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Functional Characterization Of The Cholera Toxin Promoter Of vibrio Cholerae

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FUNCTIONAL CHARACTERIZATION OF THE CHOLERA TOXIN PROMOTER OF *VIBRIO CHOLERAE*

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GENERAL INTRODUCTION

Cholera is a disease caused by the human pathogen *Vibrio cholerae*. It is characterized by voluminous watery diarrhea that is followed by dehydration and loss of electrolytes. If left untreated, patients can go into hypotensive shock, possibly followed by death, referred to as cholera gravis. Cholera patients can shed up to 1 L per hour of what is known as rice-water stool because of its similarity in appearance to water that has been used to wash rice. Other symptoms exhibited by infected individuals include sunken eyes, clammy skin, and loss of skin turgor. Treatment includes IV fluids as well as oral rehydration and, when properly and promptly administered, can improve mortality rates from more than 50% to less than 2% (164).

Cholera likely originated in the Indian subcontinent as there are descriptions of a cholera-like disease in Sanskrit dating back to 5 BC. The first cholera described beyond India was along trade routes beginning in 1817, when the disease spread throughout Southeast Asia then to the Middle East, East Africa and the Mediterranean. After this initial spread, six cholera pandemics were described between 1817 and 1923. The seventh pandemic, which started in 1961, continues to the present day. The WHO estimates three to five million cases of cholera and over 100,000 deaths annually, although the true burden may never be known as cases are vastly underreported (164). The areas currently affected by cholera are predominately in Asia and Africa where the bacterium is endemic. Specifically, outbreaks arise in regions with poor access to clean water and sanitation. In 2010, an outbreak occurred in Haiti following a severe earthquake that caused a perfect storm of the breakdown of health infrastructure, very high population density and poor sanitation following the effects of the earthquake. As cholera had not been described in

Haiti for at least 100 years, the population had no immunity to the disease. The result has been over a half million cases, 7,000 deaths, and likely a permanent public health problem (26).

Extensive studies on cholera have led to substantial contributions to the field of microbiology. In 1854, London physician John Snow identified the link between cholera and the ingestion of contaminated water which was a breakthrough epidemiological study at the time (173). During the same time period, Italian anatomist Filippo Pacini identified a possible causative agent isolated from cholera patients' intestines (12). He named these microbes Vibrios and described them as comma-shaped under a microscope although his work at the time was not widely accepted. Later, in 1883, Robert Koch rediscovered the organism and identified it as being the bacterium responsible for cholera and named it Kommabazillen (84). The name *Vibrio comma* was used for several years before Pacini was recognized as the original discoverer and the bacterium was renamed *Vibrio cholerae*.

Vibrio cholerae is classified by its liposaccharide (LPS) O antigen. Although there are over 200 serogroups of *V. cholerae* based on the O antigen that have been isolated from the environment, the O1 serogroup is responsible for all seven cholera pandemics (54, 164). The O139 serogroup also caused outbreaks of cholera beginning in 1992 and these outbreaks are sometimes referred to as the eighth cholera pandemic (14, 48, 139). The O1 serogroup contains two biotypes: the classical and El Tor. The classical biotype was responsible for the first six global pandemics. In 1961, the seventh pandemic began with the El Tor biotype (54, 164). This *V. cholerae* O1 biotype was named after the El Tor quarantine station in Sinai where it was first isolated. More recent

work determined that the O139 strains that caused cholera were derivatives of O1 El Tor strains (15, 132). The O1 serogroup can be further divided into three serotypes called Inaba, Ogawa, and Hikojima. These are based on antigenic groups A, B, and C found in the O antigen. Inaba produces groups A and B with a small amount of C while Ogawa produces only A and C (164). Hikojima produces all three but is rare and unstable.

V. cholerae naturally survives in aquatic environments where cholera is endemic. Survival in this environment can be either in a free-living state or in association with a variety of organisms including copepods, shellfish, vertebrate fish, and insect egg masses (20, 30, 71, 88, 169). Producing a chitinase allows it to attach to chitinous surfaces such as crustacean shells and utilize them as carbon and nitrogen sources (8, 124, 140). *V. cholerae* is capable of creating biofilms in the aquatic environment, enhancing its survival on a variety of surfaces (3). The biofilms are thought to be one way that humans ingest a high infectious dose of *V. cholerae* and, because bacteria in biofilms can survive acidic environments better, it also provides a possible mechanism for the bacteria to survive the acidity of the human stomach (126). In addition to being able to survive on chitin, *V. cholerae* can also survive in a 'viable but non-culturable' (VBNC) state in which they are unable to be cultured in standard laboratory media but still maintain basic metabolic function (45, 64, 70). Lastly, the bacterium may also survive in a "rugose" or "wrinkled" colony state where they are surrounded by an exopolymer of carbohydrates that makes them less susceptible to chlorine and other disinfectants while maintaining their full virulence capacity (135).

Transmission into a human host from contaminated food or water requires an infectious dose of approximately 10^8 bacteria (92). Prior to being ingested the bacteria

are motile and have active chemotaxis genes. After ingestion, the bacteria migrate to the duodenum of the upper small intestine and down-regulate genes that regulate motility and chemotaxis. Motility was thought to be an important to aid in establishing the infection by getting the bacteria to the preferred point of colonization in the upper small intestine. However, there is some controversy in the area of chemotaxis and motility and their roles in facilitating infection and colonization. In regards to motility, there are conflicting results regarding the classical strain but El Tor depends on motility for colonization (62, 98, 107, 160). Strains with defective chemotaxis systems are able to colonize infant mouse guts albeit in different locations throughout the small intestine than wild-type strains (59, 60).

While the bacteria are colonizing the small intestine, dramatic changes occur in gene expression within *V. cholerae*. The motility genes are downregulated and the virulence genes are upregulated at this point of the life cycle. At the late stage of infection, the bacteria initiate the "mucosal escape response" in which they detach from the epithelium, downregulate virulence, upregulate motility, and escape the host through the stool (145). The bacteria are reintroduced back into the environment in a hyperinfectious state; fewer than 100 bacteria can cause infection (22, 144, 198). This explains how outbreaks can magnify at a high rate.

A major turning point in *Vibrio cholerae* pathogenesis research was the discovery of the cholera toxin in 1959 by S.N. De (42). Using ligated ileal loops of adult rabbits, he was able to demonstrate that cell-free culture filtrates produce the characteristic "rice water" stool in the intestine. Confirmation followed by intravenously injecting Evans Blue which is known to bind to plasma proteins and mimic albumin. The contents of the

lumen after being exposed to the cell-free cultures were subsequently colored blue, signifying the increased permeability of the intestinal capillaries.

Cholera toxin was further characterized in the 1960s by Richard Finkelstein who isolated and purified the toxin (56-58). Two "versions" of the toxin were found: the "cholaragen" and the "choleragenoid" (56). The choleragen was shown to be highly active in inducing experimental cholera in rabbit models. The choleragenoid was also isolated and found to contribute to cholera symptoms but itself was not toxic. Since the original discovery, we now know that CT is a classical AB_5 toxin which means it has one 28 kDa A "heavy" or toxic-active subunit and several B "light" subunits with a combined size of 56 kDa. The choleragen that was discovered earlier was the AB_5 holotoxin while the choleragenoid was the B_5 subunits. During the same time period, the cell membrane receptor of CT was identified by King & van Heyningen in 1973 as being ganglioside galactosyl-*N*-acetylgalactosaminyl-(sialyl)-galactosylglucosylceramide (GM_1) (95). Further studies by other groups confirmed that GM_1 is indeed the receptor for CT by showing that CT binds to GM_1 in equimolar proportions and indicating there is a direct correlation between cellular GM_1 and the number of CT receptors (82, 83). Additonally, adding exogenous GM_1 to cell membranes increases the number of binding receptors and consequently amplifies the action of CT (37, 82). Finally, when CT is bound to GM_1, it prevents tritiation via galactose oxidase of GM_1 by sodium [^{3}H] borohydride reduction (137).

CT structure studies have further expanded our knowledge about the toxin. As with other AB_5 toxins such as Shiga toxin and pertussis, it contains one active subunit surrounded by a stable ringlike pentamer of B subunits. CT is also closely related to the

heat-labile enterotoxin (LT) produced by enterotoxigenic *E. coli* (ETEC) (38). The A subunit is originally a single polypeptide chain but it undergoes proteolytic modification to generate two fragments, A1 and A2. These subunits remain linked by a disulfide bond and the ADP ribosylating activity of the toxin remains in the A1 subunit. The CT crystal structure also provided clues as to the mechanism of translocation of the A1 subunit into the intestinal cells (199). The A2 subunit contains a unique KDEL sorting signal at the carboxyl terminus that extends past the B subunits when CT is in the holotoxin form. The B subunits bind to GM_1 gangliosides; B subunits can bind up to five gangliosides at a time on the cell surface although it has been shown that a toxin molecule may only need to bind to one receptor to gain entry into the cell (91). Additionally, *V. cholerae* produces a neuraminidase which has been hypothesized to enhance CT binding by catalyzing gangliosides into GM_1, providing more binding sites and greater fluid excretion (82, 95). The toxin associates with lipid rafts then enters the cell via endocytosis and is sorted to the ER by retrograde transport, meaning it goes from the early endosome to the Golgi complex then to the ER. In the ER, the A1 chain is unfolded but then refolds when it is released into the cytosol to avoid ubiquitin. The A1 chain was widely believed to need unfolding before entering the cytosol; however, it has since been determined that a protein-disulfide isomerase (PDI), a so-called “unfoldase”, is required for disassembly of the CT holotoxin to release but not unfold the A1 chain (180).

CTA1 is activated by ADP-ribosylation factors (ARFs) and catalyzes the ADP ribosylation of GSα component of adenylate cyclase (AC). Afterwards, the AC stays in the GTP-bound state, which increases AC activity and consequently increases intracellular cAMP. This increased concentration of cAMP decreases sodium uptake by

the cell and increases chloride expulsion by the cystic fibrosis trans-membrane conductance regulator (CFTR). The combination of decreased sodium uptake and chloride secretion skews the direction of water flow from normally entering the cell to being secreted from it. The result is massive water loss to the lumen and the subsequent characteristic voluminous watery diarrhea associated with the cholera disease.

Patients that have cholera get treatment through oral rehydration solution (ORS) and, in severe cases, intravenous infusion of fluid. ORS is used to replace the electrolytes that have been excreted in the stool and also contains glucose and potassium to aid in the absorption of sodium and water (17, 134, 178). Antibiotics can be used to help shorten the duration of the disease and reduce the amount of ORS needed, but are not essential for survival (115). Human volunteer studies provide the strongest evidence for infection-derived immunity, which can last at least three years (24, 25, 111, 112). This is supported by the frequency of infections among different regions. In areas where cholera is endemic, those most susceptible to infection are children aged 2-9 years old as well as women aged 15-35 years (65). Areas with little or no prior exposure to cholera experience cases in all age groups (65, 81).

There has been a considerable amount of work done to develop an effective cholera vaccine, dating back to the 1890s. A parenteral cellular killed vaccine was created in the 1960s but was mostly ineffective in children under 5 and adults were only protected for up to 18 months after immunization (11, 136). Later, it was discovered that CT itself acts as a mucosal adjuvant and the B subunit alone can trigger immune responses (51, 165). This led to the development of the oral cellular killed vaccine, which has a 50% efficacy after three years (34). However, this vaccine required at least

two doses and protection does not last more than four years, similar to a naturally acquired immunity, and children aged 2-5 years were protected for an even shorter amount of time (34, 185). Similar results were seen with a live-attenuated vaccine (161). The whole-cell killed vaccines are currently used in regions endemic with cholera. Further research is being carried out to make vaccines more affordable, effective for young children, and to induce a long-term immunity (16).

The key differences between Vibrios responsible for pandemics and those that are not is the presence of two important virulence factors: CT and the toxin coregulated pilus (TCP). The CT A and B subunits are encoded by the *ctxAB* locus, which is carried within the genome of the CTXΦ, a filamentous lysogenic bacteriophage. After acquisition by horizontal gene transfer, the CTXΦ site-specifically integrates into the *V. cholerae* chromosome. The entire CTX genetic element is a 6.9-kb DNA segment and is historically referred to as the "virulence cassette". It is divided into two regions: the core and the RS2 domain (186). The core of the CTX genetic element contains genes encoding CT as well as major and minor phage coat proteins, Psh, Cep, OrfU, and Ace and a protein required for CTXΦ assembly, Zot (186). The RS2 region encodes genes *rstA*, *rstB*, and *rstR* responsible for replication, integration, and regulation, respectively (187).

The TCP is a type IV pilus that is absolutely required for intestinal colonization of mammals by *V. cholerae* as demonstrated by infant mouse and rabbit cholera models and human volunteer studies (76, 163, 181). The genes required for TCP production and assembly are located on the Vibrio pathogenicity island (VPI), which itself may be a mobile element (93). Non-pathogenic Vibrios can also become toxigenic via mobile

elements in extracellular CTXΦ particles. Waldor and Mekalanos identified in *V. cholerae* that filamentous bacteriophages are involved in lysogenic conversion of bacterial pathogens and horizontal gene transfer (186). These results also support the co-evolution of the TCP and CTXΦ, for which TCP is a receptor. CT and TCP are both regulated by the same virulence cascade that is activated by environmental cues in the gastrointestinal tract, therefore providing an ideal niche for the movement of genetic elements (186).

The TCP is composed of TcpA pilin subunits. Once produced, TCPs induce aggregation of *V. cholerae* cells on the surface of epithelial cells, creating microcolonies (97). *In vivo* colonization of infant mice revealed microcolonies on the epithelial cell surfaces and field-emission scanning electron microscopy (FESEM) visualized the bacteria surrounded by pilin matrices (103). This autoagglutination may serve to protect bacteria from the intestinal environment, which contains complement, defensins, and bile, and is similar to the protective role of pilin in ETEC (33). In mutant studies where *tcpA* is not expressed, *V. cholerae* is unable to colonize both humans and infant mice and cannot cause disease (76, 182).

TcpA is just one of several genes that belong to what is referred to as the *tcp* operon, which includes a total of 12 genes. TcpA is processed during secretion by TcpJ, a prepilin peptidase (94). TcpF is another protein that is part of the operon which is required for intestinal colonization and is secreted by the TCP apparatus (96). Many of the *tcp* operon genes are necessary for the assembly of a viable pilus (19).

The VPI also contains several genes that are co-regulated with the *tcp* and *ctxAB* operons. These co-regulated genes include *acfA*, *acfB*, *acfC*, and *acfD*, which encode

accessory colonization factors that are required for optimal intestinal colonization in the infant mouse model and may be important for chemotaxis and environmental signaling (52, 151). Additional co-regulated genes found on the VPI include *tarA*, *tagA*, *aldA* and *tcpI*. A small regulatory RNA located upstream from *tcpI*, TarA regulates *ptsG*, encoding a glucose transporter and *V. cholerae* mutants lacking TarA have decreased colonization in infant mice compared to wild type (159). *tagA* encodes a secreted protease that cleaves mucin, which may aid *V. cholerae* in colonization (179). *aldA* has an unknown role in pathogenesis but encodes an aldehyde dehydrogenase (149). The role of *tcpI* is equally unclear but it encodes a putative methyl-accepting chemotaxis protein (72).

The regulation of virulence in *V. cholerae* is complex and depends on a number of different regulators and environmental signals. The virulence gene cascade responsible for the transcription of *tcp* and *ctxAB* is often called the "ToxR regulon" because ToxR was the first protein identified as a positive regulator. It is now known that virulence is controlled by a cascade of multiple positive regulators, with ToxR being on one level of the cascade. The major role of ToxR is to induce production of ToxT, the regulatory protein directly responsible for the transcription of most of the virulence genes in *V. cholerae*.

The presence of ToxT within *V. cholerae* is the result of a series of activated proteins upstream of the virulence cascade (Fig 1). The transcription of *toxT* depends on the activity of two integral membrane protein pairs, ToxR/ToxS and TcpP/TcpH (44, 73, 78, 105). ToxR dimerizes with ToxS for stability and directly binds to the *toxT* promoter (43, 49, 152). In addition to activating the transcription of *toxT*, ToxR also regulates *ompU* and *ompT*, which encode outer membrane porins (Omp). ToxR activates *ompU*

and represses *ompT* transcription (35, 113, 130). OmpU is highly expressed during host infection and is the dominant porin in the presence of bile and in minimal media when supplied with specific amino acids (106, 156, 175). This response to bile has a protective effect for the bacteria when they are in the small intestine during infection (114). OmpT is the dominant and more permeable porin under nutrient-limiting conditions and during environmental growth (114, 130) .

The other membrane protein pair that is required for *toxT* transcription is the TcpP/TcpH dimer (73, 105). TcpP is an integral membrane protein similar to ToxR that is stabilized and protected from proteolysis by TcpH (9, 23). TcpP binds to the *toxT* promoter between positions -54 and -32 relative to the transcriptional start site while ToxR binds closely upstream between positions -104 and -68 (105). The interaction between ToxR and TcpP at the *toxT* promoter is not well understood and the current model suggests that ToxR recruits TcpP to the promoter through protein-protein interactions. However, TcpP alone can activate *toxT* transcription if it is over expressed (73, 104, 105, 138). Therefore, the presence of ToxR dictates the mechanism by which *toxT* is activated by TcpP (67).

Production of TcpP/TcpH is mediated by the binding of AphA, a winged helix DNA binding protein, and AphB, a LysR-type regulator, to the *tcpPH* promoter (41, 99, 102, 172). These two proteins have overlapping binding sites, -101 to -71 relative to the transcription start site for AphA and -78 to -43 for AphB, on the *tcpPH* promoter and directly interact with each other to activate transcription (100). Production of AphA is controlled by quorum-sensing signals and its intracellular expression decreases with high cell density, indicating AphA has an important role in translating environmental cues into

virulence gene expression (101, 128). Quorum sensing master regulator HapR represses AphA production by binding to the *aphA* promoter and repressing its transcription (101). The result is levels of AphA that are too low to activate the transcription of *tcpPH*. HapR also regulates biofilm formation by repressing the VPS (Vibrio polysaccharide) operon which encodes the exopolysaccharide of biofilms (200). HapR positively controls HapA, a secreted hemagglutinin (HA) and protease involved in detaching bacteria from intestinal epithelial cells during the "mucosal escape response" (55, 90).

The activity of HapR is controlled by at least three different quorum sensing systems (109). The first system includes the autoinducer CAI-1 and the two component sensor-kinase CqsS. The second system involves autoinducer AI-2, LuxP binding protein, and LuxQ, a two component sensor. At low cell density, LuxO is phosphorylated and activated leading to the production of four regulatory sRNAs, Qrr1-4, which destabilize *hapR* mRNA in the presence of the chaperone protein Hfq (110). The third system serves to inhibit the global regulator CsrA which can activate LuxO. The VarS/VarA sensor kinase pair activates three sRNAs (CsrBCD) which leads to the inhibition of CsrA (109). These quorum sensing systems are important for regulation of virulence based on cell density. At high cell density, HapR is produced and virulence gene expression decreases, while at low cell density, HapR protein levels are very low and virulence genes are expressed. However, the requirement of *hapR*-based quorum-sensing for virulence varies in different *V. cholerae* strains. For example, some El Tor biotype strains possess a natural frame-shift mutation in *hapR* that renders it nonfunctional (201).

toxT transcription is activated by TcpP/H and ToxR/S by binding to the *toxT* promoter (44, 73, 78, 105). However, once ToxT protein is expressed within the bacterial cells, a longer transcript that contains *toxT* is produced from the *tcp* operon through ToxT activity and starts an autoregulatory loop, producing more ToxT and further inducing virulence (78, 195). The deactivation of ToxT and consequently the interruption of the autoregulatory loop involves decreasing activation at both the *toxT* promoter and the *tcpA* promoter where ToxT autoregulates. TcpP, which binds to the *toxT* promoter, is inhibited by cAMP-CRP based on the availability of glucose as a carbon source (100). TcpP is also degraded by the protease YaeL and another unknown protease despite having TcpH to prevent proteolysis (122).

One model for the down-regulation of virulence is the degradation of ToxT (2). Recent studies have shown that, when *V. cholerae is* grown in non-virulence inducing conditions, ToxT undergoes degradation. This proteolysis disrupts the ToxT autoregulatory loop and subsequently deactivates virulence. The proteolytic cleavage in ToxT has been localized to a position between amino acids 100-110, a region of ToxT that was not resolved in the crystal structure but has also been implicated in binding to ToxT effectors (2, 118).

ToxT is a member of the AraC/XylS family of transcriptional regulatory proteins found in gram negative bacteria (79). It contains a sequence of 100 amino acids in the C-terminal domain (CTD) with two helix-turn-helix domains that is common in other AraC family proteins (61, 78, 183). The remaining 176 amino acids comprise the N-terminal domain (NTD) of ToxT and do not share any homology with other proteins. The ToxT NTD is thought to be important in protein dimerization or effector binding that can

modify ToxT activity. LexA domain swapping experiments and two-hybrid analyses suggested that dimerization of ToxT NTD alone occurs; however these studies did not include the CTD of ToxT (31, 155, 170). Crystal structure studies and alanine substitution experiments with ToxT reveal that certain residues in the NTD have a role in virulence gene expression (31, 118).

There is some controversy regarding ToxT binding to DNA as monomer or a dimer. Within the AraC/XylS family of proteins, there are examples of proteins that bind as monomers such as MarA, SoxS, and Rob and proteins that bind as dimers including AraC and RhaS (50, 74, 121, 193). As mentioned above, dimerization studies with ToxT have been done using LexA fusion assays and bacterial two hybrid assays and suggest that the ToxT NTD is able to dimerize independent of the CTD (31, 155). Additionally, virstatin, a small molecule ToxT inhibitor, has been shown to inhibit activity at certain ToxT-driven promoters but not all of them; ToxT dimerization has been proposed to be inhibited by virstatin (170). The variability in these results suggests it is not obvious that ToxT needs to dimerize before binding to DNA. This is demonstrated by the *aldA* promoter that contains only one ToxT binding site, therefore requiring only one ToxT monomer for transcriptional activation (192). Additionally, if DNA is inserted between two ToxT DNA binding sites to rotate them relative to each other DNA binding by ToxT is not affected (162,163). These results strongly suggest that ToxT binds to DNA as a monomer, although ToxT dimerization may occur after DNA binding and may be important for ToxT activity.

Both positive and negative ToxT effectors have been identified. Bile has a negative effect on ToxT activity, as do specific unsaturated fatty acids (UFA) such as

oleic acid, as well as chemical compounds virstatin and capsaicin. All of these negative effectors decrease both ToxT dependent *tcp* and *ctxAB* transcription (29, 70, 86, 87, 168, 170). The crystal structure of ToxT has a *cis*-palmitoleate UFA buried in the NTD that has been proposed to inhibit ToxT activity by locking it in a structure that is unable to dimerize (118). ToxT also responds to the positive effector bicarbonate, found in high concentrations in the upper small intestine, which increases the expression of both CT and TCP within *V. cholerae* when added to culture medium (1). This supports the human volunteer studies which found that supplementing inocula with sodium bicarbonate reduce the infectious dose of *V. cholerae* from 10^8 to 10^4 (24).

When ToxT is produced in *V. cholerae*, it activates the transcription of virulence genes by binding to DNA elements that are referred to as toxboxes (191). These are degenerate thirteen base pair DNA sequences that are present in the promoters of the virulence genes that ToxT activates. The consensus sequence of ToxT binding sites was first proposed by Withey and DiRita after analyzing ToxT binding sites in *acfA* and *acfD* (190). These are two inverted repeat binding sites that ToxT binds to between the promoters of *acfA* and *acfD*. In addition to identifying a consensus sequence, mutations to one binding site did not affect binding to another. Coupled with the observation that insertional mutations of 5 or 10 base pairs did not affect ToxT binding to DNA, the hypothesis that ToxT binds to toxboxes as a monomer was proposed. However, both binding sites were required for activation by ToxT. Similar studies were performed with the promoters of *tagA* and *aldA* (192). The *tagA* promoter analysis revealed two toxboxes arranged as an inverted repeat while *aldA* has only one toxbox. This further

confirms that ToxT binds as a monomer but also complicates the mechanism by which ToxT activates transcription.

Further studies were performed using the *tcpA* promoter to characterize toxboxes. Thus far, the only common features among the toxboxes was the presence of a T tract of nucleotides at the 5' end of the binding site and that they are located upstream of the -35 promoter element (Fig 2). The *tcpA* promoter has a few potential ToxT binding sites that fit the toxbox requirements and experimentation was done to investigate which of these are directly involved in ToxT binding. The result was the identification of two binding sites that, unlike the previously characterized promoters, were orientated as direct repeats (191). When comparing the toxboxes from the various promoters, there is an obvious variability in ToxT binding requirements (Fig 3). Even though the toxboxes are all upstream of the -35 promoter element, the exact position of their proximal endpoint relative to the transcriptional start site varies from -44 to -62. ToxT either has one or two binding sites within the promoter and when two are present, they are either direct or indirect repeats. Therefore, identifying toxboxes in other promoters, such as *ctxAB*, is impossible to do without further experimentation.

AraC/XylS family of proteins often bind to degenerate binding sites and examples include the MarA, SoxS and Rob proteins which bind to the same 20 base pair sequence (69, 121). Consistent with toxboxes, these binding sites are A/T rich but a significant difference is that they are found in both class I and class II promoters. Class I promoters have transcriptional regulator DNA binding sites upstream of the -35 promoter element while class II promoters binding regions overlap with the -35 region. The location of activator protein binding to DNA can dictate which subunit of the RNA polymerase

(RNAP) will be used for interaction (21). Class I promoter activator proteins generally interact with the α-subunit of RNAP while class II promoters interact with the σ-subunit, and in some cases, both the σ-subunit and α-subunit (80, 89). However, many activator proteins are not exclusive to one promoter class. For example, SoxS, which has been found to interact with both of the RNAP subunits, can bind to both class I and class II promoters (197). There are two mechanisms by which this can occur: "prerecruitment" and recruitment pre-binding. Prerecruitment suggests that activator proteins interact with RNAP prior to DNA binding and then the complex searches for binding sites within promoters. This is the proposed pathway for SoxS-dependent transcriptional activation (68, 120). Pre-binding, in contrast, recruits RNAP to the DNA after the activator protein has already bound to its appropriate DNA binding sites near the promoter, as seen with SoxS at the *micF* and *nfo* promoters and as has been suggested for other promoters (157).

Many AraC/XylS family proteins also increase transcription by antirepression. Typically this involves H-NS, a global transcriptional repressor commonly found in Gram-negative bacteria. H-NS preferentially binds to intrinsically curved AT rich regions of xenogeneic DNA which are located in or near virulence genes for many bacteria (119, 142, 143, 148, 153, 184, 194). H-NS represses transcription by inducing hairpin-like structures that bridge two double strands of DNA and either prevent RNAP from binding or prevent transcription if RNAP is able to bind (polymerase trapping) (39, 40, 167). H-NS is abundant within bacteria and overcoming its repression is vital to activating transcription of virulence genes.

The mechanism by which the AraC/XylS family of proteins counteracts the repression by H-NS varies for different promoters (176). One obvious mechanism

involves displacing H-NS from the promoter completely by transcriptional activator competition. An example of this in *Salmonella* is SlyA/RovA, which competes for binding with H-NS at *hlyE* (117). At other promoters that are activated by SlyA/RovA, complete dissociation of H-NS is not observed but rather the H-NS-DNA complex is altered to allow other transcription factors to interact with RNAP (75, 141, 150). In some pathogenic *E. coli* strains, H-NST, a protein similar to the N-terminal portion of H-NS, has been shown to behave in a dominant negative action and can inhibit H-NS from dimerizing and forming complexes that repress promoter function (7, 189). Temperature can also mediate H-NS repression as investigated at the *virF* locus in *Shigella* (153, 154). At room temperature, two H-NS binding sites are aligned properly at an intrinsically bent region of the *virF* promoter but the binding sites are misaligned when the temperature is increased and H-NS repression is alleviated (53, 153).

V. cholerae H-NS is encoded by the *vicH* locus (13). H-NS is capable of silencing virulence gene expression at the *tcpA*, *toxT*, and *ctxAB* promoters, all of which are AT-rich and present on mobile elements within the bacterial chromosome (63, 146, 196). In addition to repressing virulence genes, H-NS also has a negative effect on *flrA* and *rpoN*, genes involved in motility and *hapA*, encoding HA/protease (171, 188). H-NS is expressed in the cells throughout the life cycle and H-NS antirepression is key to expressing genes vital for virulence.

H-NS was first determined to have a major influence in the ToxR regulon by Nye *et al* (146). Using classical *V. cholerae* Δ*hns* strains, they demonstrated that expression of *toxT*, *tcpA*, and *ctxAB* increased significantly under both inducing and non-inducing conditions. In wild-type *V. cholerae* strains, virulence transcription is induced in a

laboratory setting by growth at 30□ C and in LB with a starting pH of 6.5. Non-inducing conditions raise the LB pH to 8.5 and the temperature to 37□ C. By having such a dramatic effect in non-permissible conditions, it suggests that H-NS represses transcription at these promoters under normal conditions. Additionally, expression of *ctxAB* increased under non-inducing conditions as well as in a Δ*toxT* strain, further solidifying that H-NS has a direct influence on the *ctxAB* promoter (146).

The antagonism of H-NS by ToxT was investigated by Yu *et al*., and the mechanism by which ToxT activates transcription was further elucidated (196). This study included nested deletions of the *ctxAB* promoter and coupled them with DNase I footprinting to identify which region of the DNA was important for binding and activating transcription. The region extending to -76 relative to the transcriptional start site is important for *ctxAB* activation and the footprints revealed ToxT binding from -111 to -41 at high ToxT concentrations and -118 to -112 and -40 to -13 at low concentrations. c*txAB* activation was also analyzed in *E. coli* to determine the effects of H-NS and ToxT on the promoter. In accordance with findings by Nye *et al*., the *E. coli hns*- strain also had much higher activation of *ctxAB* than wild type (146). This suggests that ToxT needs to counteract H-NS repression to activate transcription. Additionally, this group also performed an *in vitro* transcription assay and found that ToxT was required for transcription of both *ctxAB* and *tcpA* in the presence of RNAP, indicating that ToxT, like other AraC family proteins, interacts with the RNAP to activate transcription. With these findings, the group proposed a model for the activation of *ctxAB* in which H-NS binding represses transcription from the promoter and that ToxT displaces H-NS, the de-

repression step, and interacts with RNAP, the true activation step, to activate transcription (196).

The mechanism by which H-NS represses the *ctxAB* promoter is similar to other bacteria in that in addition to binding DNA, H-NS also needs to oligomerize and occupy multiple consecutive sites near the promoter (147). Though previous studies had examined its role in repressing transcription, the region of DNA H-NS binds was determined by Stonehouse *et al* (177). Using DNase I footprinting and promoter deletion analysis, H-NS binding encompasses the ToxT binding region previously discussed, the -35 promoter element and two regions downstream of the +1 transcriptional start site. With H-NS overlapping with the ToxT binding site, the previous model describing transcriptional activation of *ctxAB* by ToxT displacing H-NS is still feasible. However, the identification of ToxT and H-NS binding sites by DNase I footprinting has its limitations. The *ctxAB* promoter is A/T rich which can interfere with DNase I cleavage resulting in possible low resolution footprints. Identifying specific binding sites of ToxT and H-NS at *ctxAB* will further characterize the mechanism by which transcription of *ctxAB* is activated and aid in understanding the interplay of these two proteins.

The studies described in this dissertation build upon the above work to generate a more complete picture of how CT production is controlled by ToxT and H-NS. The DNA binding sites were identified and characterized for ToxT and H-NS at the cholera toxin promoter. Combining high resolution copper-phenanthroline DNA footprinting with site-directed mutagenesis, *ctxAB* transcription activation is now better understood in the context of ToxT and H-NS. Having a better understanding of the mechanism by

which *ctxAB* is activated can potentially lead to novel therapeutic approaches for managing cholera outbreaks in the future.

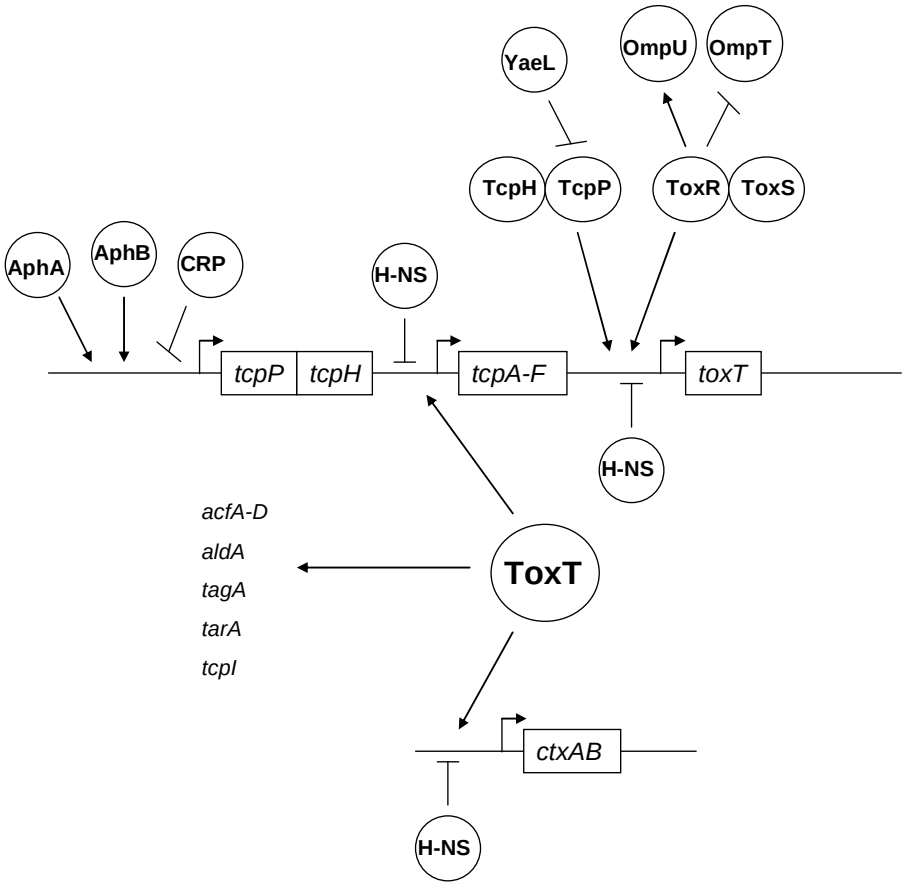


Figure 1: Virulence regulatory network in *V. cholerae*. The circles represent proteins and the rectangles are a schematic of genes found on the DNA with bent arrows showing transcriptional start sites. The solid arrows indicate positive regulation by the indicated protein while the blunt-ended lines highlight negative regulation in the promoter regions upstream of the transcriptional start sites.

tcpA:	TATTTTTTTAATA
	CATTTTTTGCTGT
acfA/acfD:	AATTTTTAAAAAT
	CATTTTGTTAAAT
tagA:	AATTTTAAGTTAA
	TGTTTTTTTAATG
aldA:	TGTTTTTTTAAAT
tcpI	TATTTTCCTAAAG
	CGTTTTAAATAGT
tarA:	TGTTTTTTTTAAT
	TATTTTTTTTAAC
tarB:	TGTTTTTTTTAAG
	TATTTTTTTAACG

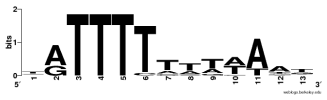
toxbox 

Figure 2: Alignment of ToxT binding sites. The toxbox sequences of ToxT regulated genes are indicated. The gene names are identified on the left side and the consensus sequence is shown in the form of a weblogo (36) at the bottom of the figure.

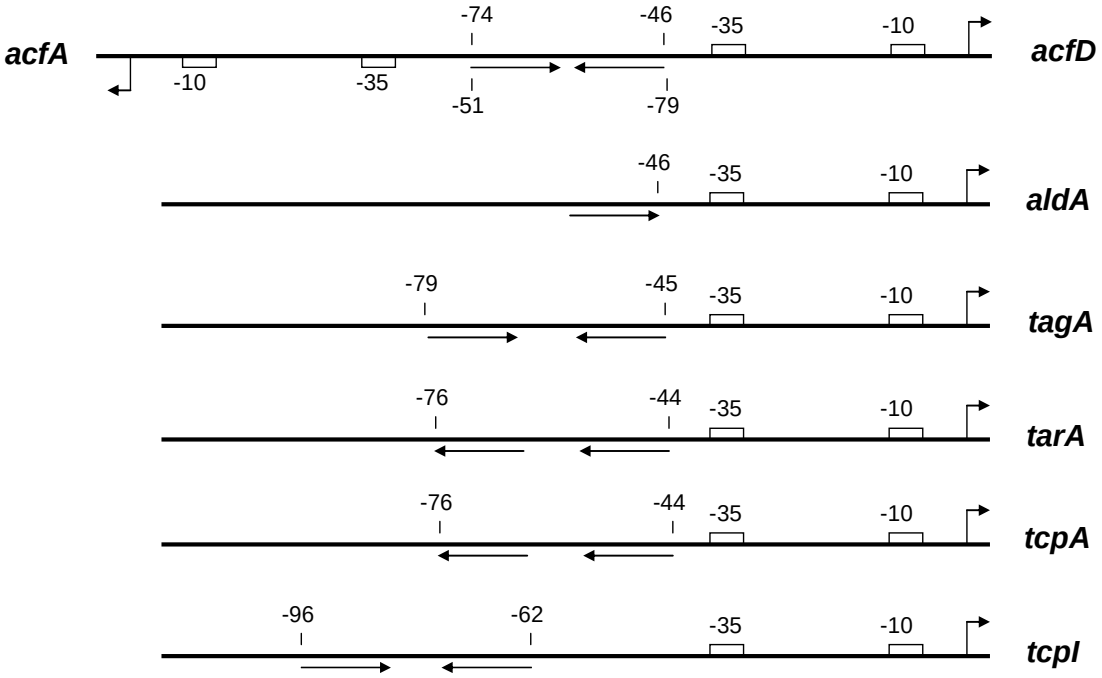


Figure 3: Toxboxes within ToxT-activated promoters in *V. cholerae*.

CHAPTER ONE

Identification and Characterization of the Functional Toxboxes in the *Vibrio cholerae* Cholera Toxin Promoter

ABSTRACT

Following consumption of contaminated food or water by a human host, the *Vibrio cholerae* bacterium produces virulence factors including cholera toxin (CT), which directly causes voluminous diarrhea, producing cholera. A complex regulatory network controls virulence gene expression and responds to various environmental signals and transcription factors. Ultimately ToxT, a member of the AraC/XylS transcription regulator family, is responsible for activating transcription of the virulence genes. ToxT-regulated promoters all contain one or more copies of the toxbox, a 13 base pair DNA sequence that ToxT recognizes. Nucleotides two through seven of the toxbox sequence are well conserved and contain an invariant tract of four consecutive T nucleotides, whereas the remainder of the toxbox sequence is not highly conserved other than being A/T rich. The binding of ToxT to toxboxes is required to activate the transcription of virulence genes and toxboxes have been characterized in several virulence gene promoters. However, the toxboxes required for activating transcription from the cholera toxin promoter, *PctxAB* have not been identified. *PctxAB* contains a series of heptad repeats (GATTTTT) each of which matches the 5' end of the toxbox consensus sequence and is a potential binding site for ToxT. Using site-directed mutagenesis and high resolution Copper-Phenanthroline footprinting, we have identified the functional toxboxes required for ToxT activation of *PctxAB*. Our findings suggest that

ToxT binds to only two toxboxes within *PctxAB*, despite the presence of several other potential ToxT binding sites within the promoter. Both toxboxes are essential for DNA binding and full activation of *ctxAB* transcription.

INTRODUCTION

Vibrio cholerae is a curved, gram-negative noninvasive bacterium responsible for the severe diarrheal disease cholera. *V. cholerae* is found predominately in coastal regions and is transmitted to humans by ingestion of contaminated water (54). The resulting infection is characterized by voluminous fluid loss leading to extreme dehydration if left untreated. Of the more than 200 *V. cholerae* serogroups present in the environment, only the O1 and O139 serogroups are associated with epidemic disease (158, 164). The O1 serogroup is further divided into classical and El Tor biotypes based upon phenotypic differences (48, 164). The current cholera pandemic, caused by El Tor *V. cholerae*, has persisted since 1961 and is estimated to affect approximately 5 million people annually (48, 164).

The diarrhea characteristic of cholera is directly caused by the secretion of cholera toxin (CT) in the upper small intestine. CT is a classic AB_5 toxin containing one active A subunit and 5 binding B subunits, which form a pentameric ring structure (64, 174). CT binds to the GM1 ganglioside of epithelial cells in the upper small intestine, allowing the active subunit to be translocated into the cells, where it is activated by proteolysis (32, 108). The resulting active A_1 subunit ADP ribosylates the regulatory G protein $G\alpha s$, which results in constitutive activity of adenylate cyclase, increasing cAMP levels within the cells and resulting in secretion of sodium, chloride, and water into the lumen (174).

V. cholerae virulence gene expression is controlled by a complex network of transcription regulators that is historically referred to as the ToxR regulon because that protein was the first to be discovered (123, 151). However, the direct activator of virulence gene expression is ToxT, whose expression depends upon ToxR (78, 79, 104). A pair of integral membrane proteins, ToxR and TcpP, in association with their respective cofactors, ToxS and TcpH, bind to the promoter region upstream of *toxT* and activate its transcription (44, 73, 78, 79, 105). Once produced, ToxT is responsible for activating transcription of the virulence genes necessary for pathogenesis. These virulence genes include the *ctxAB* genes encoding CT, which are located within the genome of a lysogenic bacteriophage, CTXΦ (85, 105, 186, 190–192, 196).

ToxT is a 32 kDa member of the AraC/XylS family of proteins, having a 100 amino acid family domain in the C-terminus that contains two helix-turn-helix domains for DNA binding (61, 78, 183). The N-terminal 176 amino acids of ToxT form another domain, the ToxT NTD, which does not have significant sequence similarity to any other proteins. However, the ToxT NTD has been proposed to be important for effector binding and dimerization (31, 170, 190, 191). The ToxT crystal structure revealed some structural similarity between the ToxT NTD and the AraC NTD, which is responsible for binding of AraC to its effector, arabinose, and for AraC dimerization (118). ToxT was monomeric in the crystals used for structural studies, and there is significant evidence that ToxT binds DNA as a monomer (190-192). However, bacterial two-hybrid studies and LexA fusion experiments revealed that the ToxT NTD is capable of dimerization when separated from the CTD, and ToxT dimerization after DNA binding may be important for transcription activation of some virulence genes (31, 155, 170). The ToxT

crystal structure also contained a buried unsaturated fatty acid, cis-palmytoleic acid, which has been proposed to be a negative ToxT effector (118). Addition of unsaturated fatty acids or bile to *V. cholerae* growth medium causes a reduction in virulence gene expression (28, 70) .

ToxT binds to 13 base pair sequences called toxboxes which are located upstream of all the genes whose transcription it activates (191). These are characterized by a well conserved 5' portion containing a poly-T tract and a degenerate 3' portion that is generally A/T rich. In addition to having somewhat degenerate sequences, toxboxes also vary in configuration and location relative to the transcriptional start site (10, 190-192). However, toxboxes are invariably located upstream of the -35 sequence recognized by RNA polymerase (RNAP) σ^{70}, suggesting that ToxT interacts with the RNA polymerase α subunit C-terminal domains (α-CTDs) to activate transcription (21). The different configurations of toxboxes likely dictate varying interactions with the two RNA polymerase α-CTDs (191).

The ToxT-responsive toxboxes at *V. cholerae* virulence genes have been identified at every virulence promoter except arguably the most important, *ctxAB* (190-192). Previous DNase I footprinting studies localized the ToxT binding region within P*ctx* to between -111 and -41 relative to the transcription start site (196). Within this region, there are a series of heptad repeats of GATTTTT which fit the highly conserved 5' segment of the toxbox consensus sequence (191). The number of these repeats varies among the O1 biotypes; classical strain O395 has 6 perfect direct repeats, whereas most El Tor strains have 3 direct repeats. Nested deletion analysis of the *ctxAB* promoter (P*ctxAB*) determined that the region extending from the transcription start site upstream

to -76 was sufficient for transcriptional activation by ToxT, correlating with the three heptad repeats proximal to the promoter being involved in ToxT binding (196). However, the exact binding sites remain unidentified, as there are several potential toxboxes within this sequence.

In this study, we used a combination of site-directed mutagenesis and high resolution copper- phenanthroline (CP) footprinting to characterize the ToxT binding sites that control *ctxAB* transcription. The location of the toxboxes was further confirmed by electrophoretic mobility shift assays that assessed the effects of toxbox mutations on DNA binding by ToxT. Our results suggest that there are two functional toxboxes located upstream of *ctxAB* that are required for control of the promoter.

MATERIALS AND METHODS

***V. cholerae* strains and plasmids**. The strains used in this study were *Vibrio cholerae* classical biotype strain O395 and its Δ*toxT* derivative (JW150) (27). P*ctxAB::lacZ* fusions for β-galactosidase assays were constructed on plasmid pTL61T (116) in strains O395 and Δ*toxT*. The strains were grown at 37ºC in Luria broth (LB) medium for overnight cultures or in LB adjusted to start at pH 6.5 at 30ºC for inducing conditions. Promoter constructs of *ctxAB* were constructed using WT O395 colonies as a template for PCR. All promoter constructs were cloned between HindIII and XbaI sites of pTL61T (116). Antibiotic concentrations were 100 μg/ml ampicillin and 100 μg/ml streptomycin. Plasmid sequences were confirmed by the University of Michigan DNA sequencing core and Genewiz. *V. cholerae* was transformed with plasmid DNA by electroporation using a Bio-Rad MicroPulser.

DNA manipulation. Plasmids were purified using Promega Wizard Plus Miniprep kits. PCR was performed using *Taq* DNA polymerase (Denville Scientific) as specified by the manufacturer in an Eppendorf Mastercycler gradient thermocycler. Restriction enzymes were purchased from New England Biolabs and used as specified by the manufacturer.

β-galactosidase assays. *Vibrio cholerae* strains were grown overnight at 37ºC in LB then subcultured at a 1:40 dilution into fresh inducing medium and grown for 3 hours at 30ºC with vigorous aeration. Bacteria were then placed on ice with the addition of 0.5 mg/ml chloramphenicol. Assays were performed using the procedure described by Miller (127).

Protein purification. ToxT-MBP was purified from *E. coli* strain JM109 with the plasmid pMALC2e containing the ToxT-MBP construct. *E. coli* was grown overnight at 37□ C then subcultured 1:40 into fresh LB and grown at 37□ C until OD_{600} reached 0.5. The culture was induced for 3 hours by addition of IPTG to 0.25 mM. Bacterial cells were collected by centrifugation then resuspended in buffer containing 20 mM Tris pH 8.0. The cells were French pressed and the lysate was centrifuged at 12,000 rpm for 10 minutes. The supernatant was passed over an amylose column (New England Biolabs) using a peristaltic pump. The column was washed with 20 mM Tris pH 8.0 buffer three times before the protein was eluted with 20-1 mL fractions of 20 mM Tris pH 8.0 and 10 mM maltose. Samples were analyzed by SDS-PAGE and elutions containing ToxT-MBP were dialyzed against a solution containing 50 mM Na_2HPO_4, pH 8.0; 10 mM Tris, pH 8.0; 100 mM NaCl then again against the same solution with 20% glycerol and aliquots

were frozen at -70 C. Protein concentration was determined using ThermoScientific Protein Assay Reagent as per manufacturer's directions.

Electrophoretic Mobility Shift Assays (EMSA). DNA probes were produced by PCR using plasmid templates containing appropriate promoter fragments with one unlabeled primer and one primer end labeled with γ-^{32}P (PerkinElmer) by T4 polynucleotide kinase (New England Biolabs). The assays were set up in a final volume of 30 μl with varying concentrations of ToxT-MBP, 10 μg/ml salmon sperm DNA, 100 ng of labeled DNA probe, and binding buffer with a final concentration of 10 mM Tris, pH 7.4; 1 mM EDTA, pH 7.0; 100 mM KCl, 1 mM DTT, 0.3 mg/ml bovine serum albumin (BSA) and 10% glycerol. The binding reactions were incubated at 30□ C for 30 minutes prior to loading into a 6% acrylamide gel at 4□ C. Gels were dried then analyzed by autoradiography.

CP Footprinting. CP footprinting was performed as previously described (190-192). Chemical cleavage was done in gel after separation of free DNA and bound ToxT-DNA complex by EMSA. Polyhistidine-tagged ToxT was purified as previously described (196). The ratio of ToxT to DNA used was adjusted empirically such that approximately 50% of labeled DNA formed a bound complex with ToxT. The sequence ladder was created using SequiTherm EXCEL II DNA Sequencing Kit (Epicentre) with the same ^{32}P-end –labeled primer used make the PCR products for EMSAs to minimize offset reactions as per manufacturer's instructions.

RESULTS

General map of ToxT binding. We began our investigation of the requirements for ToxT binding to P*ctxAB* in *V. cholerae* by analyzing the DNA sequence. The most notable feature of P*ctxAB* is the presence of heptad repeat sequences, TTTTGAT (Fig. 4), which have been previously proposed to be binding sites for transcriptional activators such as ToxR and ToxT (27, 113, 125, 129, 177, 196). The number of perfect heptad repeats differs among *V. cholerae* strains; classical biotype *V. cholerae* typically have six repeats, whereas El Tor biotype *V. cholerae* typically have only three. If written in a slightly different way, the sequence of each heptad repeat, GATTTTT, is consistent with the 5' end of the toxbox consensus sequence illustrated in Figure 2 by a sequence logo (36, 166, 191). In classical strain O395, used in all of the experiments described here, there are six perfect heptad repeats, followed by one repeat having two substitutions proximal to the promoter (Fig. 4). Therefore, several potential toxboxes could be identified by sequence analysis, but experimentation was required to determine which toxboxes are functional at P*ctxAB.*

To pinpoint the location of the functional toxboxes within P*ctxAB,* we performed site-directed mutagenesis of the heptad repeat sequences. In our initial analysis, we created double point mutations within each individual heptad repeat to produce a large effect on ToxT activity and clarify the most important repeat sequences. The poly-T tract within a heptad repeat, corresponding to the highly conserved T tract present in toxboxes, was interrupted by mutating the fourth and fifth position nucleotides from thymidines to cytosines. The mutant promoter constructs were cloned in pTL61T (116), a vector containing a multi-restriction enzyme cloning site upstream of a promoter-less *lacZ* gene, allowing us to measure P*ctxAB* activity in Miller units by β-galactosidase activity (127).

These constructs were transformed into wild type and Δ*toxT* strains of classical *V. cholerae* strain O395, assessed for ToxT-dependent activity and compared to wild type P*ctxAB::lacZ*. In addition, we included a truncated mutant promoter, pJW211, which extends to -76 relative to the transcription start site and thus includes only the promoter-proximal three heptad repeats (Fig. 4). Previous studies found that constructs extending to -76 were fully activated by ToxT but shorter constructs were not activated by ToxT (196), indicating the DNA sequences required for ToxT activity are located between -76 and the -35 box. As shown in Fig. 5, the truncated promoter that extends only to -76 was not only activated by ToxT, the fold difference in transcription induced by ToxT was twice that of full length P*ctxAB*, indicating that this truncated promoter is fully functional.

The results of our double mutagenesis experiments provided the first evidence for the location of functional toxboxes at P*ctxAB* (Fig. 5). Mutagenesis of repeats 1 and 2, at -97/-96 and -90/-89 relative to the transcriptional start site, caused no defects in ToxT-dependent transcription, which is consistent with the previous work showing only sequences downstream of -76 are required for ToxT activity (196). Mutagenesis of repeat 3 at -83/-82 caused decreased transcription with or without ToxT; however the fold difference between wild type and Δ*toxT* was consistent with WT P*ctxAB* constructs, indicating ToxT could still function. In contrast to these results, mutagenesis of repeat 4 at -76/-75 caused a complete loss of ToxT activity, suggesting this sequence may be necessary for ToxT dependent transcription activation. Mutagenesis of repeat 5 at -69/-68 also caused a complete loss of ToxT activity. Furthermore, the ToxT-independent transcriptional activity in this mutant strain was twice as high as in the WT P*ctxAB* strain,

suggesting this sequence may comprise part of a repressor binding site or may play a structural role that is favorable for ToxT-independent transcription when mutated. Mutagenesis of repeat 6 at -62/-61 reduced overall transcription levels but did not cause a significant defect in ToxT-dependent transcription, suggesting that it probably does not have a crucial role in ToxT binding. Mutagenesis of the imperfect heptad repeat 7 at positions -55/-54, which has substitutions at the two 3' nucleotides, resulted in significantly reduced ToxT activity, suggesting it does have an important role. Finally, the region downstream of the heptad repeats, at positions -52/-51, was included in the mutagenesis analysis because of its rich A/T content, similar to the somewhat degenerate 3' portion of the consensus toxbox sequence. Mutations to -52/-51 caused a complete loss of activation by ToxT, suggesting this sequence is also necessary for ToxT activity and therefore this region was further analyzed in subsequent experiments.

The above results indicate that ToxT activation of P*ctxAB* transcription requires the region downstream of -76 and are consistent with previous P*ctxAB* studies by Yu et al. (196). However, we now see that, at most, only repeats 4 (-76/-75), 5 (-69/-68), and 7 (-55/-54) as well as the A/T rich region immediately downstream of the heptad repeats are the necessary components for ToxT activity.

Comprehensive site-directed mutagenesis of P*ctxAB*. To characterize more precisely which nucleotides are necessary for ToxT activity within P*ctxAB*, single point mutations were created at each position ranging from -79 to -39, coinciding with the 5' end of repeat 4 and extending through the A/T rich region downstream of the heptad repeats (Fig. 2). Each A or T nucleotide was changed to a G or C, respectively, and each G or C was changed to an A. These mutant promoter constructs were cloned in pTL61T

and transformed into wild type O395 and its Δ*toxT* derivative. Promoter activity was measured by β-galactosidase activity and results are shown in Fig. 6.

This analysis identified numerous individual base-pairs that are important for ToxT activity. Any single point mutations within heptad repeat 5 abrogated ToxT activation of P*ctxAB* transcription, indicating that this sequence is essential for ToxT activity. Similarly, mutagenesis of the T tract within heptad repeat 6 also abrogated ToxT activity. Surprisingly, single point mutagenesis of heptad repeat 4 had little effect on ToxT activity with the exception of positions -75 and -76, which caused decreased ToxT activation when mutated. These results are consistent with the double point mutagenesis studies described above, in which mutations at positions -76/-75 abrogated ToxT activity. However, these results also suggest that the remainder of repeat 4 is not important for ToxT activity.

Mutagenesis of imperfect heptad repeat 7 did not cause significant defects in ToxT activity. However, the A/T-rich region downstream of the heptad repeat 7 revealed that some of these base-pairs are important for ToxT activity. Mutations between -52 and -45, and also -43, and -41 caused significant defects in ToxT-dependent transcription of P*ctxAB*, suggesting this region is important for ToxT function.

Previous work at other ToxT-activated promoters characterized the toxbox as a somewhat degenerate 13 base pair sequence with a conserved poly-T tract near the 5' end (Fig. 2) (190-192). The P*ctxAB* mutagenesis experiments described here revealed a clear region required for ToxT activity between -72 and -59, comprising repeats 5 and 6, that we designate toxbox 1 (Arrows in Figs. 4-6). Additionally, single point mutagenesis of the A/T-rich region downstream of the heptad repeats caused some defects in activation,

suggesting a second ToxT binding site, toxbox 2, between -58 and -46 (Figs. 4-6), that apparently has less sequence specificity. Interestingly, the mutation at position -53, which changes a C nucleotide to the consensus toxbox T nucleotide at this position, resulted in elevated transcription (Fig. 6).

Copper-phenanthroline footprinting of ToxT at P*ctxAB*. To complement our genetic analysis and confirm the locations of ToxT binding, we performed *in vitro* DNA footprinting experiments. Previous studies using DNase I footprinting identified a region of ToxT protection from -111 to -41 upstream of P*ctxAB* (196). However, DNase I footprinting is problematic at P*ctxAB* due to the presence of numerous A tracts, which interfere with DNase I cleavage even in the absence of bound proteins. To achieve higher resolution footprinting of ToxT on P*ctxAB*, we performed copper 1,10-phenanthroline (CP) footprinting analysis, which not only generates a higher resolution image of the base-pairs protected by ToxT but is also largely insensitive to DNA sequence. This technique was previously used to characterize ToxT binding at the *tcpA*, *aldD*, *acfA*, *acfD* and *tagA* promoters (190-192).

The CP footprint of ToxT at P*ctxAB* revealed two distinct regions of protection. The upstream region encompassing toxbox1, spanning -72 to -60 and including heptad repeats 5 and 6, is very strongly protected by ToxT (Fig. 7). These data correlate distinctly with the results of the mutagenesis experiments described above, suggesting it is an authentic toxbox that is required for DNA binding and P*ctxAB* activation by ToxT. The second region of protection, within toxbox2, ranges from -58 to -49 (ATTTCAAAT). This includes imperfect heptad repeat 7 and the A/T-rich region directly downstream of the heptad repeats that mutagenesis indicated may be important

for ToxT activity (Figs 5,6). In general the protection of this region was much weaker than protection observed at toxbox1. In particular, positions -51 and -52 were found to be important for ToxT activity and are somewhat protected in the CP footprint (dots in Fig. 7). However, positions -48 to -45, which also caused significant defects when mutated, are not visibly protected. These CP footprinting experiments were performed with both the full length P*ctxAB*, which includes all seven heptad repeats, and with the truncated promoter, pJW211, which includes only repeats 4-7 (Fig. 7); results are shown for the latter but were essentially identical for both constructs (data not shown).

These results suggest that P*ctxAB* contains two toxboxes, both of which are generally consistent with the previously described toxbox consensus sequence. The P*ctxAB* toxboxes are also consistent with other ToxT-activated virulence genes in both number and relative distance from the transcriptional start site (190-192).

ToxT binding to wild-type and mutant P*ctxAB* constructs. The genetic and biochemical analyses described above narrowed down the region of ToxT binding to two specific binding sites that are consistent with the toxbox consensus sequence. However, the footprinting experiments showed relatively weak protection of toxbox2, calling into question whether it is truly a ToxT binding site or instead a region possibly important for contact between RNA polymerase and ToxT. To confirm that mutations to these designated toxboxes cause defects in DNA binding by ToxT, we performed EMSAs using DNA probes that contain the double point mutations created for general mapping of ToxT binding (Fig. 5). ToxT binding to DNA was compared between wild-type P*ctxAB* and the mutant promoter sequences (Fig. 8). In these experiments the first lane of each gel contains DNA probe only and the subsequent lanes from left to right have increasing

amounts of ToxT. As ToxT concentration was increased in combination with the wild-type probe, two different shifted species were observed. This observation is consistent with one ToxT monomer occupying one toxbox at lower [ToxT] and then a second ToxT monomer occupying the second toxbox at a higher [ToxT], producing the slower migrating species. In contrast, the mutant promoter sequences shown in panels A-D of Fig. 8 did not produce the slower migrating band, even at the highest [ToxT], suggesting that only the non-mutated toxbox could be occupied. This result is evident with all the mutants that alter one of the two toxboxes we identified by mutational analysis, verifying their importance for ToxT binding.

To confirm that the abrogation of ToxT binding to toxboxes is specific to mutations within the identified toxboxes, we analyzed ToxT binding to a probe with mutations at -76/-75. These mutations are located within heptad repeat 4 upstream of toxbox 1 and caused a defect in ToxT-dependent transcription activation in β-galactosidase assays (Fig. 5). However, when comparing EMSA of the -76/-75 mutant probe to EMSA of wild-type probe, no difference is evident, suggesting the defects in transcription activation caused by the -76/-75 mutations are perhaps due to other factors such as reduced RNA polymerase interaction with DNA and not the result of decreased ToxT binding.

The above results are consistent with our designation of two toxboxes within P*ctxAB* being correct. To test our hypothesis that disrupting both toxboxes would eliminate ToxT binding, we performed EMSAs using probes with both toxboxes mutated, at positions -69/-68 and -55/-54 (Fig. 8F). A very weak shifted band was observed in these experiments that did not significantly increase in intensity as [ToxT] was increased.

These results suggest that ToxT is unable to bind specifically to probe having mutations in both toxboxes even at higher ToxT concentrations. This *in vitro* result is supported by *in vivo* β-galactosidase assays on P*ctxAB::lacZ* containing the double toxbox mutations in WT and Δ*toxT* O395 backgrounds, which produced 1776 ± 22.79 and 1755 ± 89.35 Miller units of activity, respectively.

DISCUSSION

The experiments described here were designed to characterize the DNA sequence requirements for ToxT to activate transcription of *ctxAB*, resulting in production of CT and subsequently diarrhea in cholera patients. Previous studies characterized the ToxT binding sites, or toxboxes, at several other known ToxT-activated promoters, but detailed information about the functional toxboxes at *ctxAB*, arguably the most important virulence locus in *V. cholerae*, remained lacking (Fig. 2) (190-192). The presence of GATTTTT heptad repeat sequences, each of which resembles the conserved 5' portion of a toxbox (191), made identification of the functional ToxT binding sites impossible without further experimentation. Double and single point mutations were made within the GATTTTT heptad repeats to identify which of the seven repeats within the classical *V. cholerae ctxAB* promoter are vital for transcription activation and these results were verified by CP footprinting and EMSA experiments using purified DNA and ToxT.

Results from the double point mutation experiments provided a general outline of the requirements for ToxT binding to the *ctxAB* promoter. The mutations to heptad repeats 4, 5, 6, and 7, as well as the A/T-rich region downstream of the repeats, caused severe defects in ToxT-dependent transcriptional activity, strongly suggesting that these

sequences are important for ToxT binding. In addition to abrogating ToxT activation of *ctxAB*, the mutations to repeat 5 also increased ToxT-independent transcription. This result could be due to disruption of an H-NS binding site previously identified by Stonehouse *et al*. (177), which would prevent repression of *ctxAB* expression by H-NS. H-NS preferentially binds to A/T rich regions such as this one which cause DNA to be intrinsically curved, and interrupting this stretch of nucleotides with a G or C may prevent H-NS from binding at nucleation sites and oligomerizing along the DNA (47, 143). Another possible explanation is that altering the DNA curvature may enhance interaction of RNA polymerase with the promoter region, diminishing the requirement for ToxT to activate transcription. The difference in DNA curvature may also explain the decreased expression observed when heptad repeat 3 was mutated. In this case the overall transcription magnitude decreased but the fold difference in expression between wild type and Δ*toxT* strains was similar to that of wild-type P*ctxAB* constructs, indicating ToxT activity was not affected by the mutations.

The DNA sequence requirements for ToxT activity at *ctxAB* were determined at higher resolution using *ctxAB::lacZ* constructs with single point mutations in the region between -79 and -39. Individual point mutations within a region spanning -72 to -59 caused severe defects in ToxT dependent activity, with the exception of positions -65 and -64. This 13 base pair sequence, which we designated toxbox1, is consistent with previously characterized ToxT binding sites in both sequence and proximity to the transcriptional start site (10, 190-192). Interestingly, there are no single point mutations within toxbox1 that significantly increased ToxT-independent activity, unlike the double point mutation within heptad repeat 5. This suggests that a single nucleotide change from

a thymidine to a cytosine is not enough to enhance ToxT independent transcription by whatever mechanism is responsible for this effect. However, this does not rule out the possibility that mutations to nucleotides other than cytosine may be sufficient to enhance ToxT independent transcription.

Unlike the mutations that led us to identify toxbox1, the single point mutations that led us to identify toxbox2 did not reveal such an obvious contiguous region important for ToxT binding. Only six mutations, at positions -52, -51, and the region from -48 to -45, caused significant decreases in ToxT-dependent transcription. This difference between the two toxboxes and ToxT sequence requirements is visualized in the CP footprinting experiments. These results indicate two separate regions of DNA protection by ToxT: -72 to -60 (toxbox1) and -58 to -49 (toxbox2). These regions strongly correlate with the results from the mutagenesis experiments. For toxbox 1, mutation to eleven out of the thirteen nucleotides caused severe defects in transcription activation and this segment is strongly protected by ToxT in the footprint. For toxbox 2, mutation to only six nucleotides caused significant defects in activation, and only eight nucleotides were protected, albeit weakly, by ToxT in the footprint. Combining these results with the mutagenesis experiments, we designate toxbox 2 as spanning the region between -58 to -46. This is consistent with some other ToxT-activated promoters in which toxboxes most proximal to the -35 promoter element are less specific in their sequence requirements than toxboxes located distally (190-192). Furthermore the orientation and position of this toxbox most closely resemble the single toxbox at the *aldA* promoter, which produces relatively weak activation (41).

The sequences of each of the toxboxes identified within P*ctxAB* fit the consensus sequence, although toxbox2 has a variation at position 6, which is part of the conserved T tract in every other toxbox (191). This change from T to C in toxbox2 could explain the weaker protection in footprinting experiments, and it is notable that mutating that position to the consensus T resulted in higher transcription levels.

Designation of the functional toxboxes was confirmed by EMSA experiments that compared ToxT binding to wild-type or double point mutant P*ctxAB* DNA probes. Mutations that are within the identified toxboxes visibly altered ToxT binding when compared to WT P*ctxAB.* The absence of the second, slower migrating ToxT-bound species suggests that ToxT could only occupy the non-mutated toxbox and is unable to bind to the mutated toxbox, supporting the *in vivo* transcriptional activation experiments of the double point P*ctxAB* mutants. Additionally, double point mutations within both toxboxes resulted in the complete abrogation of ToxT binding and transcription activation *in vivo* as expected. The EMSAs also support the hypothesis that mutations within heptad repeat 4 do not disrupt the ToxT binding region as the results in Figure 5 suggested. Instead, this region of P*ctxAB* may be important for interaction of ToxT bound to toxbox1 with the α-CTDs of RNA polymerase and the mutations negatively affected this interaction, resulting in lower transcription activation in the presence of ToxT.

The P*ctxAB* toxboxes are located upstream of the -35 promoter element, classifying it as a class I promoter (21). This is also the case for the toxboxes identified in every other ToxT-activated promoter that has been characterized (190-192). Class I promoters require an interaction between the activator protein and the α-CTD of RNAP for transcriptional activation (21). Because two toxboxes were identified within P*ctxAB*,

we hypothesize there is a specific interaction between two ToxT monomers and two α-CTDs. Our previous ToxT and α-CTD interaction models propose that when two toxboxes are present, there are two distinct points of interaction between individual ToxT monomers and α-CTD (191). An alternative hypothesis is that one ToxT monomer contacts RNA polymerase and the other ToxT monomer stabilizes this interaction, possibly by ToxT dimerization. The mutagenesis experiments illustrated that mutating one toxbox, particularly toxbox1, is sufficient to decrease overall transcription, suggesting that ToxT must occupy both toxboxes for full activation. The weak protection conferred by ToxT to toxbox2 raises the possibility that interaction with α-CTD may be important for enhanced binding to this sequence by ToxT. Another possible explanation for the weak footprint observed at toxbox2 is that a positive ToxT effector, such as bicarbonate, is required to increase binding specificity (1); future experiments will determine if either of these possibilities is indeed the case.

In this study, we focused on the classical biotype strain O395 *V. cholerae*, which contains six perfect GATTTTT repeats and one imperfect repeat. However, other strains possess a varying number of repeats. El Tor biotype strains generally contain only three of the heptad repeats but otherwise retain the same DNA sequence as classical strain O395 at P*ctxAB*. The absence of the distal heptad repeats does not negatively impact ToxT activated transcription as the toxboxes we identified in O395 encompass the heptad repeats that are most proximal to the transcriptional start site and would be included in the El Tor promoter region. ToxT was also not observed to bind to the distal heptad repeats in the footprinting experiments (data not shown). The significance of the distal heptad repeats in classical biotype *V. cholerae* is still unclear but they may play a role in

H-NS binding, may contribute to the curvature of the DNA, or could be important for the ToxR mediated activation of P*ctxAB* observed in the presence of bile, which was only observed in classical biotype (86).

In summary, we have characterized the specific sequence requirements for binding to P*ctxAB* and transcription activation by ToxT. The DNA sequences of the identified toxboxes are consistent with the consensus toxbox in that they are degenerate but contain the 5′ poly-T tracts common among all known ToxT DNA binding sites (190-192). The toxboxes in P*ctxAB* are also consistent with other ToxT-activated promoters in their positioning relative to the transcriptional start site (10, 190-192). Alhough ToxT is a flexible transcription activator in regard to sequence requirements, configuration, and number of binding sites, it has specific requirements for activation of P*ctxAB* and a single mutation within one the two toxboxes is enough to severely decrease transcription activation by ToxT.

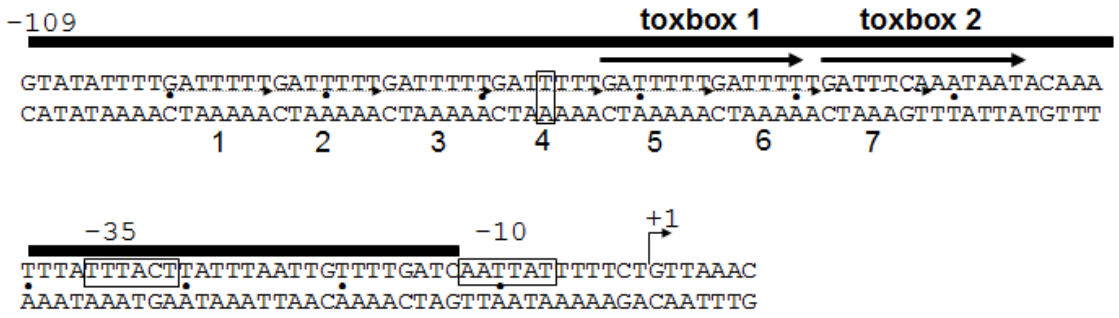


Figure 4: ToxT binding to the *ctxAB* promoter. The black bar over the sequence indicates DNase I footprinting protection by ToxT (196). ToxT binding sites, toxbox1 and toxbox2, determined in this study by mutagenesis and copper-1,10-phenanthroline footprinting, are illustrated by the arrows. The base-pair at -76 is boxed to indicate the endpoint of the minimal *ctxAB* construct that is activated by ToxT. Heptad repeats are numbered underneath the sequence and indicated by dotted arrows; imperfect repeat 7 is indicated by a dashed arrow. The transcriptional start site is indicated by a bent arrow and the putative -10 and -35 promoter elements are boxed.

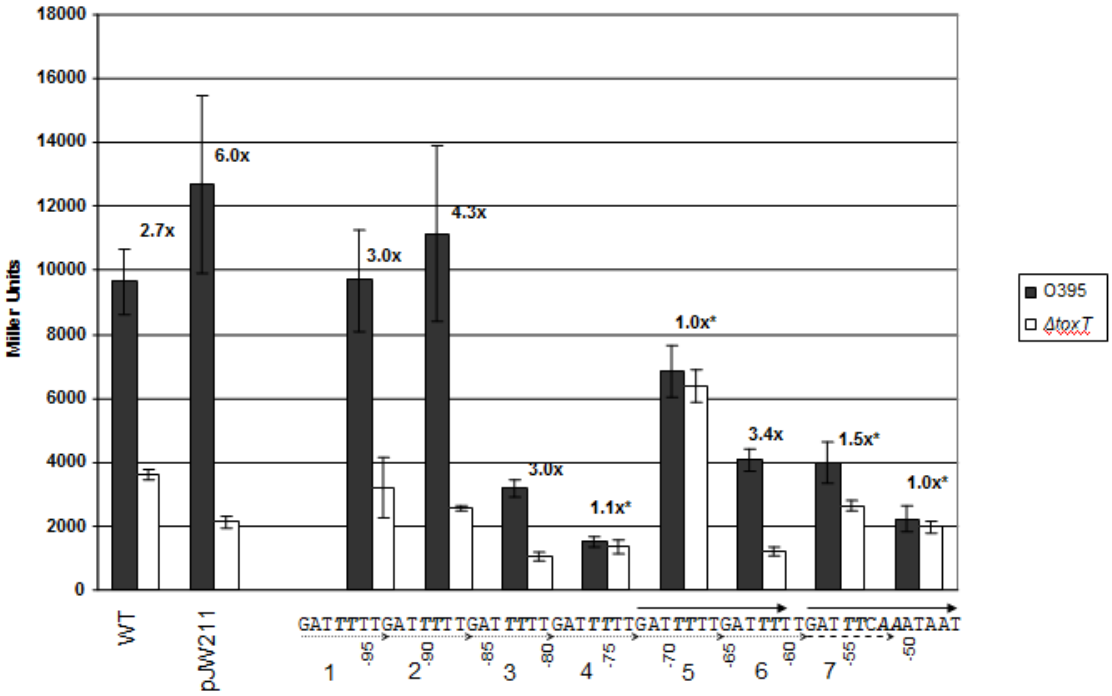


Figure 5: Effects of double point mutations to heptad repeats on *ctxAB* transcription. β-galactosidase results from *ctxAB::lacZ* double point mutations are shown. Results from the WT full length promoter strain are at the far left. The truncated promoter extending only to -76 is marked as p211. The double point mutations are indicated in italics within the sequence. Heptad repeat are numbered and shown as dotted arrows; imperfect repeat 7 is indicated by a dashed arrow. The black bars indicate O395 WT *toxT* strains and the grey bars are O395 Δ*toxT* strains. The fold difference in β-galactosidase between WT and Δ*toxT* strains is labeled above each promoter. Each experiment was repeated a minimum of three times and the data shown are mean values with the standard deviation indicated by error bars.

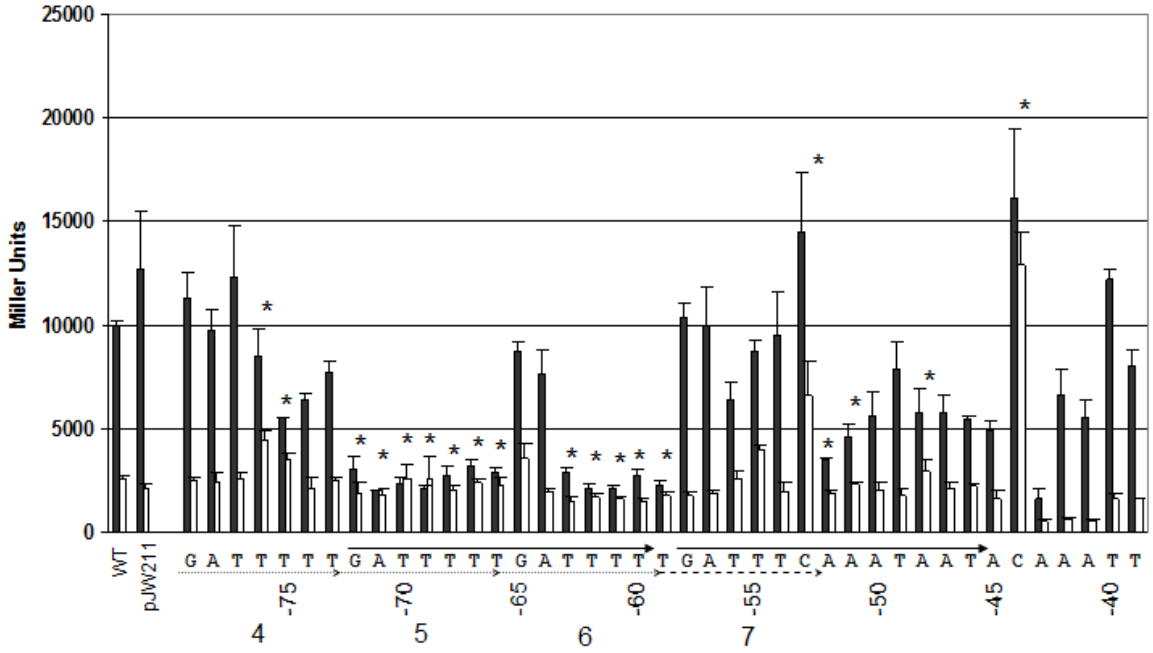


Figure 6: Effects of single point mutations on *ctxAB* transcription. β-galactosidase results from *ctxAB::lacZ* single point mutations are shown. Results from full length WT and truncated WT (p211) promoter constructs are shown at the far left. Individual mutation results correlate with the nucleotide underneath the x-axis. Heptad repeats included in the mutagenesis are numbered and indicated by dotted arrows; imperfect repeat 7 is illustrated by a dashed arrow. Black bars are O395 WT *toxT* strains and white bars are O395 Δ*toxT* strains. The asterisks indicate statistically significant differences in β-galactosidase results between WT O395 P*ctxAB* and mutant O395 P*ctxAB* strains where according to Student's *t*-test ($P<0.03$).

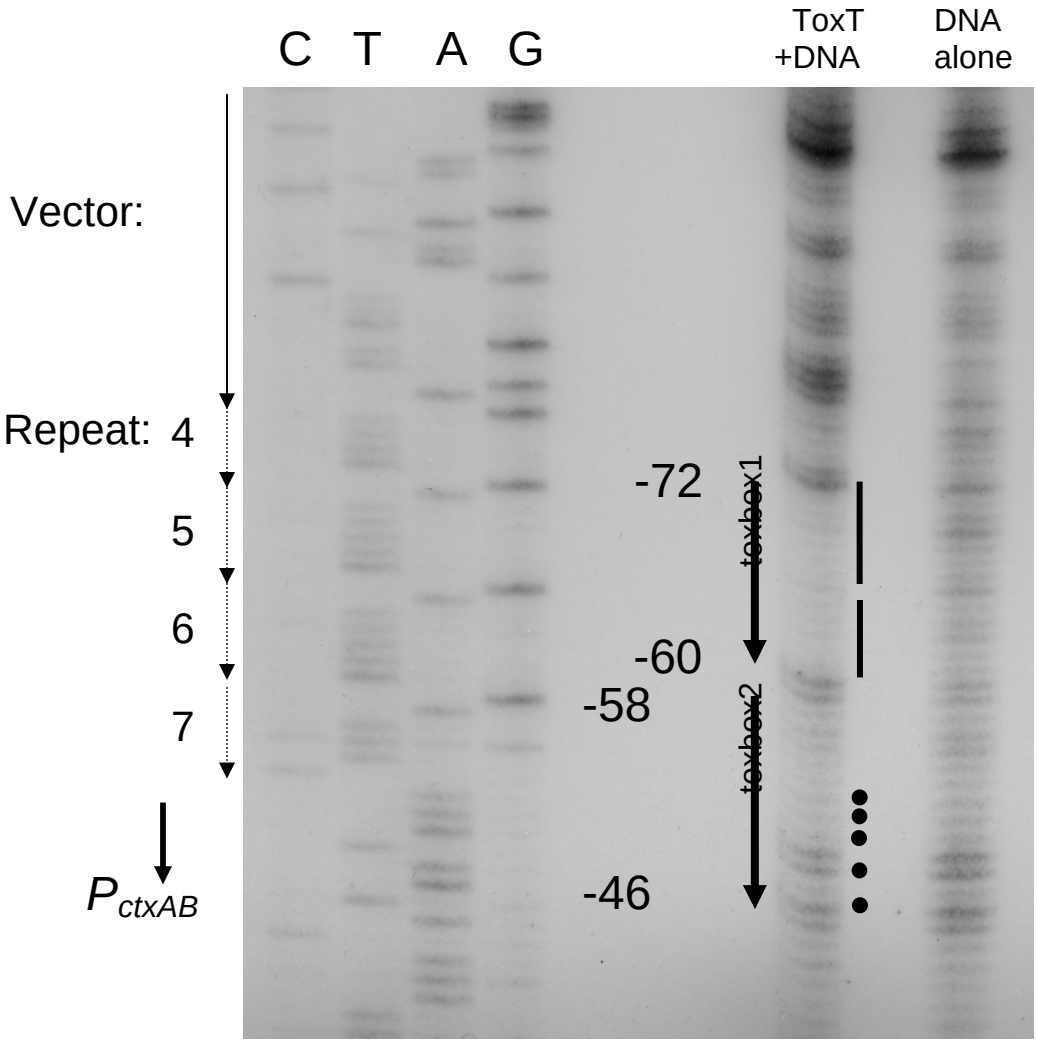


Figure 7: CP footprinting of ToxT on P*ctxAB*. Toxbox 1 and toxbox 2 are indicated by solid arrows with the correlating numerical position from the transcriptional start site. 'C, T, A, G' on the top left of the figure refers to the nucleotide lanes of the sequencing ladder. The dotted arrows and numbers show the locations of the GATTTTT repeats. The solid lines and black dots to the right of the toxboxes indicate the locations of ToxT-dependent transcriptional defects identified in the single point mutagenesis experiments.

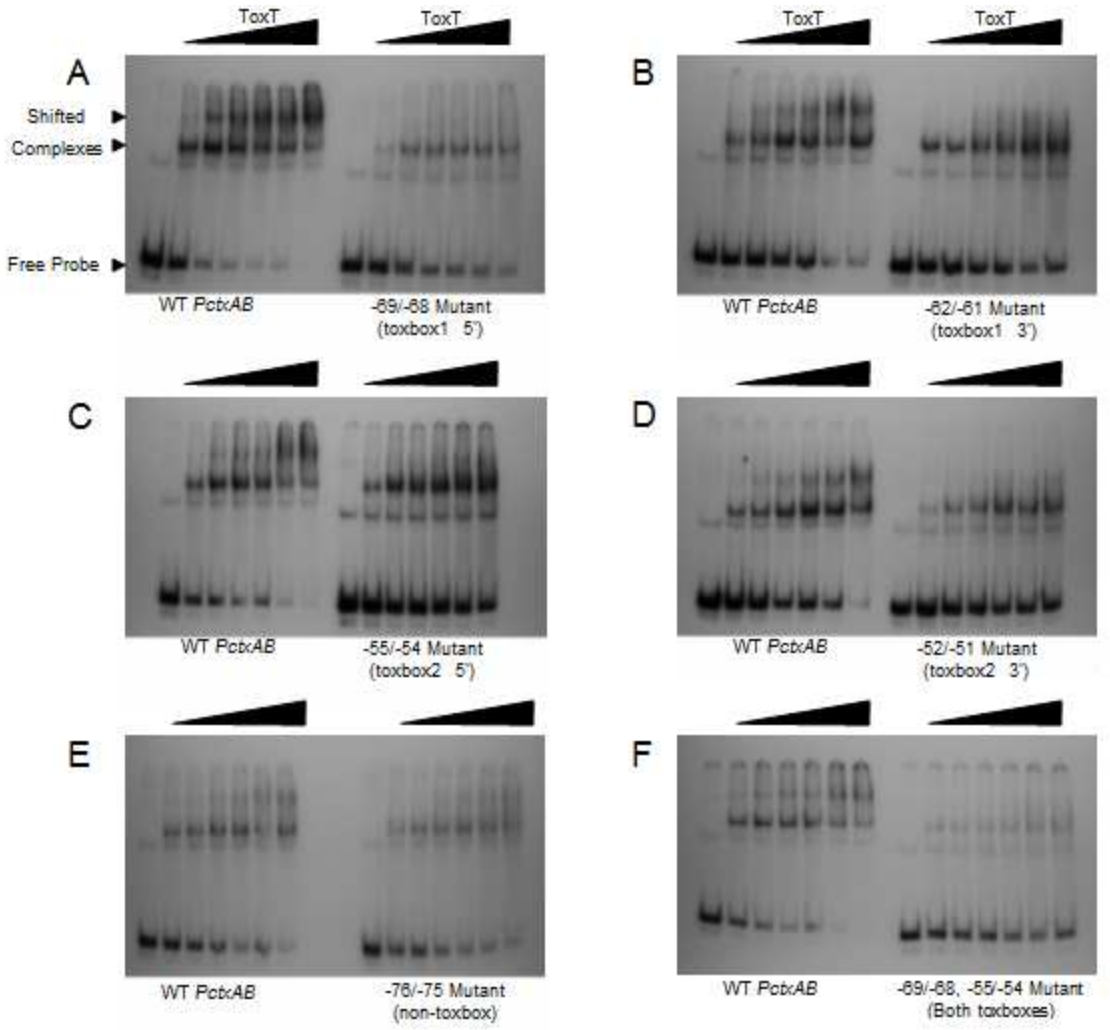


Figure 8: ToxT binding to wild-type and mutant P*ctxAB* constructs. Electrophoretic mobility shift assays (EMSAs) were carried out with each mutant P*ctxAB* construct as indicated below the right panel of each EMSA. Lane one of each gel is free probe with no ToxT present. ToxT-MBP concentrations increase across the gel from left to right as indicated by the black triangle. ToxT-MBP concentrations used in each EMSA are 2.3 nM, 4.6 nM, 6.9 nM, 9.2 nM, 11.5 nM, and 13.8 nM.

CHAPTER 2

Differential control of cholera toxin production in classical and El Tor biotype *V. cholerae* is mediated by H-NS binding to heptad repeat sequences

ABSTRACT

The bacterium *Vibrio cholerae* infects human hosts following ingestion of contaminated food or water and causes the severe diarrheal disease cholera. The watery diarrhea that is characteristic of the disease is directly caused by production of cholera toxin (CT). The regulatory network that controls CT and other virulence factors is complex. However, a single transcriptional activator protein, ToxT, directly binds to virulence gene promoters and activates their transcription. Previously, we identified two ToxT binding sites, or toxboxes, within the cholera toxin promoter (P*ctxAB*). The toxboxes overlap with the two downstream-most of the six GATTTTT heptad repeats found within P*ctxAB.* These heptad repeats were previously found to be within a large region bound by H-NS, a transcriptional repressor expressed in Gram-negative bacteria. The current model for control of P*ctxAB* transcription requires H-NS displacement from the DNA by ToxT and then the subsequent activation of transcription by ToxT by contacting RNA polymerase (RNAP). The goal of this study was to determine more precisely where H-NS binds to P*ctxAB* and test the hypothesis that ToxT completely displaces H-NS from the P*ctxAB* promoter before activating transcription. Results described here suggest that H-NS binds only to the region of P*ctxAB* encompassing the heptad repeats. Interestingly, the *V. cholerae* classical biotype has six heptad repeats at P*ctxAB* while the El Tor biotype has only three, suggesting a varying degree of H-NS-mediated repression at each of these promoters. The results from promoter mutagenesis

and *in vitro* DNA binding experiments in this study call for a revision of the current model involving H-NS and ToxT at P*ctxAB*.

INTRODUCTION

Cholera is a severe diarrheal disease that affects an estimated 5 million people annually. Cholera is caused by the aquatic bacterium *Vibrio cholerae,* a Gram negative curved bacillus that is ubiquitous in coastal regions. *V. cholerae* infection is the result of ingesting contaminated food or water (54). If left untreated, cholera can cause severe dehydration and death in more than 50% of cases but that number falls to approximately 1% when patients are treated with oral rehydration solution and IV fluids. Although more than 200 *V. cholerae* serogroups are present in the aquatic environment, epidemic cholera is only associated with the O1 and O139 serogroups (158, 164). The current, ongoing seventh cholera pandemic, originating in 1961, is caused by the El Tor biotype of the O1 serogroup (164). The El Tor biotype is differentiated from the classical biotype, which was ostensibly responsible for the first six cholera pandemics, by a variety of factors including antibiotic resistance, production of hemolysin, and presence of mobile genetic elements (48).

Pathogenic *V. cholerae* requires two major virulence factors to cause cholera: cholera toxin (CT), which is directly responsible for producing the characteristic diarrhea, and toxin-coregulated pilus (TCP), which is required for intestinal colonization. CT is a classical AB_5 toxin that is encoded by *ctxAB*, located within the genome of the CTXΦ lysogenic bacteriophage (64, 186). CT binds via the pentameric B subunits to GM1 gangliosides found on the intestinal epithelial cells and activates cAMP production by

translocation of the ADP-ribosylating A_1 subunit into the cell (32, 174). This subsequently leads to secretion of water and ions into the intestinal lumen (174). TCP is a type IV pilus encoded by genes on the *V. cholerae* pathogenicity island (VPI). TCP is important for microcolony formation and serves as a receptor for CTX Φ (85, 186).

V. cholerae virulence gene regulation is very complex and consists of a cascade of positive transcription activators along with transcription repressors and post-transcriptional regulators (123). Environmental cues such as the availability of nutrients, low pH, and anaerobiosis trigger the positive cascade and increase the intracellular production of proteins AphA and AphB (101, 128). These two proteins directly interact and activate the transcription of *tcpPH* (99-102). TcpP and its cofactor TcpH along with ToxR and its respective cofactor ToxS are integral membrane protein pairs that comprise the second level of the positive cascade and are responsible for the transcription of *toxT* (44, 73, 78, 79, 105). ToxT, a 32-kDa transcriptional regulator that belongs to the AraC/XylS family, is directly responsible for activating the transcription of *ctxAB* and *tcp*, as well as other virulence genes (78, 79, 85). Virulence gene expression levels are also affected by the presence of unsaturated fatty acids and bile, which decrease expression levels by decreasing ToxT activity, or by bicarbonate, which has been shown to enhance ToxT activity (1, 70, 86, 87, 168).

We recently have characterized the ToxT binding sites, referred to as toxboxes, found within the *ctxAB* promoter (P*ctxAB*) (45). Toxboxes are 13 base pair degenerate DNA sequences that are located upstream of the -35 promoter element of all genes whose transcription ToxT activates (191). The toxboxes at individual genes have some variability in their positions from the transcriptional start site and configuration (10, 190-

192). One common feature, however, is the presence of a 5' poly(T) tract that is found in every toxbox. P*ctxAB* contains several potential ToxT binding sites that fit the toxbox consensus sequence and also directly overlap with where H-NS, a transcriptional repressor, is thought to bind DNA and act as a repressor of P*ctxAB* (177).

H-NS is a 15-kDa histone-like nucleoid associated protein. It binds to xenogeneic DNA that has been acquired through horizontal transfer and represses transcription by oligomerizing along the DNA (119, 142, 162). H-NS can form bridges to prevent transcription activators from binding DNA and some studies also indicate that H-NS can halt active transcription by trapping the RNA polymerase (RNAP) (39, 40, 167). Additionally, the expression of genes that are modulated by H-NS have been found to be responsive to environmental signals such as osmolarity, pH, and temperature (4-6, 46). There are many models that detail the derepression of H-NS by DNA binding proteins and the relationship between H-NS and transcriptional activators has been characterized in various Gram-negative bacteria including *Escherichia coli* and *Salmonella enterica* (see review(176)).

The interplay between ToxT and H-NS in *V. cholerae* has been integral in understanding the expression of the virulence factors TCP and specifically CT (146, 177, 196). The current model indicates that H-NS, which is expressed constitutively within the bacteria, binds to the *ctxAB* promoter's A/T rich regions under non-virulence inducing conditions. Under virulence condions, ToxT is expressed and displaces H-NS from the promoter to activate transcription of *ctxAB* (196). Stonehouse *et al*. proposed that the degree of displacement is dependent on the level of ToxT in the cell (177). At

low levels, H-NS still binds to the promoter but is completely displaced by ToxT when the appropriate [ToxT] is achieved within the cell.

The recently acquired detailed knowledge of the functional toxboxes in P*ctxAB* further elucidates the complex relationship between H-NS and ToxT. In this study, we have identified the specific binding sites of H-NS using high resolution copper-phenanthroline DNA footprinting. We then characterized the interplay between ToxT and H-NS by *ctxAB* promoter expression studies in *E. coli* and competitive electrophoretic mobility shift assays (EMSA) and propose a revised model to define the transcription regulation of *ctxAB* by ToxT and H-NS.

MATERIALS AND METHODS

***E. coli* strains and plasmids.** The *E. coli* K5971 strain and derivatives used in these studies were previously used by Yu *et al.*, (196). The strains either contain an inducible *toxT*-encoding plasmid (pMMTT) or the vector alone (pMMB208). P*ctxAB:lacZ* fusions for β-galactosidase assays were previously constructed and the full-length mutant promoter constructs created in this study were cloned into pTL61T using XbaI and HindIII sites of the plasmid (45). The constructs were transformed into the *E. coli* strains by electroporation using a Bio-Rad MicroPulser. The strains were grown at 37º C in Luria broth (LB) medium for overnight cultures or in LB adjusted to start at pH 6.5 at 30º C for inducing conditions. Antibiotic concentrations were 100 μg/ml ampicillin and 30 μg/ml chloramphenicol. Plasmid sequences were confirmed by commercial DNA sequencing (Genewiz.)

DNA manipulation. Plasmids were purified using Promega Wizard Plus Miniprep kits. PCR was performed using *Taq* DNA polymerase (Denville Scientific) as specified by the manufacturer in an Eppendorf Mastercycler gradient thermocycler. Restriction enzymes were purchased from New England Biolabs and used as specified by the manufacturer.

β-galactosidase assays. *E. coli* strains were grown overnight at 37º C in LB then subcultured at a 1:40 dilution into fresh inducing medium and grown for 3 hours at 30º C with vigorous aeration with the addition of 1mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Bacteria were then placed on ice with the addition of 0.5 mg/ml chloramphenicol. Assays were performed using the procedure described by Miller (127).

Protein purification. Polyhistidine tagged H-NS (H-NS-His) was purified from *E. coli* strain JM109 with the plasmid pBAD32 containing the H-NS-His construct. *E. coli* was grown overnight at 37º C then subcultured 1:40 into fresh LB and grown at 37º C until OD_{600} reached 0.7. The culture was induced for 3 hours by addition of arabinose to 0.2%. Bacterial cells were collected by centrifugation then resuspended in buffer containing 50mM Na_2HPO_4, pH 8.0; 300 mM NaCl; 10 mM imidazole; 20 mM β-mercaptoethanol and 25 mg lysozyme. The cells were French pressed and the lysate was centrifuged at 12,000 rpm for 10 minutes. The supernatant was collected and 4 mL of Ni-NTA resin (Sigma) was added. The cells were mixed overnight at 4º C then packed into a column with the resin. The column flow-through was collected and the resin was washed three times with 50mM Na_2HPO_4, pH 8.0; 300 mM NaCl; 20 mM imidazole. H-NS-His was eluted using 50mM Na_2HPO_4, pH 8.0; 300 mM NaCl; 250 mM imidazole in six 1 mL fractions. The samples were analyzed by SDS-PAGE and the fractions

containing the protein were added to a dialysis cassette (Thermo Scientific) and were dialyzed against a solution containing 50 mM Na_2HPO_4, pH 8.0; 10 mM Tris, pH 8.0; 100 mM NaCl then again against the same solution with 20% glycerol and aliquots were frozen at -70º C. Protein concentration was determined using ThermoScientific Protein Assay Reagent as per manufacturer's directions.

Electrophoretic Mobility Shift Assays (EMSA). DNA probes were produced by PCR using plasmid templates containing appropriate promoter fragments with one unlabeled primer and one primer end labeled with γ-^{32}P (PerkinElmer) by T4 polynucleotide kinase (New England Biolabs) or 5'-end labeled with Fluorescein (Sigma). The assays were set up in a final volume of 30 µl with varying concentrations of ToxT-MBP and H-NS-His, 10 µg/ml salmon sperm DNA, 100 ng of labeled DNA probe, and binding buffer with a final concentration of 10 mM Tris, pH 7.4; 1 mM EDTA, pH 7.0; 100 mM KCl, 1 mM DTT, 0.3 mg/ml bovine serum albumin (BSA) and 10% glycerol. The binding reactions were incubated at 30□ C for 30 minutes prior to loading into a 6% acrylamide gel at 4º C. Gels with γ-^{32}P were dried then analyzed by autoradiography. Gels containing Fluorescein-labeled DNA were visualized using Safe Imager 2.0 (Invitrogen).

CP Footprinting. CP footprinting was performed as previously described (190-192). Chemical cleavage was done in gel after separation of free DNA and bound H-NS-DNA complex by EMSA. Polyhistidine-tagged H-NS was purified as described above. The ratio of H-NS to DNA used was adjusted empirically such that approximately 50% of labeled DNA formed a bound complex with H-NS. The sequence ladder was created using SequiTherm EXCEL II DNA Sequencing Kit (Epicentre) with the same ^{32}P-end –

labeled primer used make the PCR products for EMSAs to minimize offset reactions as per manufacturer's instructions.

RESULTS

Identification of H-NS binding sites by copper-phenanthroline footprinting. Previous studies on P*ctxAB* indicated that H-NS binds to a large region encompassing the GATTTTT heptad repeats characteristic of the *ctxAB* promoter (Fig. 9), as well as to regions both upstream and downstream of these repeats (177). This region includes ToxT binding sites and the -35 promoter element. These studies utilized DNase I footprinting to identify the preferential binding regions for H-NS. This method of DNA footprinting poorly cleaves A/T rich regions due to the narrowing of the minor groove in A tracts, making results difficult to interpret in a very A/T rich sequence such as that found at P*ctxAB*. To determine the exact DNA binding locations of H-NS, we used a higher resolution copper-1, 10- phenanthroline (CP) footprinting technique that previously identified ToxT binding sites at *ctxAB, tcpA, aldD, acfA*, and *tagA* promoters (45, 190-192). This technique is largely sequence-independent and allows for more specific characterization of protein contact sites on DNA.

Our DNA footprinting results significantly narrowed down the region of H-NS binding at P*ctxAB* from the previously published DNase I results. Using the full length classical biotype promoter of *ctxAB*, which contains six full heptad repeats and a seventh, imperfect repeat, we were able to visualize H-NS protection from cleavage by CP in the region from -100 to -59 relative to the transcriptional start site (Fig. 10). This protected region includes only one of the previously characterized toxboxes that spans -72 to -60.

Neither the other, promoter-proximal toxbox between -58 and -46 nor the -35 promoter element were protected in our experiments as had been previously described (177). Additionally, the H-NS footprint contains hypersensitive sites that are likely to be the result of DNA bending, causing the DNA to be more susceptible to cleavage by CP. The H-NS binding region also appears to be contiguous with no unprotected sites in the area of protection, suggesting that H-NS does oligomerize along the DNA when binding.

Control of P*ctxAB* by ToxT and H-NS in *E. coli*. In our previous study that characterized the ToxT binding sites of P*ctxAB* we created mutant promoter constructs fused to *lacZ* in reporter plasmid pTL61T. These mutant constructs altered individual GATTTTT heptad repeats that were potential ToxT binding sites by replacing two of the T nucleotides with two C nucleotides (45). Based on the CP footprinting data that indicated where H-NS binds to P*ctxAB*, we used these same mutant constructs to help characterize the relationship between H-NS and ToxT by measuring promoter activity in β–galactosidase assays. We also analyzed two different length P*ctxAB* constructs, the full length that extends to -182 and contains all six perfect heptad repeats that are found in classical biotype strain O395 and a truncated version that extends to -76 and has only three heptad repeats; the latter resembles the El Tor biotype P*ctxAB*. Yu and DiRita found that P*ctxAB* constructs extending to -76 relative to the transcription start site can be fully activated by ToxT (196) and our previous work confirmed this to be true (11).

To further understand the roles that ToxT and H-NS play at P*ctxAB*, experiments with *hns* mutants were required. H-NS in *V. cholerae* is encoded by *vicH*, however, these mutants in *V. cholerae* are apparently unstable as we were unable to delete *vicH* without acquiring secondary mutations. Instead, we carried out promoter activity experiments in

an *E. coli* background as had been done previously (196). For these experiments we used the *E. coli* WT strain K5971 and *E. coli hns*$^-$ strain K5972 that Yu and DiRita used to characterize ToxT and H-NS at P*ctxAB*. These strains carry plasmids that either encode inducible *toxT* or an empty vector control (77, 78, 133). These *E. coli* strains allow us to assess promoter activity in the presence and absence of both ToxT and H-NS and have the additional advantage of eliminating confounding *V. cholerae* factors that act directly on the *ctxAB* promoter such as ToxR (131).

The result of these experiments using the full length *ctxAB* promoter construct, which contains all six of the heptad repeats, indicated the level of repression that H-NS manifests on the promoter (Fig. 11). In the WT *E. coli* strain that expresses H-NS there is little promoter activity without ToxT. However, once ToxT is expressed, there is a 5.43 fold difference in expression, confirming that P*ctxAB* activity is activated by ToxT in these *E. coli* strains (Table 1A). This activation level accounts for both H-NS derepression and true activation by ToxT, as proposed by Yu and DiRita (196). In the *E. coli hns*$^-$ background, when ToxT is not expressed, the expression level is similar to the level produced by ToxT-dependent activation in *E. coli hns*$^+$. Expressing ToxT in *E. coli hns*$^-$ background then increases promoter activity to more than twice that observed when H-NS is present in the cells. This result shows that true activation by ToxT, presumably by contacting RNAP, occurs in the absence of H-NS as previously reported (196).

Next we examined the effects of mutations to the heptad repeat sequences on control of *ctxAB* expression by ToxT and H-NS illustrated in Figure 11 and listed the fold differences between the strains in Table 1A. Mutation of the first heptad repeat at -97/-96 resulted in expression levels not significantly different from those observed with the

wild-type construct. Mutation of heptad repeat 2 at -90/-89 resulted in loss of H-NS repression. There is no significant difference in the expression levels +/- ToxT in the WT *hns* and *hns*$^-$ backgrounds. This suggests that this repeat sequence is essential for H-NS repression of P*ctxAB*. Mutation to heptad repeat 3 caused an unexpected loss of ToxT-dependent *ctxAB* expression but did not affect H-NS repression. These mutations are not near the toxboxes and caused no defect in ToxT-dependent expression in *V. cholerae* (45). Mutation to repeats 4 and 5, at positions -76/-75 and -69/-68, respectively, resulted in elevated basal expression levels that resemble the expression levels of the WT construct in the *hns*$^-$ background. However, H-NS was still an active repressor of these constructs, as expression levels were even higher in the *hns*$^-$ background.

This result suggests two possibilities: 1) these repeats may be important for H-NS binding because the promoter activity independent of ToxT is increased in the mutants when compared to WT P*ctxAB* and is consistent with previous promoter expression studies in *V. cholerae* with these mutant constructs; or 2) these mutations improve binding by RNAP or improve DNA contact sites for the RNAP α-subunit C-terminal domains. The mutations to repeats 4 and 5 also caused a loss of ToxT-dependent transcription as previously observed in *V. cholerae* (45). Mutations to repeat 6 at -62/-61, within the most downstream of the H-NS binding region we observed in the footprinting experiments, caused decreases in both ToxT-dependent transcription and H-NS repression. Mutations to imperfect repeat 7 at -55/-54 and the downstream region at -52/-51 are similar in that in the *hns*$^+$ background, there is no ToxT-dependent activation. This is consistent with results from *V. cholerae* since these are mutations in toxbox 2. However, because these mutations are downstream of the H-NS binding region in the

footprint experiment, there is evidence of H-NS dependent repression similar to WT P*ctxAB*.

Comparison of H-NS effects on classical and El Tor P*ctxAB*. Because the number of heptad repeats differs between the classical and El Tor *V. cholerae* biotypes, we explored whether P*ctxAB* constructs that only contain three of the repeats (ala El Tor) would behave differently from the constructs having six repeats (ala classical.) These constructs include the T-tract of repeat 4 and complete repeats 5-7. The footprinting experiments indicated that H-NS binds to the region of DNA including repeats 4-6 but not the imperfect repeat 7 immediately downstream. Eliminating the upstream-most repeats removes half of the H-NS DNA binding region and results in elimination of H-NS repression in the WT short construct (Fig. 12). The background activity of the WT promoter increases by 4000 Miller units as compared to the full-length construct, to the same level as was observed in the *hns*$^-$ background. When ToxT is expressed within the cells, expression greatly increased to levels much higher than those observed using the full-length promoter.

Mutagenesis of repeat 4 in the shortened P*ctxAB* resulted in markedly different expression patterns than those observed using the full-length version of the promoter. The expression levels are similar in WT *E. coli* and in the *hns*$^-$ *E. coli* backgrounds, indicating H-NS has no effect on this construct, whereas H-NS was clearly acting as a repressor in the full length construct which is also seen in the fold differences between the strains (Table 1). ToxT cannot activate transcription from this shortened mutant construct as we also observed using the full-length construct. Mutagenesis of repeat 5 also caused dramatic differences in activity between the shortened and full-length

constructs. The shortened mutant promoter produced a dramatic increase in ToxT-independent activity. This suggests that this mutation may either alter the local DNA structure, making it more favorable for activation, or may facilitate better RNAP binding. Mutagenesis of repeats 6 and 7 produced similar results in the shortened promoter and the full-length promoter. However, overall expression was much higher in WT *E. coli* in the shortened promoter.

Competitive DNA binding by ToxT and H-NS. To further understand the interplay between ToxT and H-NS at P*ctxAB*, we peformed competitive EMSAs using WT, full length P*ctxAB*, i.e. containing all seven heptad repeats, as the probe. In Figure 13, the first lane of the gel is DNA probe only. Lane 2 contains 23 nM ToxT bound to DNA with two separate bands indicating the occupancy of either one or both toxboxes. Lane 3 contains 260 nM H-NS bound to DNA, and this complex migrates further down the gel than the ToxT/DNA complexes. In lanes 4 and 5 the concentration of ToxT used is 14 nM the concentration of H-NS used was 170 nM and 260 nM, respectively. The band corresponding to the H-NS/DNA complex is still present in these lanes, as is the band corresponding to the ToxT/DNA complex. However, in lanes 6 and 7, when the amount of ToxT was increased to 23 nM, there is a supershift that appears when H-NS is added, suggesting both ToxT and H-NS are simultaneously bound to the DNA. When the concentration of H-NS was increased (lane 7), the supershift was not prominent and the H-NS/DNA band starts to reappear. To confirm that the supershift seen does indeed contain both ToxT and H-NS, we performed the same EMSA but using a primer 5' end-labeled with fluorescein to make the DNA probe. The supershift band was visualized, excised, and sent for mass spectrometry analysis, which confirmed that both proteins

were present. Conversely, we repeated the EMSA using the shorter El Tor WT P*ctxAB* as a probe (Fig 13B). Using the same protein and DNA concentrations, the supershift that was visible in the O395 P*ctxAB* EMSA was not present when the El Tor probe was used. Instead, only the two bands representing bound toxboxes appear. This is consistent with our findings from the promoter-reporter experiments in *E. coli* that H-NS cannot repress this construct containing only three heptad repeat sequences.

DISCUSSION

The experiments in this study were designed to elucidate the complex mechanism under which transcription of *ctxAB* is activated with respect to ToxT and H-NS. The cholera toxin promoter is an A/T rich region of DNA for which H-NS has a strong affinity (142). In our previous work, we used the CP footprinting technique as well as site directed mutagenesis experiments to successfully characterize ToxT binding sites at P*ctxAB* (45). Here, using similar methods, the H-NS binding area in P*ctxAB* was identified which allowed us to better understand the role it plays in regulating *ctxAB*.

DNase I footprinting at P*ctxAB* previously revealed that H-NS binds to a very large region of the DNA that includes areas both upstream and downstream of the GATTTTT repeats found in the promoter (177). CP footprinting with H-NS revealed protection in the region spanning from -100 to -59, which only includes the six perfect heptad GATTTTT repeats in the promoter (Fig. 10). Interestingly, this region of DNA overlaps with toxbox 1, where ToxT binds from -72 to -60 but not toxbox 2, which is located immediately downstream from -58 to -49. Currently, it is accepted that ToxT needs to displace H-NS from P*ctxAB* in order to activate transcription. However, with

such a small region of overlap between the two proteins observed in our experiments, the extent of the displacement was not clear (196).

V. cholerae biotypes El Tor and O395 have a varying number of heptad repeats within P*ctxAB*, three and six perfect repeats, respectively. To investigate the differences in H-NS repression between the two biotypes, double point mutations were created in each of the GATTTTT repeats in both full length (classical) and shortened (El Tor) P*ctxAB* and analyzed by β-galactosidase assays in *E. coli* strains that have an inducible *toxT*-encoded plasmid (Figs. 11,12). When comparing the two versions of the promoter, the overall background levels of activation increased in the shortened promoter to levels matching those of *hns*- in the full length promoter. Because half of the known H-NS binding sites are not present in the shortened promoter and H-NS functions by oligomerizing on DNA, it is likely that H-NS does not have the same repressive effect in the shortened promoter, as also indicated by the doubling of fold differences between the WT H-NS and *hns*- background *E. coli* in the context of ToxT activation (Table 1). Additional binding sites, like those found in the full length promoter, allow for H-NS to repress transcriptional activation to a greater extent. This is evident in the WT versions of both promoters; the classical promoter has an overall increase in transcription in *hns*- while there is no significant difference when H-NS is not present in the El Tor promoter (Table 1). Double point mutations at repeats 2 and 6 have an effect on H-NS repression in the O395 P*ctxAB* and this could be due to interrupting important H-NS oligomerization domains. This is also seen at the *virF* promoter in *Shigella* and *proU* in *E. coli* (18, 153). Lastly, it is possible that the El Tor P*ctxAB* does not provide enough H-NS binding sites for it to have an effective repressive role. This is not due to H-NS's inability to bind to

the El Tor promoter; EMSAs were performed using the shortened promoter as the probe and H-NS is able to bind without any apparent binding differences when compared to the full length promoter (Fig. 13). Additionally, the bound H-NS-DNA band appears at low concentrations of ToxT when it is only bound to one toxbox and not both toxboxes, suggesting protein competition for DNA binding at the shortened promoter.

The observed differences in H-NS dependent repression of *ctxAB* in the classical and El Tor strains calls for a revision of the current model regarding transcriptional activation of P*ctxAB*. The current model, devised by Yu *et al*., states that H-NS binding to DNA needs to be completely displaced by ToxT before transcription can be activated (196). However, this model did not take into account variable H-NS binding sites found among *V. cholerae* biotypes and lacked the new knowledge of where ToxT binds at P*ctxAB*. We suggest an updated model for transcription activation of *ctxAB* based on our experiments (Fig. 14). ToxT and H-NS only overlap from -72 to -60 relative to the transcriptional start site which coincides with toxbox 1. ToxT displaces H-NS from this binding region in both promoters but not completely from the full length promoter, with evidence from both the competitive binding EMSA and the mass spectrometry results.

In addition to the differences between the promoters, it is interesting to note that the mutation at repeat 5 causes a similar effect on both the shortened and full length promoters. The promoters in *E. coli hns*- strains have an elevated level of ToxT-independent expression. This trend is also seen when this mutant promoter is in *V. cholerae*, suggesting that this specific mutation changes the curvature of the DNA to make it favorable for transcription, possibly by enhancing RNAP binding.

These studies were conducted in *E. coli* to avoid other *V. cholerae* factors that may have an effect on P*ctxAB*. One such factor is ToxR, which is known to have an effect on P*ctxAB* transcription in the presence of bile in O395. Recently, a ToxR consensus sequence was characterized and found to have multiple potential binding sites in P*ctxAB* coinciding with the heptad repeats (66). Mutations to repeat 6 and the imperfect repeat 7 have been shown to have a defect in ToxR-dependent activation and overlap with ToxT binding sites although the other repeats have not been investigated. ToxR is also thought to displace H-NS at the *toxT* promoter and it is possible for it to have a similar role in P*ctxAB* (66, 146).

In summary, we have produced a higher resolution image of the H-NS binding region in P*ctxAB*, a large A/T rich stretch of DNA that fits the requirements for H-NS binding. The binding region was also characterized in two *ctxAB* promoter constructs having different numbers of heptad repeats, corresponding to differences between the classical and El Tor P*ctxAB*. These promoters have different requirements for activation and the mechanism by which H-NS represses activation. Additionally, mutations to these promoters can drastically effect not only ToxT-dependent activation but H-NS-mediated repression.

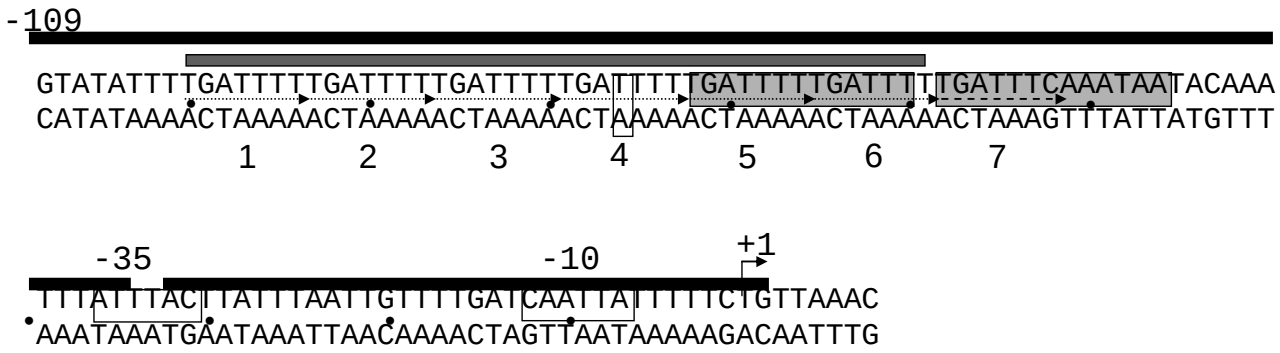


Figure 9: Map of the *ctxAB* promoter region in O395. The solid black bar above the P*ctxAB* sequence indicates the DNase I footprinting protection by H-NS (177). The gray bar illustrates binding by H-NS characterized by copper-1,10-phenanthroline in this study. The heptad repeats are designated by dotted arrows and numbered underneath the sequence; imperfect repeat 7 is designated by a dashed arrow. The shaded gray boxes highlight previously identified toxboxes (45). The shortened promoter used in this study extends to the boxed nucleotide at position -76. The putative -35 and -10 promoter elements are also boxed and the transcriptional start site is indicated by the bent arrow.

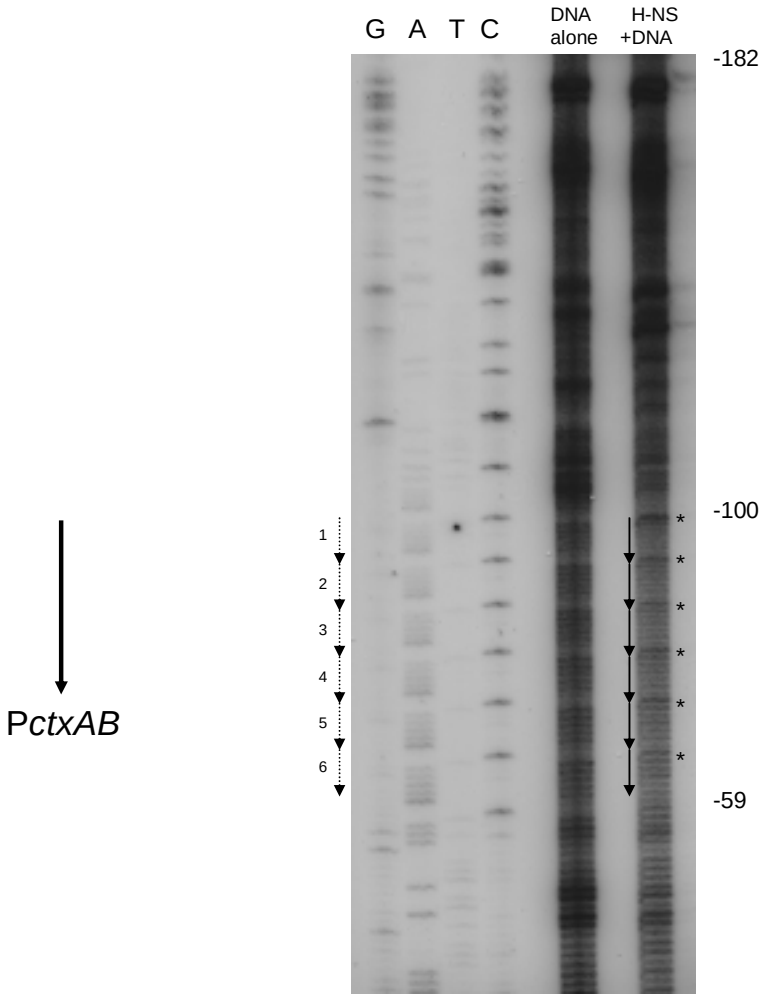


Figure 10: CP footprinting of H-NS on P*ctxAB*. H-NS binding is indicated by the solid arrows and the associated numbers correlate to the position from the transcriptional start site. The asterisks highlight hypersensitive sites in the footprint that suggest DNA bending at those nucleotides. "G," "A," "T," and "C" at the top of the figure refer to the nucleotide in the lane of the sequencing ladder. The dotted arrows represent each of the numbered GATTTTT heptad repeats. The footprint was created using the full-length O395 promoter construct and extends to position -180.

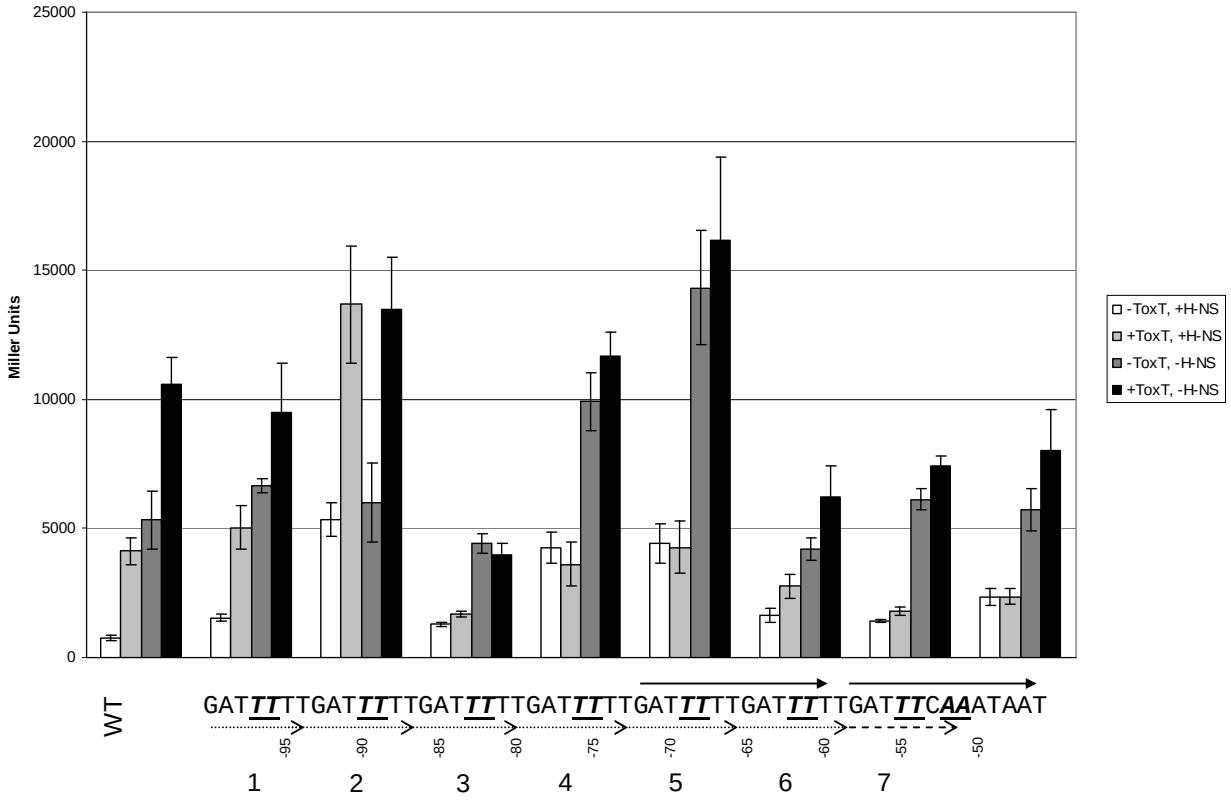


Figure 11: Effects of double point mutations on full-length P*ctxAB* transcription in *E. coli*. The graph indicates the β-galactosidase results from *ctxAB::lacZ* promoter constructs. The WT promoter construct is on the far left. The double point mutations are highlighted in bold text and underlined. Toxboxes are indicated by black solid arrows. The dotted arrows represent the numbered heptad repeats as well as the H-NS DNA binding region. The imperfect heptad repeat is represented by the dashed arrow. The white and light gray bars indicate WT *E. coli* K5971 with empty vector pMMB208 and *toxT*-encoding plasmid pMMTT, respectively. The dark gray and black bars represent *E. coli hns*- strains with empty vector pMMB208 and *toxT*-encoding plasmid pMMTT, respectively. Each experiment was repeated at minimum three times and the data show mean values with error bars showing standard deviation.

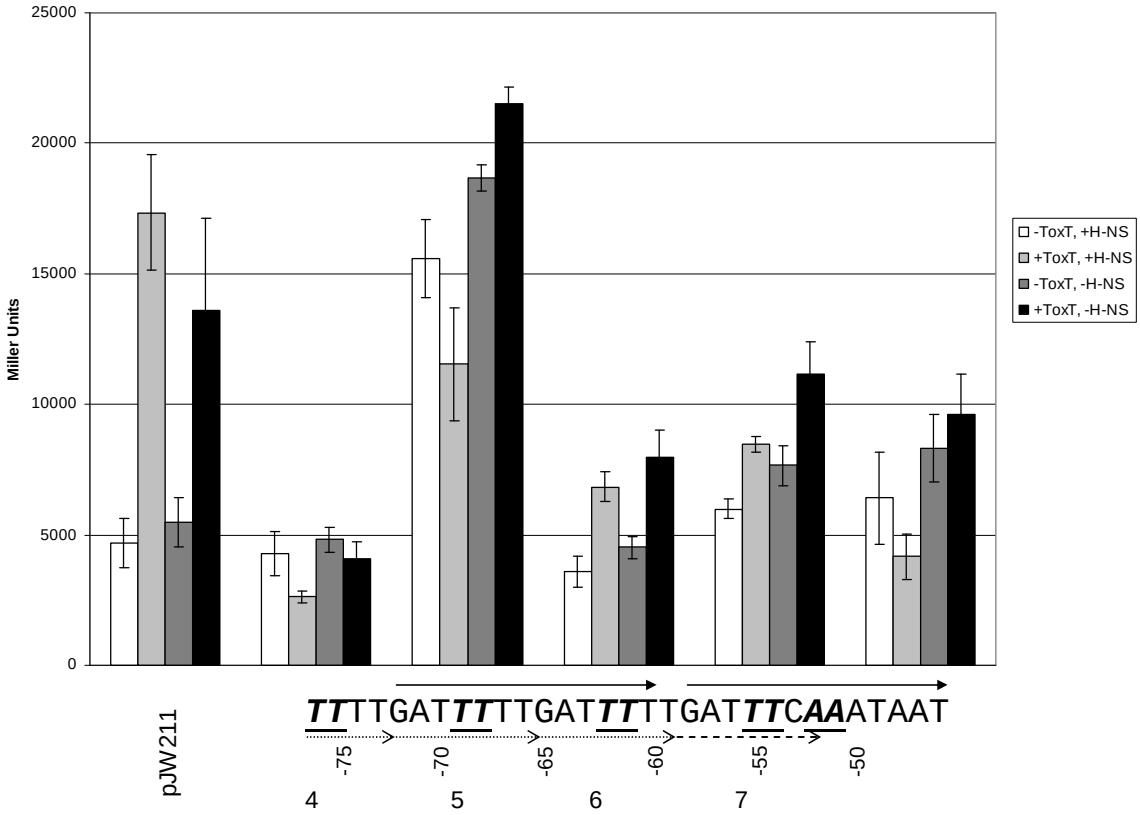


Figure 12: Effects of double point mutations on El Tor P*ctxAB* transcription in *E. coli*. β-galactosidase results from El Tor *ctxAB::lacZ* promoter constructs. The WT shortened promoter construct extends to -76 relative to the transcriptional start site is on the far left and labeled as pJW211. The double point mutations are highlighted in bold text and underlined. Toxboxes are indicated by black solid arrows. The dotted arrows represent the numbered heptad repeats as well as the H-NS DNA binding region; the imperfect repeat is represented by the dashed arrow. The white and light gray bars indicate WT *E. coli* K5971 with empty vector pMMB208 and *toxT*-encoding plasmid pMMTT, respectively. The dark gray and black bars represent *E. coli hns*- strains with empty vector pMMB208 and *toxT*-encoding plasmid pMMTT, respectively.

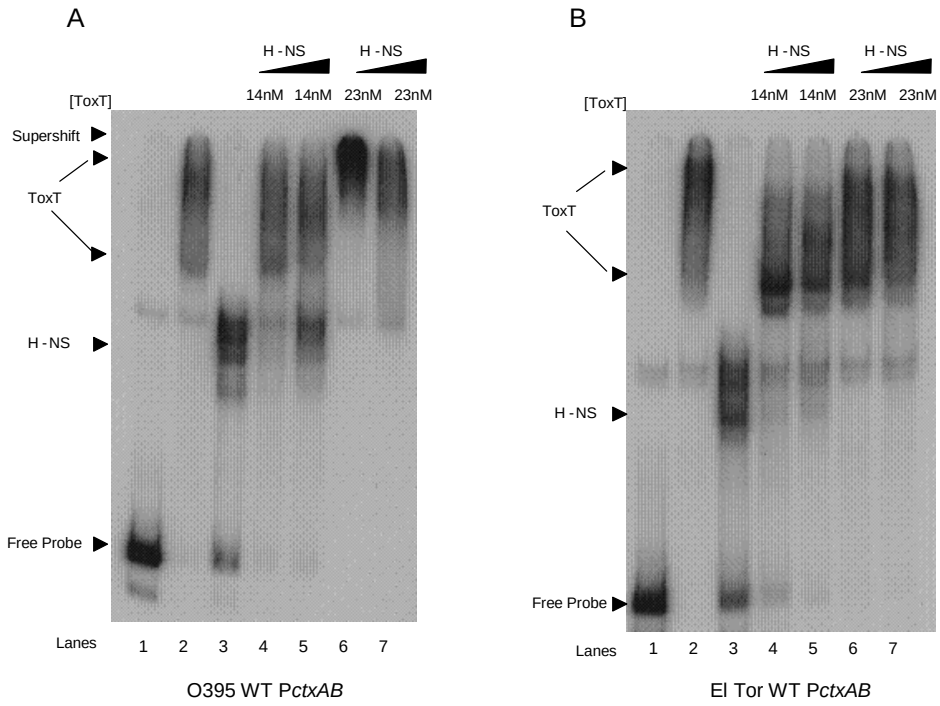


Figure 13: ToxT and H-NS binding to wild-type O395 and El Tor P*ctxAB* constructs. Electrophoretic mobility shift assays (EMSAs) were performed with the full-length WT O395 P*ctxAB* (A) and with the shortened El Tor P*ctxAB* (B). Lane 1 of each gel is the free probe without ToxT or H-NS. Lane 2 contains 23 nM ToxT-MBP and lanes 3, 5 and 7 contains 260 nM H-NS-His and the two arrows represent either one toxbox being occupied (lower arrow) or two toxboxes being occupied (upper arrow). Lanes 4 and 6 decrease the concentration of H-NS-His to 170 nM.

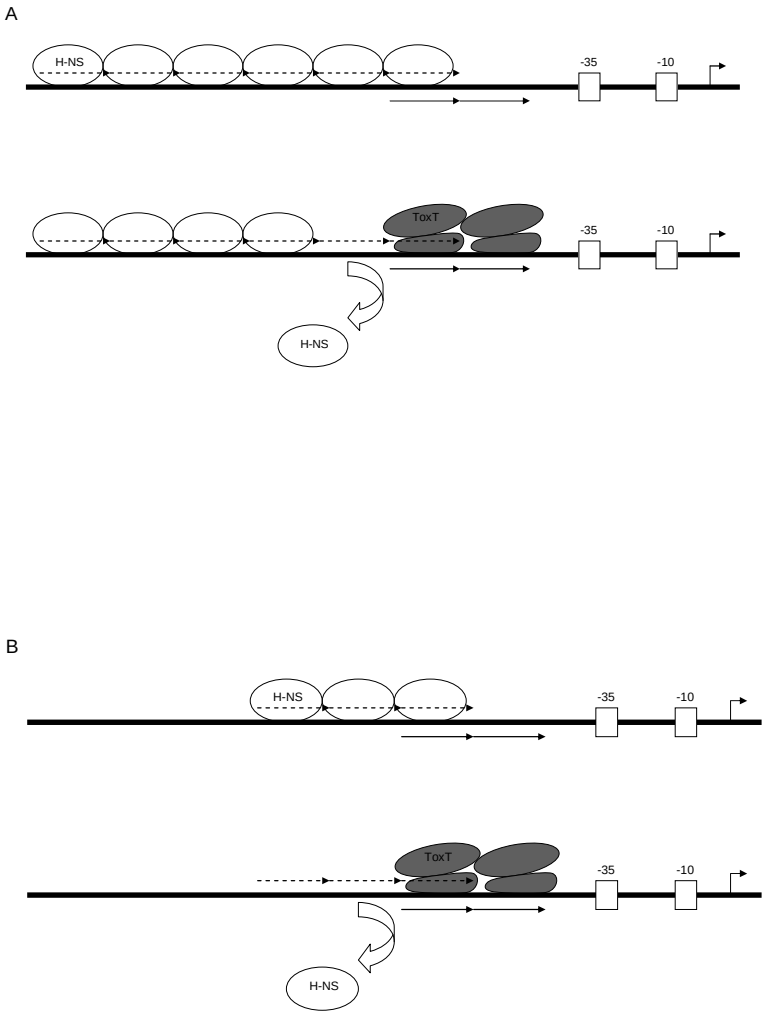


Figure 14: Model for P*ctxAB* activation in O395 and El Tor. The dashed arrows highlight the H-NS binding region and the heptad GATTTTT repeats on P*ctxAB*. The solid arrows indicate the toxboxes. The white circles represent H-NS which is bound to P*ctxAB* under normal conditions and oligomerizes along the DNA. Under ToxT-inducing conditions, ToxT, represented by the darker circles, displaces only H-NS monomers that overlap with toxbox 1 in O395 (A) or displaces H-NS completely from the promoter in El Tor (B), then subsequently binds to the toxboxes and activates transcription.

A

	ToxT/*toxT*-		H-NS/*hns*-	
	H-NS	*hns*-	ToxT	*toxT*-
WT	5.43	2.10	2.57	6.97
-96	3.24*	1.38	1.84*	4.32*
-89	2.55*	2.01	1.00*	1.11*
-82	1.31*	0.90*	2.35	3.42*
-75	0.85*	1.19	3.35	2.34*
-68	0.96*	1.13	3.81*	3.24*
-61	1.70*	1.48	2.25	2.64*
-54	1.26*	1.21	4.13*	4.29*
-51	1.00*	1.39	3.44	2.45*

B

	ToxT/*toxT*-		H-NS/*hns*-	
	H-NS	*hns*-	ToxT	*toxT*-
WT	3.78	2.21	0.69	1.20
-75	0.63*	0.86*	1.57*	1.14
-68	0.71*	1.15*	1.98*	1.20
-61	1.93*	1.80	1.18	1.27
-54	1.41*	1.47	1.32*	1.27
-51	0.66*	1.16*	2.33*	1.34

Table 1: Relative fold differences between ToxT/*toxT*- and H-NS/*hns*- strains in wild-type O395 (A) and El Tor (B) P*ctxAB* constructs. The fold differences represent the activation between ToxT/*toxT*- in H-NS and *hns*- *E. coli* strain on the left side of the tables and between H-NS/*hns*- in ToxT and *toxT*- *E. coli* strains on the right. The P*ctxAB* construct is indicated on the far left of the table. Asterisks indicate statistically significant differences between the wild-type and mutant P*ctxAB* constructs according to Student's *t* test ($P<0.03$).

CONCLUSIONS

Vibrio cholerae possesses a complex regulatory network of virulence factors that enable it to infect human hosts and subsequently cause the severe diarrheal disease cholera. Central to this cascade is ToxT, a positive transcriptional regulator that binds to and activates expression of most *V. cholerae* virulence determinants, including CT and TCP. ToxT binds to toxboxes, which are found in the promoters of each virulence gene ToxT activates. These toxboxes have been identified in many virulence gene promoters but, prior to this work, had not been characterized in P*ctxAB*. P*ctxAB* is arguably the most important promoter controlled by ToxT as *V. cholerae* cannot cause cholera if the bacteria cannot produce CT. There are several repeats within P*ctxAB* that fit the toxbox consensus sequence and previous studies had not revealed which of these are important for ToxT binding and activation of P*ctxAB* transcription. Additionally, H-NS, a global repressor, also binds to P*ctxAB* and its interplay with ToxT had not been fully characterized prior to the work described here.

The results of the work described in this dissertation indicated that ToxT binds to two toxboxes within P*ctxAB*; one that encompasses two of the repeats found in the promoter and another that is immediately downstream. This is a rather surprising finding given that each heptad repeat sequence could potentially comprise a toxbox, and the classical biotype P*ctxAB* has six perfect heptad repeat sequences followed by a seventh, imperfect repeat, and thus seven potential toxboxes. However, only the sixth and seventh repeats are recognized by ToxT both in vitro and in vivo The positioning of the toxboxes relative to the transcriptional start site classify P*ctxAB* as a class I promoter, requiring that the activator protein, ToxT, interact with the α-CTDs of the RNAP to activate

transcription. The potential DNA interaction or binding sites of the α-CTDs at P*ctxAB* is unknown, as are the points of interaction between the α-CTDs and ToxT. Future experiments could elucidate the interaction between the α-CTDs and ToxT to better understand the requirements necessary for P*ctxAB* transcription activation. The complex relationship between RNAP and activator proteins can then be used to investigate possible therapeutics against *V. cholerae* as well as other pathogens.

P*ctxAB* is also regulated by H-NS, by way of transcriptional repression. It was commonly accepted that ToxT first derepressed P*ctxAB* by entirely displacing H-NS from the promoter. This occurred by competitive binding to the DNA of ToxT, presumably because it bound to the promoter region with higher affinity than H-NS. The derepression step was followed by transcription activivation mediated by ToxT contacting RNAP. However, H-NS binding to P*ctxAB* had not previously been characterized in detail. The studies in this dissertation characterized H-NS binding sites at P*ctxAB*, revealing that there is only a small region of DNA in which H-NS and ToxT binding sites overlap. Additionally, the degree of repression exerted by H-NS is dependent on the number of heptad repeats found in P*ctxAB*; the number of heptad repeats varies between the classical and El Tor biotypes and even between individual strains of the same biotype. The observation that H-NS and ToxT binding sites have a relatively short region of overlap suggests that H-NS and ToxT may occupy P*ctxAB* simultaneously and that full displacement of H-NS may not be necessary for full transcriptional activation by ToxT. Future studies could investigate the possible role of H-NS in controlling transcription levels after ToxT binding and whether other proteins such as ToxR and RNAP, which may also bind to P*ctxAB*, are affected by H-NS repression. The knowledge from these

experiments can glean new information about the role of H-NS on virulence promoters in *V. cholerae* and many other pathogenic Gram-negative bacteria.

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ABSTRACT

FUNCTIONAL CHARACTERIZATION OF THE CHOLERA TOXIN PROMOTER OF *VIBRIO CHOLERAE*

by

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Vibrio cholerae is the bacterium responsible for the severe diarrheal disease cholera. The disease is directly caused by cholera toxin, which is secreted by the bacterium in the upper small intestinal lumen during the course of infection. Expression of cholera toxin, along with other virulence genes, is activated by the positive transcriptional regulator, ToxT. ToxT binds to DNA sequences known as toxboxes that are found within promoters of virulence genes and subsequently ToxT activates transcription. However, the toxboxes have not been previously characterized in arguably the most important virulence promoter in *V. cholerae,* the cholera toxin promoter (P*ctxAB*). Additionally, H-NS, a global transcriptional repressor found in Gram-negative bacteria including *V. cholerae,* also binds to P*ctxAB*. To activate transcription of P*ctxAB*, ToxT needs to overcome the repression mediated by H-NS. The mechanism for this, along with characterization of ToxT and H-NS binding to P*ctxAB*, is investigated in this dissertation.

Chapter one characterizes ToxT binding to P*ctxAB* and the experimental results identified two functional toxboxes in the promoter. Mutagenesis to either of the toxboxes resulted in a significant defect in ToxT-dependent transcriptional activation and ToxT binding to DNA. ToxT was also unable to bind the DNA when both toxboxes contained mutations and this led to a complete loss of ToxT activation of P*ctxAB*. Although there are other potential ToxT binding sites within the P*ctxAB* promoter, ToxT requires only these specific regions of DNA for activation.

Chapter two investigates the interplay between ToxT and H-NS at P*ctxAB*. Different *V. cholerae* biotypes contain a varying number of heptad repeats that are also H-NS binding regions and it was previously thought that H-NS needs to be completely displaced from the DNA to relieve its repressive role. However, the binding sites of ToxT and H-NS overlap in a small region of DNA and complete disassociation of H-NS from the promoter may not be necessary for P*ctxAB* activation by ToxT. Further experiments also revealed that ToxT and H-NS bind to the DNA simultaneously in promoters containing six heptad repeat sequences. This does not occur in the promoter that contains only three heptad repeat sequences. These results suggest there is a direct correlation between the number of these heptad repeat sequences and the overall repression exerted by H-NS.

AUTOBIOGRAPHICAL STATEMENT

I began my research career as an undergraduate at Wayne State University in Dr. Aleksandar Popadić's lab in the Biological Sciences department investigating the role of Hox genes in insects. I graduated from Wayne State University with a B.S. degree in Biological Sciences in 2006. In 2007, I joined the Department of Immunology and Microbiology at Wayne State University and the following year joined Dr. Jeff Withey's lab where I completed my dissertation work on the functional characterization of the cholera toxin promoter of Vibrio cholerae. In March 2013, I will be joining Dr. Mike Konkel's lab at the School of Molecular Biosciences at Washington State University as a postdoctoral fellow.