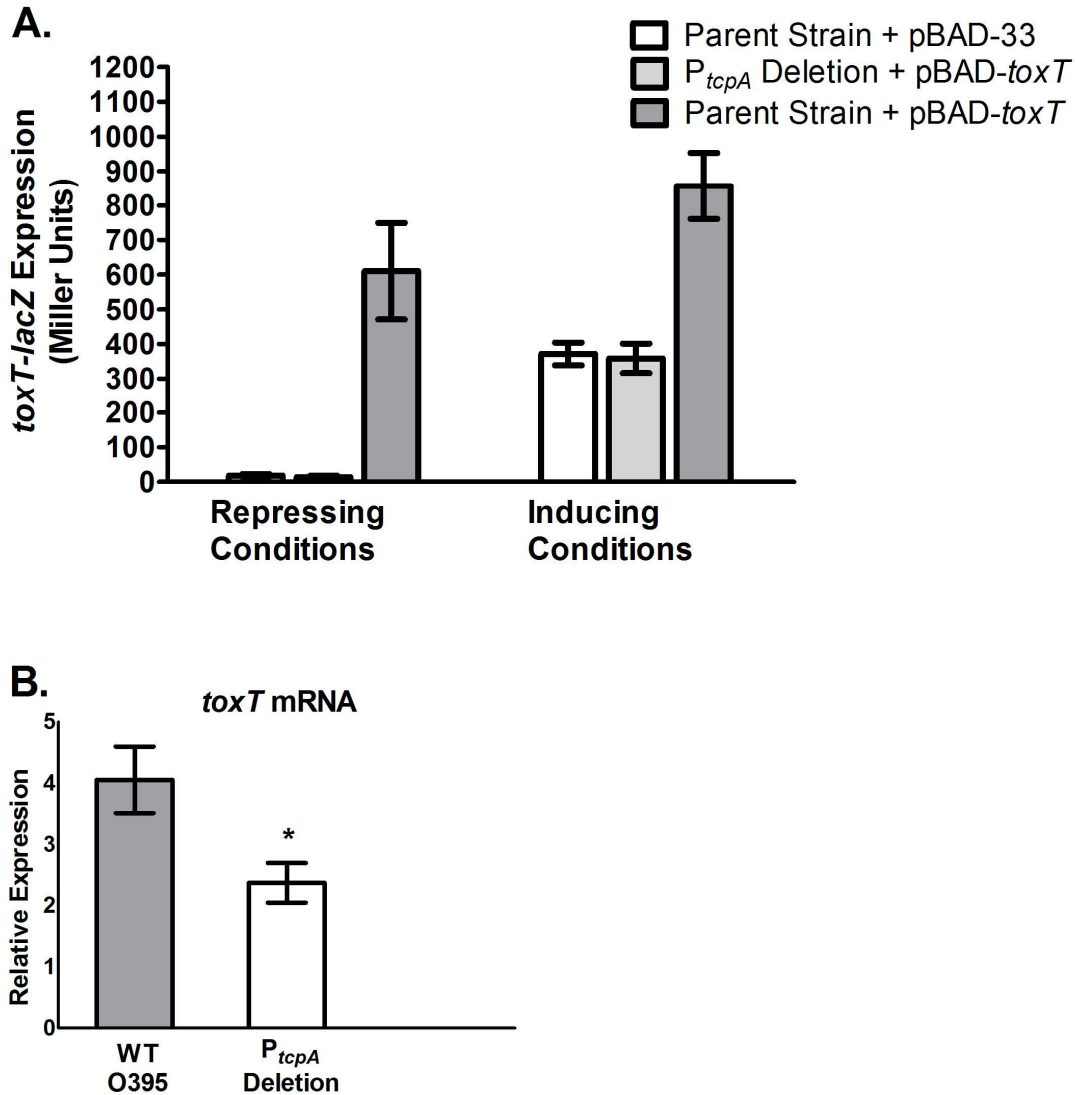


FIG. 13. Transcriptional analysis of the ToxT autoregulatory loop. (A) β -galactosidase production from the chromosomal *toxT-lacZ* fusion strain. Under inducing conditions, production of β -galactosidase occurs both through activation of the *toxT* promoter and via the autoregulatory loop by activation of the *tcpA* promoter (dark grey bars). Under repressing conditions, production of β -galactosidase occurs only through the autoregulatory loop by activation of the *tcpA* promoter (dark grey bars). In the absence of ToxT (white bars) or deletion of the *tcpA* promoter (light grey bars), production of β -galactosidase occurs only through the *toxT* promoter activation. (B) Contribution of the autoregulatory loop to the level of *toxT* mRNA. White bar is the *tcpA* promoter deletion strain. Grey bar is wild type O395 strain. *Significant difference in *toxT* mRNA level was determined by student's t-test with a p Value of < 0.02.

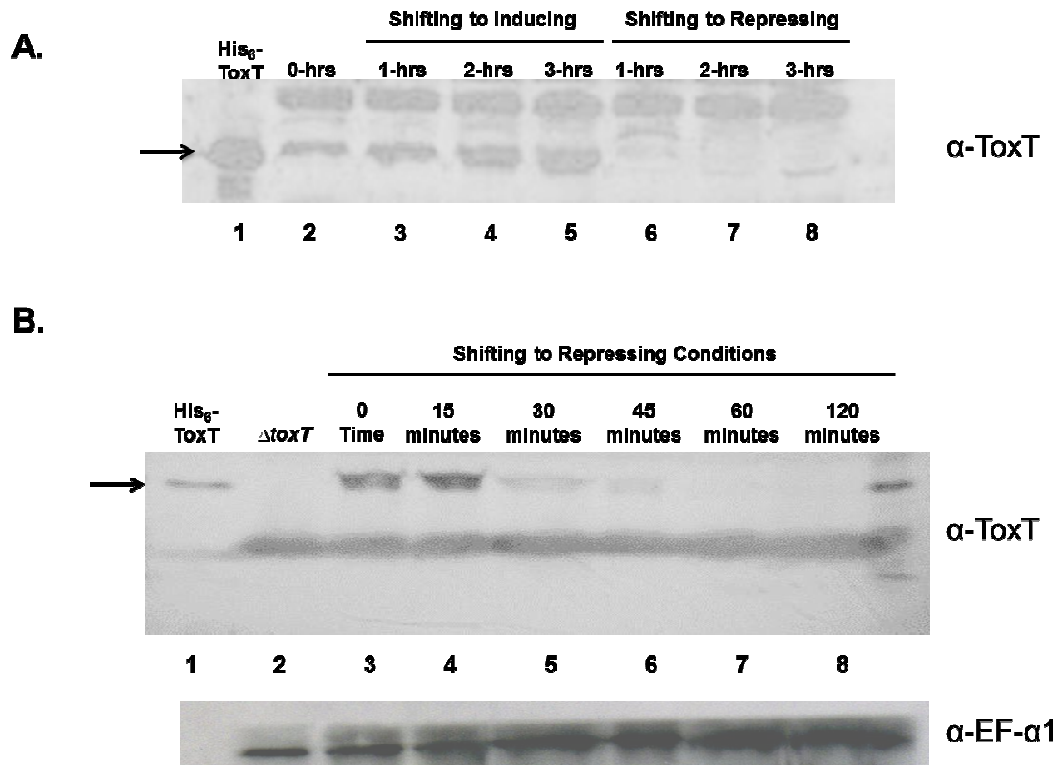


FIG. 14. The fate of ToxT protein when shifting the cells from inducing to repressing conditions. (A) ToxT was undetected after one hour post shifting from inducing to repressing growth conditions. (B) Earlier time points show the loss of ToxT protein starts within 15 minutes post shifting and ToxT was undetected after one hour. Protein loading control shows an increasing in the amount of control protein over time. Arrows indicate ToxT protein band.

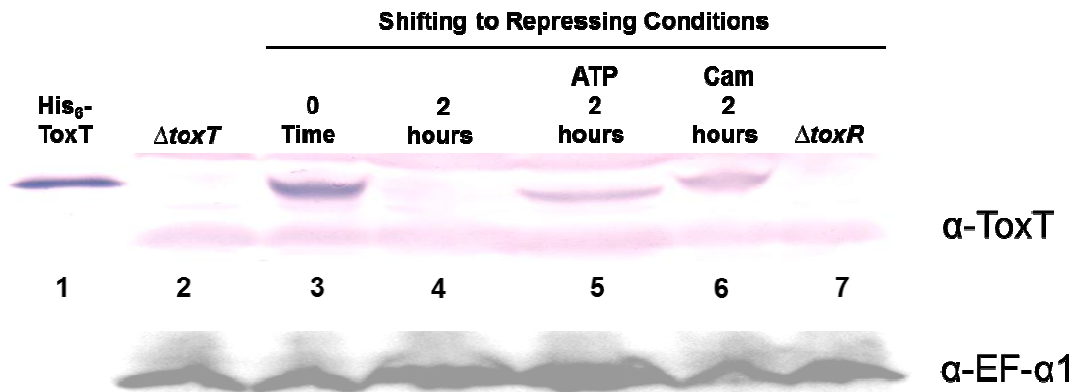


FIG. 15. ToxT fate in the absence of protein synthesis and cellular ATP. Primary cultures were grown under inducing conditions to produce ToxT (lane 2). Prior to shifting into repressing conditions, cells were either not treated (lane 4), treated with α -methylglucoside and sodium azide to deplete cellular ATP (lane 5), or treated with chloramphenicol to inhibit protein synthesis (lane 6). ToxT protein was monitored by western analysis using anti-ToxT antibody. Purified His₆-ToxT was used as a positive control, $\Delta toxT$ and $\Delta toxR$ strain backgrounds served as negative controls, and Anti-EF- α 1 was used for protein loading control.

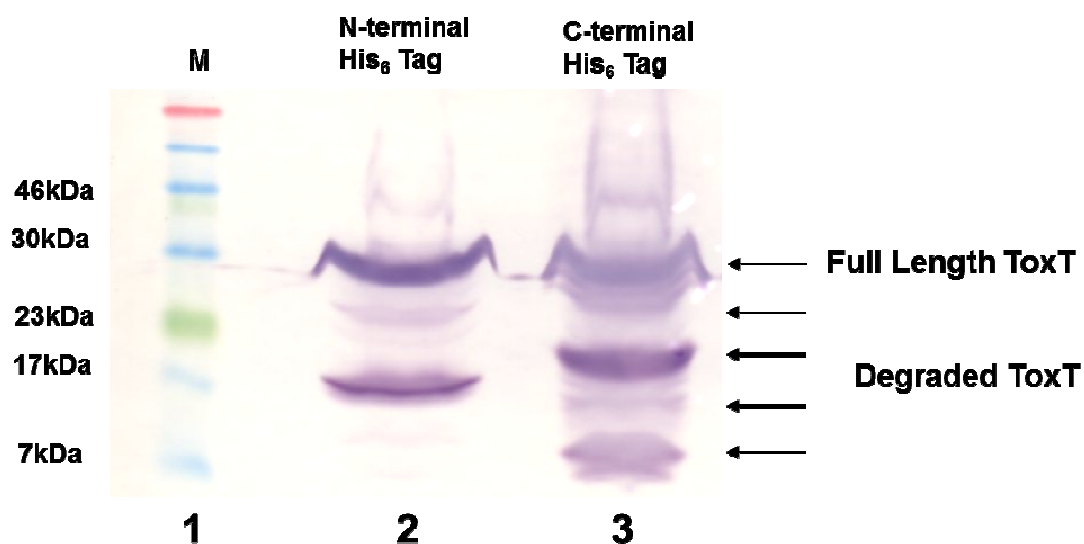


FIG. 16. Proteolytic cleavage of ToxT protein. N-terminal and C-terminal His-tagged ToxT was overexpressed while the cells were grown at 37°C in LB with starting pH 8.5 (virulence repressing conditions). Protein samples were run on 15% SDS-PAGE and His-tagged ToxT and its cleavage fragments were detected with anti-polyhistidine monoclonal antibody. Solid arrows indicate the predominant ToxT fragments, whereas light arrows indicate full length and the minor ToxT degradation fragments. M for protein marker ladder with the correspond sizes.

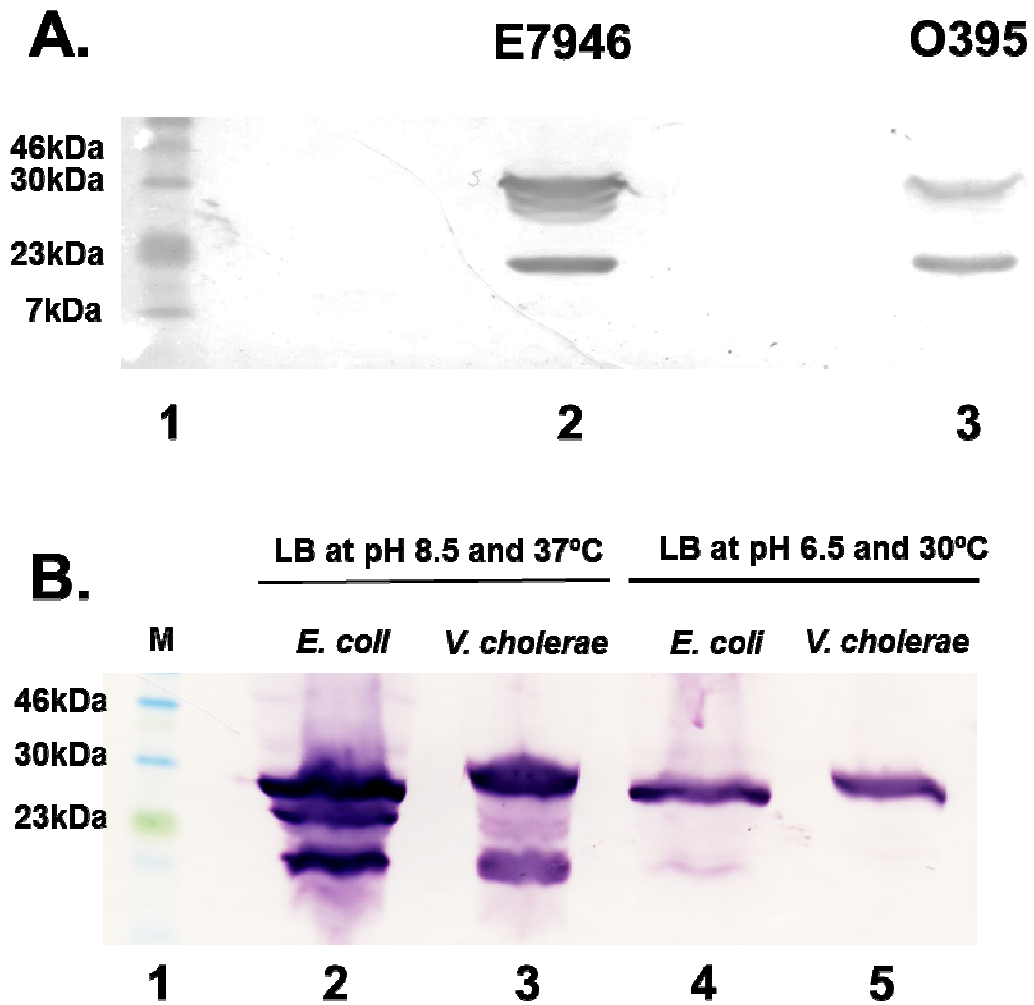


FIG. 17. ToxT degradation in both *V. cholerae* biotypes and *E. coli*. Both the classical and El Tor biotypes of *V. cholerae* (A) as well as *E. coli* K12 (B) can degrade ToxT when cultured under high pH and temperature. (B) ToxT degradation was observed when *V. cholerae* or *E. coli* were grown in either LB at pH 8.5 and 37°C or in LB at pH 6.5 and 30°C. Full length ToxT and the fragments that resulted from the C-terminal His-tagged protein degradation were detected with anti-His₆ monoclonal antibody.

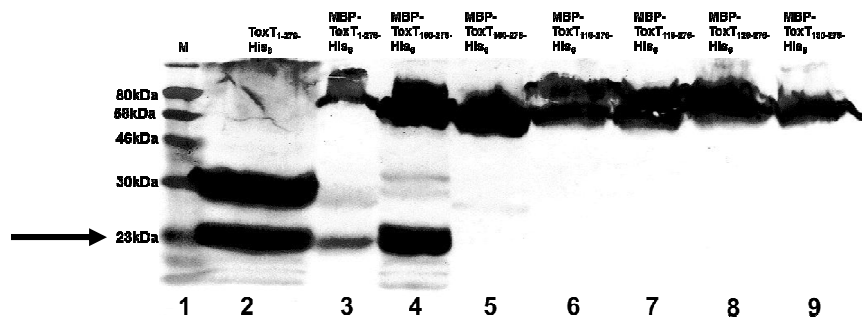


FIG. 18. ToxT motif located between amino acids 100-110 is required for ToxT degradation. When grown under virulence repressing conditions, overexpression of the translational MBP-ToxT-His₆ fusion produces a predominant degradation fragment just below the 23 kDa protein marker band (arrow) in addition to full length protein construct (lane 3) similar to the ToxT-His₆ control (lane 2). N-terminal truncation of the first 100 amino acids did not interfere with production of the degradation fragment (lane 4). However, truncation of 110 amino acids or more prevents production of the degradation fragment. Full length ToxT and the degradation protein fragment were detected with anti-His₆ antibody.

Strain or Plasmid	Discriptions	Reference or Source
Strain		
JW 419	<i>E.coli</i> K12	Lab collections
JW 75	<i>E.coli</i> SM10λpir	(162)
JW 9	<i>V.cholerae</i> classical biotype O395	Lab collections
JW 513	<i>V.cholerae</i> El Tor biotype E7946	Lab collections
JW 150	O395Δ <i>toxT</i>	(25)
JW 199	O395Δ <i>toxR</i>	(100)
JW 690	O395 <i>tcpA-lacZ</i>	(3)
JW 611	O395 <i>toxT-lacZ</i>	(65)
JW 714	JW 150 <i>tcpA-lacZ</i>	This work
JW 919	JW 611Δ <i>P_{tcpA}</i>	This work
JW 922	JW 690Δ <i>P_{tcpA}</i>	This work
JW 672	JW 611 + pBAD33	This work
JW 673	JW 611 + pJW 181	This work
JW 808	JW 714 + pJW 342	This work
JW 812	JW 714 + pJW 347	This work
JW 928	JW 919 + pJW 181	This work
JW 895	JW 513 + pJW 342	This work
JW 811	JW 419 + pJW 342	This work
JW 1076	JW 714 + pJW 412	This work
JW 1077	JW 714 + pJW 413	This work
JW 1078	JW 714 + pJW 414	This work
JW 1120	JW 714 + pJW 429	This work
JW 1121	JW 714 + pJW 430	This work
JW 1122	JW 714 + pJW 431	This work
JW 1123	JW 714 + pJW 432	This work
Expression Plasmids		
pBAD33	Arabinose-inducible plasmid	(58)
pJW 181	pBAD- <i>toxT</i>	(3)
pJW 342	pBAD- <i>toxT-his₆</i>	This work
pJW 347	pBAD- <i>his₆-toxT</i>	This work
pMAL-c-2e	IPTG-inducible plasmid	New England Biolabs
pJW 412	pMal- <i>ToxT₁₋₂₇₆-His₆</i>	This work
pJW 413	pMal- <i>ToxT₁₀₁₋₂₇₆-His₆</i>	This work
pJW 429	pMal- <i>ToxT₁₁₀₋₂₇₆-His₆</i>	This work
pJW 430	pMal- <i>ToxT₁₁₅₋₂₇₆-His₆</i>	This work
pJW 431	pMal- <i>ToxT₁₂₀₋₂₇₆-His₆</i>	This work
pJW 432	pMal- <i>ToxT₁₃₀₋₂₇₆-His₆</i>	This work
pJW 414	pMal- <i>ToxT₁₅₁₋₂₇₆-His₆</i>	This work

Table 1. List of strains and expression plasmids used in this work.

CHAPTER THREE

Genetic Screening for Bacterial Mutants in Liquid Growth Media by Fluorescence-Activated Cell Sorting

ABSTRACT

Many bacterial pathogens have defined in vitro virulence inducing conditions in liquid media which lead to production of virulence factors important during an infection. Identifying mutants that no longer respond to virulence inducing conditions will increase our understanding of bacterial pathogenesis. However, traditional genetic screens require growth on solid media. Bacteria in a single colony are in every phase of the growth curve, which complicates the analysis and make screens for growth phase-specific mutants problematic. Here, we utilize fluorescence-activated cell sorting in conjunction with random transposon mutagenesis to isolate bacteria grown in liquid media that are defective in virulence activation. This method permits analysis of an entire bacterial population in real time and selection of individual bacterial mutants with the desired gene expression profile at any time point after induction. We have used this method to identify *Vibrio cholerae* mutants defective in virulence induction.

INTRODUCTION

A common theme among bacterial pathogens is the response to host or environmental cues during an infection, followed by modulation of virulence gene expression. Isolating bacterial mutants that no longer respond to these cues

allows us to identify genes involved in virulence regulation and increases our understanding of how environmental cues contribute to virulence. Screening for regulatory mutants utilizing *lacZ* fusion technology has been successfully applied to a wide range of biological processes (157). In this method, transcriptional fusions are constructed between a promoter of interest and *lacZ*, which encodes β -galactosidase. By screening for bacteria with altered β -galactosidase production, mutations of interest can be isolated. However, this method requires culturing the bacteria on solid media to identify blue and white colonies using X-Gal. Within a single colony, bacteria in each stage of the growth curve are present, which can make identification of some mutants difficult. The lengthy growth period required to form a colony may also result in the slow accumulation of β -galactosidase, resulting in failure to identify mutants of interest.

Many bacterial pathogens have well-defined in vitro conditions that induce virulence gene expression in liquid media. Developing a screening method to isolate mutants in liquid media, in which most bacteria are in similar phases of the growth curve, and which may, in some cases, better mimic the interaction between the bacteria and the stimuli in vivo (3), would be a major improvement over traditional genetic screening methods.

Fluorescence-activated cell sorting (FACS) technology has been shown to be an efficient method to isolate specific bacterial populations (9, 139). FACS has also been used to isolate intracellular bacteria and bacteria grown during infections that modulate gene expression (18, 184), and to identify promoters

induced during infection (183); this method is generally known as differential fluorescence induction. Here, we utilized FACS technology to screen a bacterial random transposon mutant library grown in vitro in liquid medium. We used the gram-negative pathogenic bacterium *Vibrio cholerae* for our screen as its virulence inducing and repressing conditions in liquid media are well-characterized (50, 125).

V. cholerae is the causative agent of cholera and responds to environmental factors including pH, temperature, osmolarity, certain amino acids, bile, and bicarbonate to modulate virulence (57, 82, 125). Classical biotype *V. cholerae* virulence gene expression can be controlled simply by altering the starting pH of LB medium and growth temperature. In LB starting at a low pH (pH 6.5) and lower temperature (30°C) virulence gene expression is fully induced. In LB starting at a high pH (pH 8.5) and higher temperature (37°C), virulence gene expression is minimal (50, 125).

We designed a screening method that utilized FACS to identify and select bacterial mutants having lowered *V. cholerae* virulence gene transcription under virulence inducing conditions in liquid media. The *V. cholerae* strains used in this screen carry a fusion of the *tcpA* promoter region, which controls expression of the toxin-coregulated pilus, a major *V. cholerae* virulence factor, to the *gfp* gene encoding green fluorescent protein (GFP). The GFP produced from these constructs has a half life of ~20 minutes (18), so the problem of slow accumulation of GFP over time, leading to false positives, is avoided. The *V.*

cholerae mutant library was created using random transposon mutagenesis. This FACS screening method identified all genes known to positively regulate *V. cholerae* virulence gene expression as well as genes that had no previously known role in virulence gene expression. Similar screens are likely to be very useful for other bacterial pathogens having defined growth conditions in liquid media.

MATERIALS AND METHODS

Bacterial Strains, Reagents, and DNA Manipulation. *V. cholerae* classical biotype strain O395 was used as the parent strain to generate the transposon mutant library, an isogenic $\Delta toxT$ strain was used as a negative control, and *E. coli* SM10 (λ pir) was used for conjugation. All strains were maintained at -70°C in LB medium containing 20% glycerol. Bacteria were grown in LB medium containing antibiotics at the following concentrations: streptomycin, 100 µg/ml; ampicillin, 100 µg/ml; and kanamycin, 50 µg/ml. The *tcpA::gfp* fusion plasmid (pJW316) was constructed by PCR amplifying the *tcpA* promoter from *V. cholerae* O395 genomic DNA and cloned into pMW82 (18) using *SphI* and *XbaI* restriction sites. To construct the chromosomal *tcpA-gfp* fusion, *tcpA::gfp* was amplified by PCR along with 600 bp segments of the *lacZ* gene and cloned into the suicide vector pKAS32. The resulting product was moved into the *V. cholerae* *lacZ* locus by filter conjugation as previously described (162).

Transposon Mutagenesis and Identifying Site of Insertion. The suicide plasmid pFD1, which encodes the Kanamycin resistance *Himar1*-derived

transposon and *Himar1* transposase, was introduced by filter conjugation into *V. cholerae* O395 carrying pJW316. This was done by co-incubating *V. cholerae* (recipient strain) and SM10 (λ pir) (donor strain) on nitrocellulose filter paper over an LB plate for 3 hours at 37°C. Cells were then washed into LB media containing Streptomycin and Ampicillin and 2mM of isopropyl b-D-1-thiogalactopyranoside (IPTG) was added to induce the expression of the transposase. *V. cholerae* transposon mutants were selected on LB plates containing streptomycin, ampicillin, and kanamycin. The plates were incubated for 7 hours at 37°C, which was sufficient for colony formation. Resulting colonies were pooled and cultured in liquid medium for 3 hours under virulence inducing (LB at pH 6.5 and 30°C) or repressing conditions (LB at pH 8.5 and 37°C). At this point the bacteria are in logarithmic growth phase (115) and the *V. cholerae* virulence regulon is fully induced (190-192).

Direct sequencing by using genomic DNA was performed to identify the insertion site of the transposon. Briefly, genomic DNA was isolated from each mutant using the Wizard Genomic DNA Purification Kit (Promega). The DNA sequence adjacent to the transposon was obtained by using primers BP 30 (5'-ATGCATTTAATACTAGCGACGCC-3') or BP 31 (5'-CGCTCTTGAAGGGAAGTATGTTG-3'), which bind near the ends of the transposon.

Flow Cytometry and FACS. *V. cholerae* O395 strain lacking *gfp* was grown under inducing conditions for 3 hours and used to set the parameter

channels of the flow cytometer and the cell sorter. The three parameters; forward scatter (FSC), sideward scatter (SSC) and green fluorescence (515-545 nm), were set using a logarithmic scale. For flow cytometry analysis, the level of green fluorescence was measured by using a FACScan flow cytometer (Becton Dickinson) and the data were further analyzed with FlowJo analysis software. For bacterial sorting, low GFP-expressing mutants were sorted using a FACS Diva (Becton Dickinson) into LB media containing 20% glycerol and stored at -70°C. Individual colonies were recovered by plating the FACS isolates on LB plates containing ampicillin, streptomycin and kanamycin.

Statistical analysis. The percentage of GFP-expressing cells was calculated as the average of three independent experiments \pm standard deviation (S.D.) Statistical significance of the low level of *gfp*-producing mutants was determined by student's t-test with p value <0.0006.

RESULTS

***gfp* fusion constructs.** We constructed strains carrying the *tcpA::gfp* fusion on either the bacterial chromosome or on a plasmid. The plasmid-based construct is a derivative of pMW82 (18) in which the *tcpA* promoter region from classical *V. cholerae* strain O395 has been inserted upstream of *gfp* to create pJW316. Wild-type O395 and JW150, an isogenic strain containing a deletion of *toxT*, the major virulence activator, were then transformed with pJW316. Because ToxT activates transcription of all major *V. cholerae* virulence genes, including *tcpA*, we used the *tcpA* promoter region as a prototypical major

virulence promoter. Preliminary flow cytometry experiments with these strains indicated that high GFP levels were produced when bacteria were grown under virulence inducing conditions (Fig. 19A), whereas very low GFP levels were produced when bacteria were grown under virulence repressing conditions. GFP production is ToxT -dependent, as the $\Delta toxT$ strain that carries *tcpA::gfp* did not express any GFP.

Transposon mutagenesis and FACS screening. *V. cholerae* O395 carrying *tcpA::gfp* was subjected to *mariner*-based transposon mutagenesis. This method was chosen because it has proven very effective in producing mutant libraries (148). Approximately 10^5 mutant colonies were pooled after growth on LB plates containing proper antibiotic. The mutant pool was also tested for response to virulence inducing and repressing conditions by measuring GFP levels. As expected, most of the mutant pool resembled wild type *V. cholerae* and produced high GFP levels under inducing conditions and low GFP levels under repressing conditions (Fig. 19B). To identify mutants with defects in activation of *tcpA* expression we grew the mutant pool under virulence inducing conditions and screened for low GFP-expressing cells by FACS.

Identification of genes that are required for *V. cholerae* virulence gene expression. Using the FACS screen/selection described above, we sorted approximately 3×10^5 transposon mutants having low *tcpA::gfp* expression in liquid medium from a population of 6×10^8 *V. cholerae*. Individual mutants were plated and resulting colonies were re-suspended in liquid medium as before,

grown for 3 hours under virulence inducing conditions, and retested by flow cytometry to verify low GFP expression levels in liquid medium under inducing conditions. One hundred fifty-five mutants were individually tested in this manner and fifty-three transposon mutants were identified that had reproducibly low GFP expression; genes carrying the transposon insertion were identified by DNA sequencing out of the transposon. The genes containing transposon insertions included known positive virulence regulators *aphA*, *aphB*, *tcpP*, *toxR*, and *toxT* that validate our screening method (Fig. 20). In addition we identified genes encoding N-acetylmuramoyl-L-alanine amidase (*amiB*), DNA mismatch repair protein MutL (*mutL*) and DNA polymerase II (*polB*), as previously unknown positive virulence regulators (Fig. 20).

DISCUSSION

Here we describe a FACS-based screening system for identifying bacterial mutants with altered virulence gene expression in liquid growth media. Our data indicate that this screening system works well for *V. cholerae*, which has well-defined virulence inducing and repressing conditions in liquid media. In addition to experiments described above that identified mutants with defects in virulence gene expression under classical virulence inducing conditions, we have successfully performed screens for mutants that have increased virulence gene expression under classical virulence repressing growth conditions (B. H. Abuaita and J. H. Withey, unpublished data). We have also observed altered GFP expression when either classical or El Tor biotype *V. cholerae* are grown in the

presence of two natural effectors, bile and bicarbonate, and have isolated mutants that do not respond to either of these effectors (B. H. Abuaita and J. H. Withey, unpublished data). Therefore, this screen should be useful in general for identifying *V. cholerae* mutants with alterations in the virulence regulatory cascade. Our screen successfully identified all known positive virulence regulators, as well as genes not previously known to be involved in the virulence cascade. These results strongly validate the methodology of screening for bacterial mutants by FACS in liquid culture.

In addition to *V. cholerae*, this screen should prove useful for any bacterial species with defined growth conditions in liquid medium. Other bacterial pathogens having well-defined virulence-inducing conditions in liquid media include *Bacillus anthracis*, *Streptococcus pyogenes*, Enterohemorrhagic *Escherichia coli*, and *Citrobacter rodentium*, all of which stimulate virulence induction in response to the presence of CO₂/ bicarbonate in liquid media (2, 7, 22, 81, 196). Additionally, *Yersinia pestis* induces its type-three secretion system in response to low calcium levels (170), and *Bordetella pertussis* induces the BvgAS system when subcultured into media lacking MgSO₄ or nicotinic acid (32, 167). These different growth conditions have been designed to maximize virulence factor production. However, the mechanisms by which these environmental signals stimulate virulence are not well defined. Isolating bacterial mutants that no longer respond to these cues using FACS technology will increase our understanding of how these different systems work. Utilizing FACS

technology to screen for mutants grown in liquid media allows examination of an entire bacterial population (or entire mutant library) in real time and provides a significant advantage over the classical *lacZ* screens on solid media.

Our screen for *V. cholerae* mutants with defects in virulence activation identified not only all the known positive virulence regulators but also *amiB*, *mutL* and *polB*, three potential positive regulators. Both *mutL* and *polB* are involved in the DNA repair system, while *amiB* is involved in cell wall hydrolysis (13, 182). *amiB* is located adjacent to *mutL* and in *E. coli* the *mutL* promoter region is located within *amiB* (182). Because the transposon inserted near the end of *amiB*, we suspect that this mutant may have a polar effect on *mutL* expression.

The DNA repair systems could positively affect virulence induction by maintaining the integrity of the *V. cholerae* genome. Both *mutL* and *polB* are involved in DNA repair. One explanation for how mutations to these genes may affect virulence gene induction is by an increase in secondary mutations. Secondary mutations could affect known transcriptional activators (AphA, AphB, TcpP, ToxR, and/or ToxT), or alter DNA binding sites recognized by these activators. An in-frame *mutL* deletion strain constructed after identifying *mutL* in the FACS screen had the same *tcpA* expression defects as the *mutL* transposon insertion strain and also had a defect in cholera toxin production (data not shown), confirming that disrupting *mutL* causes a bona fide defect in virulence. Further work is being done to understand how the *mutL* and *polB* mutations reduce virulence induction.

Here, the FACS-based screening method was shown to be effective for identifying transposon insertions that disrupt virulence activation. However, the screen can easily be modified for other applications. Isolating transposon mutants with constitutively active virulence promoters can identify negative virulence regulators. Another application of this technique could be isolating mutants based on cell size. Different cell sizes can be distinguished and isolated by FACS based on forward and sideward scatter profiles. This type of screen would be useful for further understanding the mechanism by which different growth conditions lead to different cell sizes, as we have limited knowledge regarding the regulatory pathways that govern this process.

In summary, we were able to identify and select mutants that did not induce P_{tcpA} -*gfp* expression under virulence-inducing growth conditions in liquid using FACS. Because *V. cholerae* has well-defined virulence inducing and repressing conditions in liquid media but ill-defined virulence characteristics on solid media, this screen has proven very useful to us for many different applications. Many other bacteria, especially pathogens, also have well-defined growth conditions in liquid that induce virulence, and thus we believe FACS genetic screening will prove very useful to many labs.

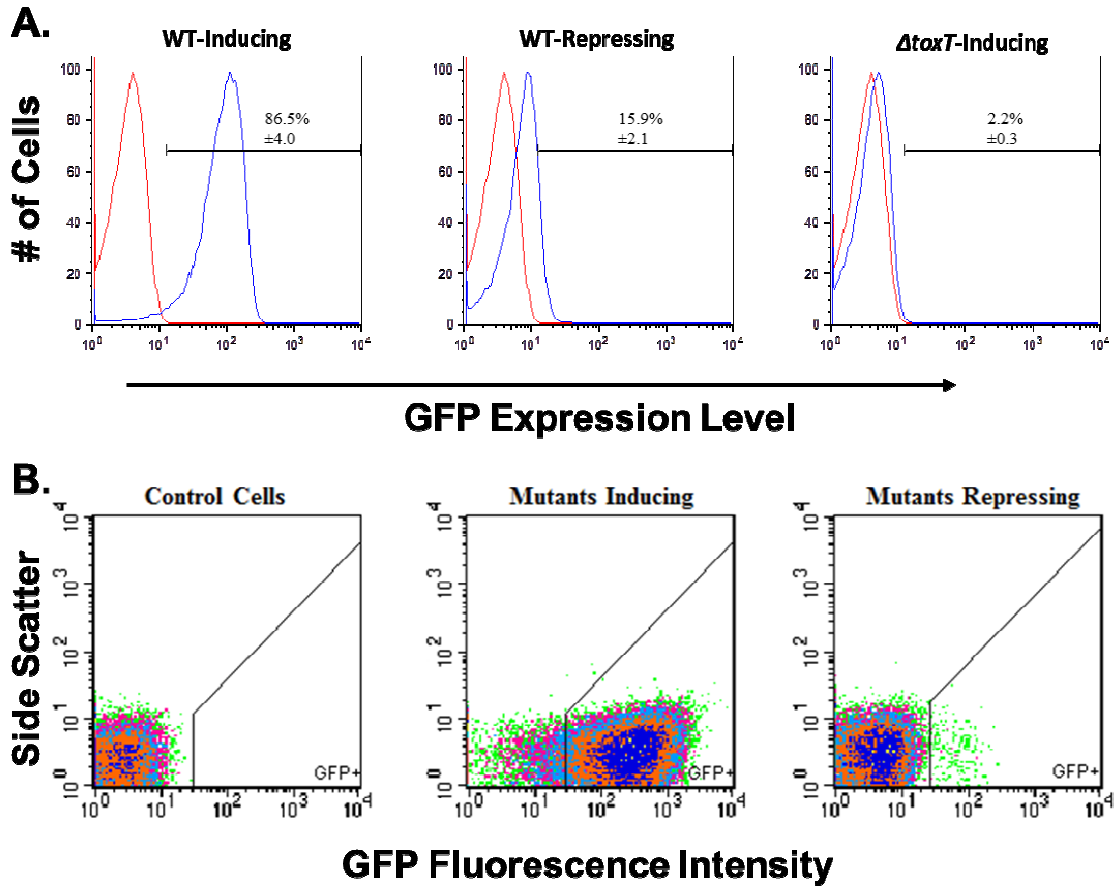


FIG. 19. GFP production under inducing and repressing conditions. (A) Histograms show the level of *gfp* fluorescence intensity of *V. cholerae* O395 and its isogenic $\Delta toxT$ strain that carry the *tcpA::gfp* fusion plasmid and grown under inducing or repressing conditions. The red histograms represent *gfp* negative *V. cholerae* grown under inducing conditions, the blue histograms represent the fluorescent bacteria. The percentage of gated cells is the average of three independent experiments with \pm S.D. (B) FACS analysis of a pool of mutants grown under inducing or repressing conditions. A wide range of *gfp* expressing mutants was produced when grown under inducing conditions as most of the mutants produce high fluorescence while some produced low *gfp*. Under repressing conditions, most of the mutants produce low *gfp* while some produce intermediate fluorescence levels.

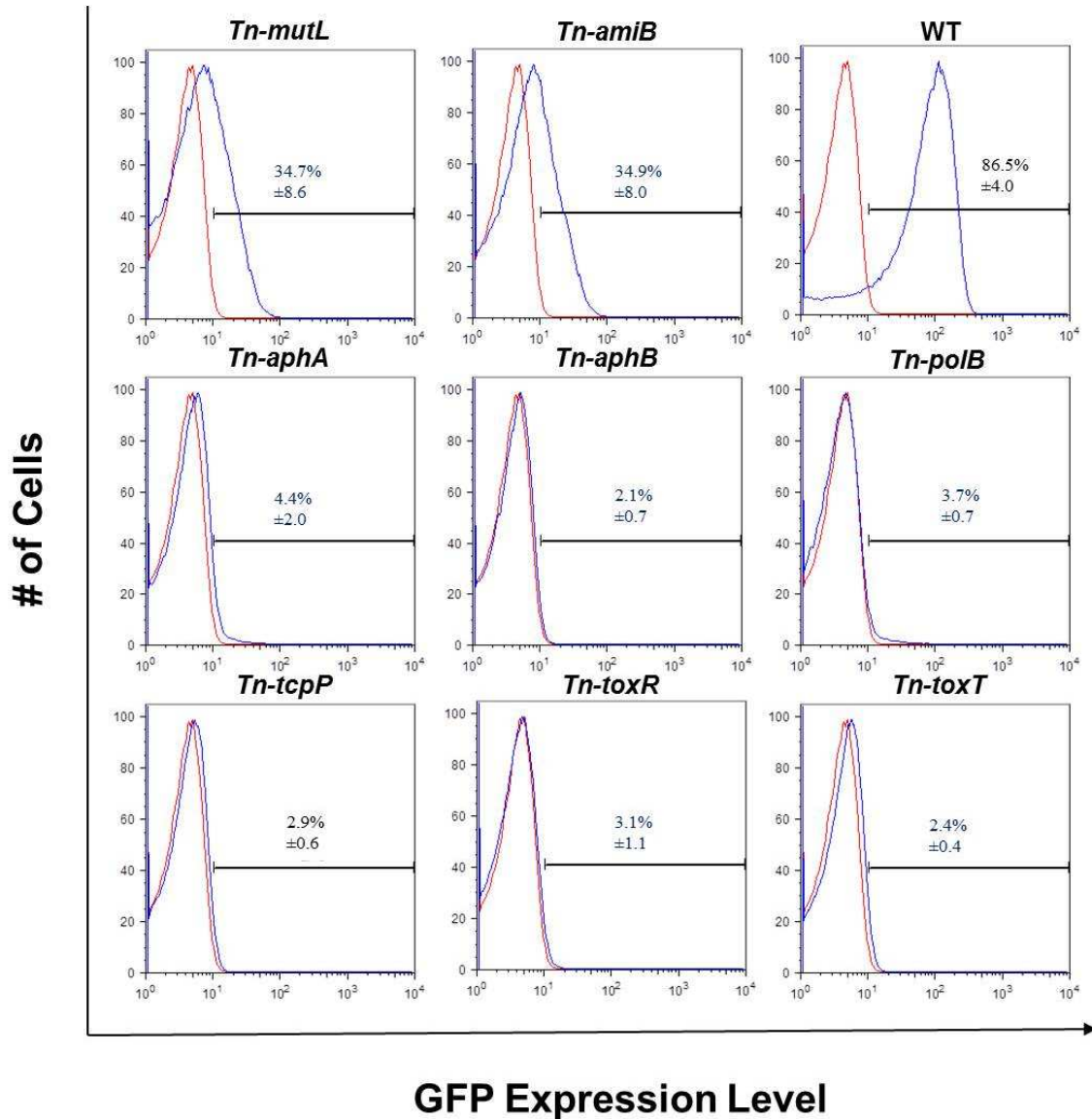


FIG. 20. Individual transposon insertion mutants exhibit a lower level of *tcp::gfp* expression when grown under inducing conditions. The red histograms show non-fluorescent *V. cholerae* grown under inducing conditions. The blue histograms represent *gfp* fluorescence intensity of different transposon insertion mutants and parent strain. The percentage of gated cells is an average of three independent experiments. Statistical significance between each individual mutant and the parent strains was determined by student's t-test with P value <0.0006.

APPENDIX A

The effect of ToxT on swarming motility

Virulence and motility are two processes that are inversely regulated by *V. cholerae*. Hyper-swarming motile bacterial strains produce lower levels of TCP and CT, while non-swarming motile bacteria produce larger amounts of TCP and CT when compared to the wild type parent strain (49). The mechanism by which one process regulates the other is not well understood. The two processes require massive amounts of energy and one explanation is that *V. cholerae* can't perform the two functions at once. Therefore, regulators must somehow activate one process while repressing the other. The primary virulence activator is ToxT and a $\Delta toxT$ strain is hyper-motile (Table 2). To further analyze the role of ToxT in the capability of the bacteria to swarm, we overexpressed ToxT *in trans* from pBAD-*toxT* in a $\Delta toxT$ strain and assessed the overall swarming motility. Different strains of *V. cholerae* were stabbed using a toothpick onto swarming plates (LB with 0.3% agar) that contain arabinose to induce ToxT expression and chloramphenicol for plasmid stability. The plates were incubated at 37°C for 16 hours and the swarming diameter for each strain was measured.

Overexpressing ToxT significantly inhibits total motility. ToxT-expressing strains produce swarming circles of 14 mm in diameter on average, whereas the control plasmid produces swarming circles 32 mm in diameter on average (Table 2). These results suggest that ToxT can repress or induce gene products that affect the swarming motility. One mechanism for this is that ToxT directly

activates the production of the TCP. Over-produced pili could lead to the formation of microcolonies, which could interfere with flageller rotation and thus reduce swarming motility. To test this possibility, we overexpressed ToxT in a $\Delta toxT$ and ΔP_{tcpA} (*tcpA* promoter deletion) strain and assessed total swarming motility. Overexpressing ToxT in this strain still inhibits swarming motility when compared to a strain that harbors the control plasmid. Double deletion of *toxT* and the *tcpA* promoter strain which harbors pBAD-*toxT* produced a 15 mm swarming diameter as apposed to a strain harboring the control plasmid, which produced 30 mm. These results suggest that the effect of ToxT on overproduction of TCP can't account for the effect of ToxT on swarming motility. To further assess whether functional ToxT is required to reduce swarming motility, we overexpressed a ToxT mutant that either has a point mutation at amino acid 52 or a truncation mutant that lost the second helix-turn-helix motif. These ToxT mutants are defective in activation of a *tcpA-lacZ* fusion (M. Bellair; unpublished data). Overexpressing ToxT _{Δ hth} mutants in a $\Delta toxT$ strain did not inhibit swarming motility, while an amino acid substitution at position 52 somewhat reduced the swarming motility by an average of 7 mm. These results suggest that functional ToxT is required for swarming motility inhibition.

In vivo virulence and motility have also been proposed to be inversely regulated. To assess whether overexpression of ToxT, which interferes with swarming motility, affects overall colonization of the infant mouse gut, we utilized two approaches. First, a strain with plasmid-based overexpression of ToxT

protein was competed with a control parent plasmid strain. Second, a constitutively ToxT-expressing strain (ToxT^C) was constructed and assessed for its ability to compete with wild type O395 for colonization of the infant mouse intestine.

The ToxT^C strain was constructed by fusing a λP_R promoter to *toxT* (λP_R -*toxT*) and inserted into the *lacZ* locus of *V. cholerae*. The new strain was confirmed for its ability to constitutively express ToxT under repressing conditions when ToxT protein is normally undetected in wild type parent strains (Fig. 21A). ToxT protein is also functionally active in the ToxT^C strain (Fig. 21B). Notably, the level of ToxT protein and its activity is still at minimum when compared to the level of ToxT protein produced by the cells that are grown under inducing conditions (Figs. 21A and B). The difference in ToxT protein is not due to lower *toxT* transcripts as real time PCR showed that the level of *toxT* transcripts are the same under inducing and repressing conditions (Fig. 21C). These results suggest that ToxT protein is regulated at a posttranscriptional level and perhaps by degradation (see Chapter 2 of this dissertation).

To test the ability of ToxT^C strains to colonize the infant mouse intestine, a competitive index was determined. An equal amount of wild type O395 and ToxT^C strains were inoculated intra-gastrically into 4-day old mice. After 16 hours of incubation at 30°C, the mice were sacrificed, their intestines were homogenized, serially diluted with LB and plated on LB plates containing streptomycin and X-Gal. White colonies (ToxT^C, lacZ-), and blue colonies (wild-

type, lacZ+) were counted after 16 hours of incubation at 30°C. *In vitro* control growth was done in which the combined inocula were plated on LB plates containing streptomycin and X-Gal and incubated at 30°C for 16 hours. The competitive index (CI) was calculated by utilizing the following formula; $CI = (\text{white/blue during infection}) / (\text{white/blue in vitro})$. Data in Fig. 22 show there was a small defect (about half fold) in the ability of the ToxT^C strain to colonize the infant mouse intestine. Similar results were obtained when ToxT protein was overexpressed from a plasmid and competed with a strain that harbors the control plasmid (Fig. 22). Collectively, these results suggest that overexpressing ToxT might not be deleterious to the cells as *V. cholerae* has multiple mechanisms to regulate its level and activity during infection.

Strain	Genetic Discriptions	Swarming Motility Diameter (mm)
JW 715	O395 Δ <i>toxT</i> + pBAD33	32 \pm 3
JW 716	O395 Δ <i>toxT</i> + pBAD- <i>toxT</i>	14 \pm 2
JW 927	O395 $\Delta\Delta$ <i>P</i> _{<i>tcpA</i>} <i>toxT</i> + pBAD33	30 \pm 3
JW 928	O395 $\Delta\Delta$ <i>P</i> _{<i>tcpA</i>} <i>toxT</i> + pBAD- <i>toxT</i>	15 \pm 3
JW 952	O395 Δ <i>toxT</i> + pBAD- <i>toxT</i> _{Δhth}	31 \pm 3
JW 953	O395 Δ <i>toxT</i> + pBAD- <i>toxT</i> _{E52A}	23 \pm 3
JW 989	O395 + pBAD33	17 \pm 2

Table 2. ToxT affects the swarming motility of *V. cholerae*. Overexpression of ToxT, ToxT mutation or a control parent plasmid in the indicative strain were assessed for effects on swarming motility. Swarming motility was measured by diameter of migration in soft agar.

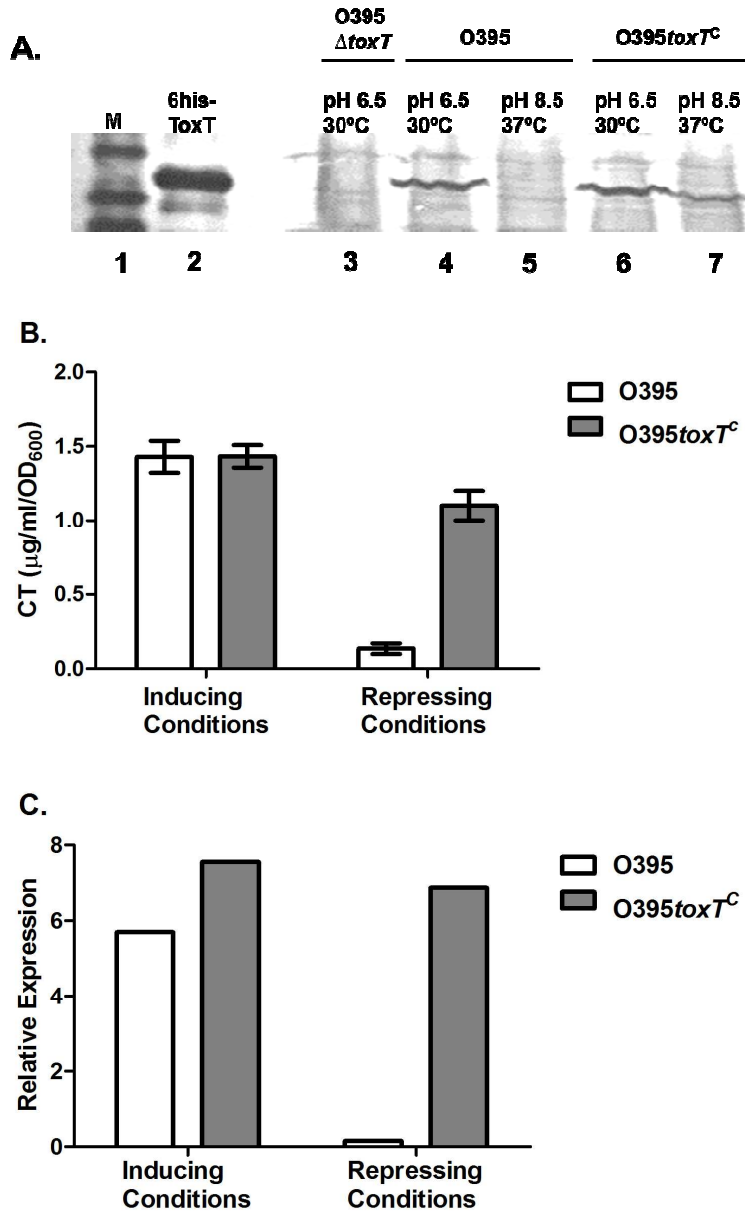


FIG. 21. Characteristics of a strain expressing ToxT constitutively (ToxT^c). Expression of ToxT protein (A) and its activity in terms of cholera toxin production (B) were measured after wild type O395 or its derivative ToxT^c strain were grown under inducing and repressing conditions. (C) The level of *toxT* mRNA that is produced by the two strains when grown under both conditions was determined by quantitative RT-PCR and normalized to the level of *rpoB* mRNA. ToxT protein was detected with anti-ToxT antibody.

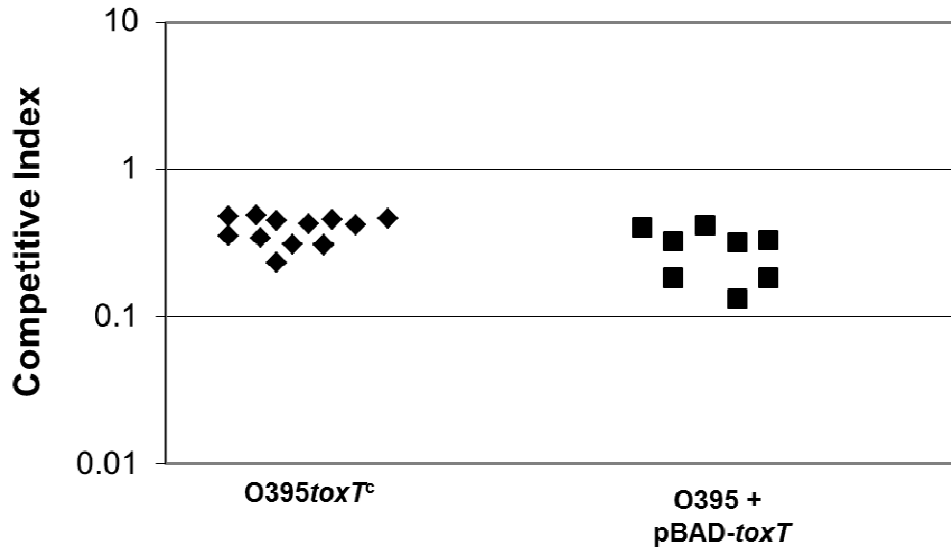


FIG. 22. Competitive indices of *V. cholerae* strains that overexpress ToxT in the infant mouse model. The constitutive ToxT expressing strain (O395toxT^C) was competed with wild-type O395 while the plasmid-based ToxT overexpressing strain (O395 + pBAD-toxT) was competed with O395 + pBAD33. Shaped boxes represent individual mice.

Appendix B

Isolating Bacterial Mutants that Affect the Activity of ToxT protein

To identify negative regulators that affect the activity of ToxT, we utilized a *tcp-gfp* fusion construct to isolate mutants with elevated ToxT activity when cultured under virulence repressing conditions. A pool of mutants was cultured under high pH and temperature, conditions under which wild type bacteria express a small amount of GFP, and high GFP expressing mutants were selected. This screen identified phosphoenol carboxykinase (*pckA*) and isocitrate dehydrogenase (*icd*) as potential negative regulators of ToxT activity. When these mutants were cultured under repressing conditions, they produced higher amounts of GFP when compared to the wild type strain (Fig. 23). Both *pckA* and *icd* are involved in central metabolism, therefore virulence and central metabolism could somehow be intertwined.

The second screen was done to isolate mutants that are required for the ToxT autoregulatory loop. We used the *toxT-lacZ* reporter strain. This strain does not produce functional ToxT. However, when ToxT is applied *in trans* and the cells are grown at high pH and temperature on plate media (virulence repressing conditions), β -galactosidase is produced through the autoregulatory loop by ToxT-dependent activation of the *tcpA* promoter. A pool of mutants of this parent strain were grown on LB plates at pH 8.5 containing arabinose, triple antibiotic (streptomycin, kanamycin, and chloramphenicol), and X-Gal. Arabinose was added to induce *toxT* expression while X-Gal was added to monitor β -

galactosidase activity. White and light blue colonies were picked for further analysis. All white colonies gave transposon insertions in the *toxT* gene. This confirms and validates our screen. Seven out of forty-four light blue colonies were isolated and lower levels of β -galactosidase were confirmed to be produced when cultured in liquid media (Fig. 24). The transposon insertions for three of the seven mutants were determined to be in *pntB*, *varA*, and *uvrC*. The *varA* gene is the response regulator that has been shown to activate three small regulatory RNAs (*csrB*, *csrC*, and *csrD*) implicated in quorum sensing (104) (Fig. 4). In addition, the *varA* deletion strain produces lower levels of TCP and CT, and is attenuated in mice (193). However, the mechanism for how *varA* negatively affects virulence is not understood. To further study the effect of VarA, an in-frame deletion of *V. cholerae varA* was constructed. Deletion of the *varA* gene produces translucent colony morphology. It is severely attenuated in mice, but produces the same levels of TCP and CT *in vitro* when it is compared with the wild type strain. Future analysis should be conducted to understand the mechanism of attenuation of the $\Delta varA$ strain *in vivo*. This type of screen should also be repeated to isolate additional mutants that affect the activity of ToxT protein.

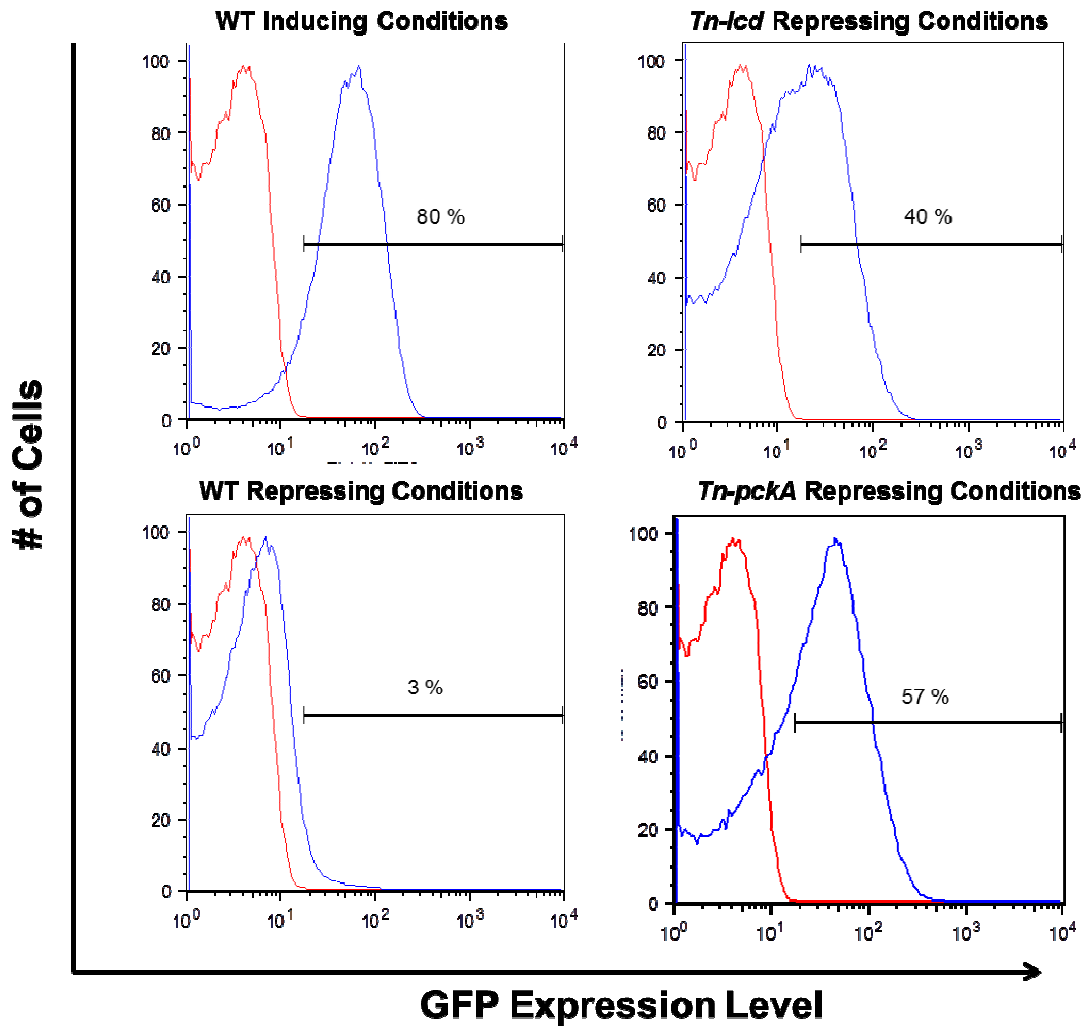


FIG. 23. *V. cholerae* mutants with elevated ToxT activity. Histograms show the level of *gfp* fluorescence intensity of *V. cholerae* O395 and transposon mutant derived strains that carry the *tcpA::gfp* fusion plasmid after growth under inducing or repressing conditions. The red histograms represent *gfp* negative *V. cholerae* grown under inducing conditions, the blue histograms represent the fluorescent bacteria. Both *pckA* and *icd* transposon mutants show elevated *tcpA::gfp* expression under repressing conditions.

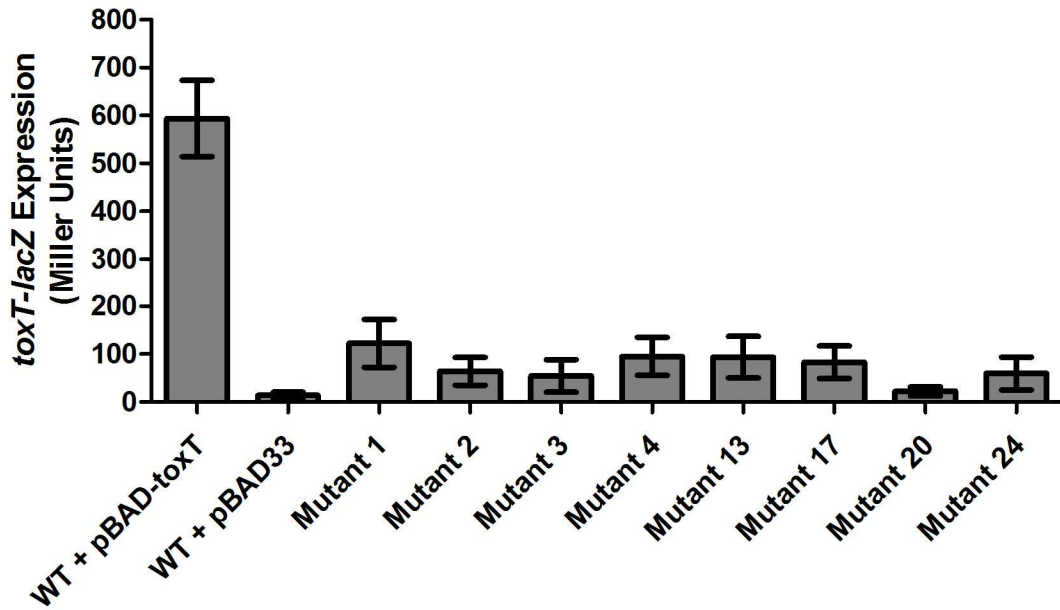


FIG. 24. Defective mutants in activation of the autoregulatory loop. Seven mutants (1, 2, 3, 4, 13, 17, and 24) showed more than three fold reduction in the ability of ToxT to activate the autoregulatory loop when compared to a wild-type parent strain. All mutant strains carry the pBAD-*toxT* plasmid and were grown under repressing conditions in the presence of 0.2% arabinose. Mutant 20 produced a basal level of β -galactosidase activity.

CONCLUSION

Vibrio cholerae, which is part of the normal aquatic flora around the world, uses a complex regulatory cascade to control expression of virulence determinants that it has acquired through horizontal gene transfer to facilitate colonization of the human intestine and to cause disease. For the past forty years, *V. cholerae* research has led to the discoveries of several regulators that are involved in the controlling virulence. Most of these studies were focused on understanding how expression of these regulators is controlled at the transcription level. However, posttranscriptional regulation of several key regulators such as TcpP and ToxT has been observed. ToxT protein is the primary transcriptional regulator that activates most virulence factors of *V. cholerae*, including TCP and CT, and can also activate itself through an autoregulatory loop of activation. The research that is presented in this dissertation highlights the regulation of ToxT protein after its initial production.

Two effectors with opposite function control the activity of ToxT protein. Addition of bile or bile components to the culture media reduces the activity of ToxT whereas addition of bicarbonate enhances ToxT activity. Bile and bicarbonate are found in the small intestine where *V. cholerae* colonizes, and could be the natural effectors or signal molecules that *V. cholerae* senses to direct the bacteria into the appropriate niche within the intestine and provide a specific time for when virulence is induced and repressed. Future work should be conducted to better understand the mechanism of reduction and enhancement of

bile and bicarbonate over the activity of ToxT. With this knowledge we would have a better understanding of how ToxT protein is regulated and design simple cheap synthetic molecules that inactivate the function of this protein.

ToxT protein is also regulated by proteolysis and this could be the mechanism for virulence termination. Early studies that monitored the *V. cholerae* transcriptome during infection led to the discovery of temporal virulence regulation. Both TCP and CT expression are induced during the early stages of infection and are repressed during the late stage of infection. We have a relatively good understanding of how virulence is turned on as many studies have been conducted to elucidate this induction. However, how virulence is terminated is not known. Virulence induction occurs through coordinate activation of the ToxR/TcpP/ToxT system in response to a still unknown *in vivo* signal. Here, ToxT degradation by proteolysis under certain conditions has been demonstrated and this could account for virulence repression during the natural course of infection. Future work that identifies the protease that is responsible for ToxT degradation would be a major scientific achievement as it would increase our knowledge and understanding of the mechanism that the bacteria utilize to control the activity of transcriptional regulators at posttranscriptional levels and perhaps through proteolysis regulation.

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ABSTRACT**POST-TRANSCRIPTIONAL REGULATION OF *VIBRIO CHOLERAE*
VIRULENCE ACTIVATOR TOXT**

by

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Vibrio cholera, the causative agent of the severe diarrheal illness cholera, uses a complex array of gene regulation to induce its virulence determinants. During the early stage of infection, and upon response to unknown signals, virulence genes are turned on. ToxT protein is the primary virulence gene transcription activator. Once ToxT is produced, it amplifies its own expression through an auto-regulatory loop and directly binds and activates expression of various virulence factors including the toxin-coregulated pilus (TCP) and cholera toxin (CT). During the late stage of infection, virulence genes are turned off and the bacteria escape the host to resume their lifestyle in the aquatic environment. While posttranscriptional regulation of ToxT has been observed, most studies were focused on revealing how *toxT* expression is initiated. Here, the regulation of ToxT protein at the posttranscriptional level has been explored.

In chapter one, experiments are presented that indicate bicarbonate is a positive effector molecule that enhances ToxT activity. Culturing the bacteria in the presence of bicarbonate increases the ToxT activity without increasing the protein level. Bicarbonate is found in the small intestine where *V. cholerae* colonize humans and could be the natural signal that the bacteria sense during the course of infection to maximally induce its virulence determinants.

In chapter two, the mechanism of virulence down-regulation as *V. cholerae* terminates its virulence expression during the late stage of infection preparing to enter back into the environment is assessed. The data suggest that virulence expression could terminate through ToxT proteolytic degradation. ToxT proteolysis was observed when culturing the bacteria at high temperature and pH, condition that has been found to repress virulence induction. Further analysis revealed that the unstructured motif which is located between amino acids 100-110 of ToxT is important for this degradation.

In the last chapter, a method of utilizing fluorescence-activated cell sorting (FACS) technology in conjunction with transposon mutagenesis is described. This method was used to isolate bacterial mutants that produce different gene expression profiles in response to environmental cues while cultured *in vitro* in liquid growth media. This technique should be applicable for isolating bacterial mutants that respond differently to chemical and physical inducers or repressors that are present in the liquid growth conditions.

AUTOBIOGRAPHICAL STATEMENT

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EDUCATION

Wayne State University School of Medicine. Detroit, MI. September, 2006 – 2010. Ph.D. Immunology and Microbiology.

GPA: 4.00

University of Michigan. Flint, MI. September, 2002 – December, 2005

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HONORS

Maize and Blue Award for Distinguished Scholarship. University of Michigan. Flint, MI. December, 2005.

PUBLICATIONS

1. **Abuaita, B. H., and J. H. Withey.** Genetic Screening for Bacterial Mutants in Liquid Growth Media by Fluorescence-Activated Cell Sorting. *J Microbiol Methods*.
2. **Abuaita, B. H., and J. H. Withey.** 2009. Bicarbonate Induces *Vibrio cholerae* virulence gene expression by enhancing ToxT activity. *Infect Immun* **77**:4111-20.
3. **Tischler, J. L., B. Abuaita, S. C. Cuthpert, C. Fage, K. Murphy, A. Saxe, E. B. Furr, J. Hedrick, J. Meyers, D. Snare, and A. R. Zand.** 2008. Simple inhibitors of histone deacetylase activity that combine features of short-chain fatty acid and hydroxamic acid inhibitors. *J Enzyme Inhib Med Chem* **23**:549-55.

LANGUAGE OTHER THAN ENGLISH

Arabic, written and spoken