Molecular analysis of ToxR-TcpP functional interaction and activation of the virulence cascade in *Vibrio cholerae*

by

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For my parents and Jason,
without whom I wouldn't have made it this far.

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Table of Contents

Dedication.....ii
Acknowledgements.....iii
List of figures.....viii
List of Tables.....xi
Abstract.....xii

Chapter 1: Introduction.....1
Cholera as a driving force for microbiology, a historical perspective.....1
Transition of *V. cholerae* from aquatic organism to pathogen.....3
Environmental signaling and the virulence cascade.....5
Structure and function of ToxR and TcpP.....10
Models of transcriptional activation of *toxT*.....18
References.....22

Chapter 2: ToxR recognizes a direct repeat element in the *toxT, ompU*, *ompT*, and *ctxA* promoters in *Vibrio cholerae* to regulate transcription.....28
Summary.....28
Introduction.....28
Results.....32
Discussion.....51
Materials and Methods.....60
References.....69

Chapter 3: The two faces of ToxR: activator of *ompU*, co-regulator of *toxT* in *Vibrio cholerae*.....73
Summary.....73
Introduction.....74
Results.....76
Discussion.....101
Materials and Methods.....109
References.....117

Chapter 4: The wing of the TosR winged helix-turn-helix domain is required for DNA binding.....121
Summary.....121
Introduction.....122
Results.....127
Discussion.....140

Materials and Methods....................143
References....................152

Chapter 5: An intramolecular periplasmic disulfide bond in TcpP prevents premature degradation and enables virulence gene expression in Vibrio cholerae....................155
Summary....................155
Introduction....................156
Results....................159
Discussion....................172
Materials and Methods....................176
References....................182

Chapter 6: Discussion....................186
Summary....................186
Promoter architecture....................187
ToxR and TcpP structure and function....................191
Transcriptional activation of the virulence cascade....................198

List of Figures:

1-1 Virulence gene activation in *V. cholerae*......6

1-2 Structural comparison of w-HTH family members......12

1-3 Models of ToxR co-activation of *toxT*......19

2-1 DNA sequence of the *V. cholerae* classical strain 0395 promooter-poximal region of the *toxT* promoter and ToxR-dependent activation of single base pair substitutions......33

2-2 ToxR binding site mutations do not affect *toxT* activation by overexpressed TcpP......36

2-3 Mutations in the putative ToxR-binding site disrupt ToxR binding to the *toxT* promoter......39

2-4 Assessment of 50% gel shifting activity of various *toxT* promoter mutants......41

2-5 ToxR fails to bind or activate a *toxT-lacZ* derivative containing the degenerate ToxR-binding sites from -69 to -56......45

2-6 ToxR binding site location is not critical for transcriptional activation of *toxT*......46

2-7 The ToxR consensus-binding site is required for ToxR-mediated activation of the *ompU* promoter......48

2-8 The ToxR consensus-binding site is required for ToxR-mediated activation of the *ctxA* promoter......50

2-9 The ToxR consensus-binding site is required for ToxR-mediated repression of the *ompT* promoter......52

2-10 Models for the role of ToxR in TcpP-mediated *toxT* activation......58

3-1 Activation of *ompU-lacZ* or *toxT-lacZ* chromosomal reporter constructs in *V. cholerae* by various ToxR mutant derivatives......79

3-2 Modeling of the N-terminal DNA-binding/transactivation domain of ToxR (residues 1-114) using threading of the ToxR sequence onto structurally related w-HTH family members......80

3-3 Gel-shift assays to assess promoter recognition by various ToxR mutant proteins identified in a random mutagenesis *ompU-sacB* selection strategy......83

3-4 Gel-shift assays to assess promoter recognition by various ToxR mutant proteins identified previousy, or in a random mutagenesis *toxT-lacZ* blue-white screen......................................88

3-5 Gel-shift assays to assess promoter recognition by putative α-loop site-directed ToxR mutant proteins..94

3-6 Assessment of TcpP protein interaction by various ToxR mutant proteins that preferentially affect *toxT* transcription...97

3-7 Assessment of ToxR/TcpP interaction by various ToxR and TcpP mutants using an adenylate cyclase-based membrane-anchored bacterial two-hybrid system...99

3-8 Model for differential activation of the *ompU* and *toxT* promoters of *V. cholerae* by ToxR 102

4-1 Model of ToxR bound to the promoter..124

4-2 Requirement of each residue in the ToxR wing on *toxT* and *ompU* activation.....................128

4-3 Location of ToxR wing residues...129

4-4 ToxR wing mutants expressed from the *toxR* chromosomal locus are not preferentially defective for *toxT* activation..131

4-5 The wing of ToxR is not required for ToxR-TcpP interaction...133

4-6 ToxR-TcpP interaction as monitored by BACTH varies...135

4-7 Certain wing residues of ToxR are required for DNA binding...137

4-8 Model of ToxR mediated co-activation of the *toxT* promoter..144

5-1 TcpP forms intramolecular and intermolecular disulfide bonds..160

5-2 TcpP-HSV is able to activate transcription of *toxT* in the presence of intramolecular or intermolecular periplasmic disulfide bonds..162

5-3 The instability of chromosomally-expressed TcpP cysteine mutants lacking a C-terminal epitope tag results in a *toxT* activation defect..164

5-4 TcpP expression in strains assessed for CT production...167

5-5 TcpP mutants with intermolecular or no periplasmic disulfide bonds are degraded through the YaeL pathway...169

5-6 Chromosomally-encoded ToxR periplasmic mutants have modest effects on *ompU* expression and minimal effects on *toxT* expression and CT production...............................171

5-7 A model for disulfide bond and TcpH-mediated protection of TcpP from degradation.......174

6-1 Orientation of ToxR binding-sites on ToxR regulated promoters..190

6-2 Model of ToxR w-HTH residues by function...192

List of Tables

2-1 Relative affinities of *toxT* promoter mutants for membranes containing or lacking ToxR.....42

2-2 Strains and plasmids.....61

2-3 Oligonucleotides used in this study.....63

3-1 Activation phenotypes of ToxR derivatives from various mutagenesis studies.....86

3-2 Activation phenotypes of ToxR α-loop mutants.....92

3-3 Affect of side chain substitutions in the wing domain on promoter activation.....96

3-4 Strains and plasmids.....110

3-5 Primers used in this study.....112

4-1 Strains and plasmids.....147

4-2 Primers used in this study.....149

5-1 Some chromosomally-encoded TcpP periplasmic cysteine mutant are severely defective for CT production.....166

5-2 Strains and plasmids.....177

5-3 Primers used in this study.....179

Abstract

Vibrio cholerae, the causative agent of cholera, regulates expression of virulence factors such as the cholera toxin and the Toxin Co-regulated Pilus through the master virulence regulator ToxT. Transcriptional activation of *toxT* is in turn co-activated by the transmembrane winged helix-turn-helix (w-HTH) transcription factors ToxR and TcpP. ToxR is a fully functional transcriptional activator and is able to directly activate transcription of the outer membrane porin encoded by *ompU*. However, ToxR does not activate transcription of *toxT* directly, but instead assists TcpP-mediated activation of the *toxT* promoter. We have found, through mutational studies that ToxR binds the *toxT* promoter 3-helical turns upstream of the promoter proximal TcpP binding site. This is in contrast to the ToxR binding site on the *ompU* promoter, which is just upstream of the -35 element and in the opposite orientation. Additionally, different faces of the ToxR protein are preferentially required for co-activation of *toxT* as compared to direct activation of *ompU*. This highlights the different functions of ToxR as a co-activator and a direct activator. Although, w-HTH proteins often interact through the wing, the wing of ToxR is not required for ToxR interaction with TcpP. However, the wing of ToxR is required for binding to, and transcriptional activation of, both *toxT* and *ompU* promoters. Finally, we investigated the regulation of TcpP stability in transcriptional activation of *toxT*. Controlled proteolytic degradation of TcpP has been previously shown to downregulate activation of *toxT* under non-inducing conditions. We have found that the periplasmic cysteines in TcpP form an intramolecular disulfide bond, and that this bond protects TcpP from proteolytic degradation, allowing TcpP to activate transcription of *toxT*. Similar periplasmic cysteine bonds are present in

ToxR, however they are not critical for stability or transcriptional activation. ToxR may co-activate the *toxT* promoter by binding to, and altering the structure of, the *toxT* promoter thereby enhancing TcpP binding. Additionally, ToxR-TcpP interactions may help recruit TcpP to the promoter. Co-activation of *toxT* by ToxR and TcpP allows for integration of many environmental signals, which insures that virulence gene expression will only be activated under appropriate conditions.

Chapter 1

Introduction

The disease cholera is characterized by profuse watery diarrhea, which is often described as rice water diarrhea due to its appearance. The fluid loss due to cholera is so dramatic that it can result in severe dehydration and even death within 48 hours of onset of symptoms. Cholera is caused by ingestion of the gram-negative bacterium *Vibrio cholerae*, and subsequent colonization of the small intestine. The profuse watery diarrhea caused by *V. cholerae* is a result of secretion of the cholera toxin (CT), which induces elevated levels of cAMP resulting in rapid ion secretion into the lumen of the intestine, followed by water to balance osmolarity. The other major virulence factor is the toxin co-regulated pilus (TCP), which allows for microcolony formation and colonization of the intestine (2). Transcription of both of these virulence factors is controlled by the master virulence regulator ToxT (5). *toxT* expression, in turn, is co-regulated by the membrane-localized transcription factors ToxR and TcpP (6-8).

Cholera as a driving force for microbiology, a historical perspective

Cholera has been reported for centuries, with descriptions appearing in ancient Greek, Arabic, and Sanskrit writings dating back to 500 BC (10, 11). During this time period cholera was primarily a seasonal endemic disease, although this would change as global travel and trade routes expanded (11, 12). Beginning in the early 19th century, pandemics of cholera spread

throughout the world, decimating populations, inducing fear, and motivating microbiological and epidemiological research. From 1817-1823 the first cholera pandemic swept out of India along the trade routes. The second pandemic, from 1829-1851, spread even further decimating Russia, reaching the Americas by 1832, and leaving no continent, except Antarctica, untouched (11, 14).

The first hint of a treatment for cholera was found during the 2nd pandemic when Scottish physician Latta had the idea of using intravenous (IV) fluids to restore the specific gravity of the blood. Due to problems with IV fluid administration and composition only 5 of the 15 patients survived, and IV therapies wouldn't be used again to treat cholera until the 1890s (11). Although Latta's technique failed, his concept was sound, as today the amount of fluid administered is determined by the volume of fluid lost through diarrhea, thereby restoring the specific gravity of the blood. Although most cases of cholera are currently treated by oral rehydration therapy, in severe cases this may be insufficient and IV fluids are administered.

In 1849, during the 2nd pandemic John Snow presented the idea that cholera could be transmitted through fecal contamination of the water supply, using one of the first applications of epidemiology to map out cases. Snow went so far as to remove the pump handle of a contaminated well in London, in order to prevent the spread of the disease. Although many people dismissed Snow's hypothesis at the time, it proved true as improvements in sanitation of drinking water during the 5th and 6th pandemic helped to protect Europe and the United States from cholera. By 1926, improvements in sanitation worldwide led many to hope that there would be no subsequent cholera pandemics.

It was during the 3rd pandemic from 1852-1859 that the bacterium was first observed and described by Filippo Pacini in Italy while examining the luminal contents of patients, although his findings were lost (11). During the 5th pandemic Koch re-discovered the bacterium *V.*

cholerae, and proved that it causes the disease cholera. Koch called for better understanding of the disease and the biology of the organism as a way to control outbreaks. Koch's observations lead him to propose that the interaction of *V. cholerae* with the environment may be critical to understanding spread of the disease, a theory that still drives much of the research on cholera today.

Despite improvements in sanitation and identification of *V. cholerae* and its major virulence factors, today we are in the midst of the 7th and 8th pandemics, resulting in endemic cholera in Asia, Africa, and parts of South America. The endemic cholera in these regions primarily affects children (<5 years) since infection does result in some protection from subsequent infections (17). Additionally, there are sporadic outbreaks among all age groups due to breakdowns in sanitation and/or introduction of the bacterium into non-endemic environments. A dramatic demonstration of this was the outbreak in Haiti following an earthquake in 2010 which affected over 500,000 people and resulted in over 7,000 deaths (18). The 7th pandemic is caused by the El Tor strain, whereas the 5th and 6th pandemics, and likely previous pandemics, were caused by the classical strain (10, 11). Both the El Tor strain and the classical strain are serotype 01, which is the only serotype, aside from 0139, that has been shown to cause cholera epidemics despite approximately 200 serotypes present in the environment. Serotype 0139 is responsible for the 8th pandemic, likely as a genetic crossover event from an El Tor strain (10).

Transition of *V. cholerae* from aquatic organism to pathogen

V. cholerae cycles between aquatic and host environments. Both *V. cholerae* pandemic strains as well as non-pandemic, non-toxigenic strains are found naturally in estuaries and other aquatic environments. *V. cholerae* survival is optimal in warm, slightly salty water, where it can

remain viable for at least 50 days (10). In freshwater, the lack of salt can be compensated for by increased organic nutrients and/or divalent cations. In non-optimal aquatic environments, *V. cholerae* enters a viable but non-culturable stage that maintains virulence if ingested (10). In aquatic environments, *V. cholerae* is primarily found on biofilms associated with chitinous organisms such as plankton and copepods (19). Copepods can be colonized by up to 10^4 *V. cholerae* cells, enough for an infectious dose (10). Since most *V. cholerae* are present in biofilms on these marine organisms, simply filtering them out with folded cloth can greatly reduce endemic cholera (20).

Upon ingestion of the *V. cholerae* the bacterium travels to the small intestine where it colonizes. A relatively high infectious dose (10^4-10^{11} bacterium depending on the study) is required likely due to acid sensitivity during passage through the stomach (10, 19). Upon ingestion, TCP is expressed, allowing *V. cholerae* microcolonies to form and colonization to occur (2). Symptoms of cholera appear 12 to 27 hours after ingestion, although some people can be asymptomatic temporary carriers (17). During the height of infection, up to 1 L/hour of fluid can be lost, resulting in severe dehydration and even death if not treated (17). This rapid dehydration is due to production of CT, an AB toxin which binds to the GM1 ganglioside of intestinal epithelial cells. Upon entry into the intestinal epithelial cell, CT is activated inducing increased levels of cAMP. This results in increased secretion of sodium chloride and bicarbonate, which, due to osmotic pressure, results in secretion of water. Along with water and electrolytes, 10^{10}-10^{12} *V. cholerae* per liter are shed in the characteristic rice water stool secreted during the height of infection, and bacteria can be shed for 1-2 weeks (17). In late stages of infection, changes in transcription, including down regulation of the ToxR regulon, and induction of flagella, Fur and c-di-GMP occur, preparing the bacterium to exit the host and

survive the environment (17, 21). The resulting *V. cholerae* released in the stool is hyper-infectious, reducing the number of *V. cholerae* cells required for infection over 100-fold. This hyper-infectivity is due in part to a decrease in chemotaxis and increase in motility (22). Hyper-infectivity lasts at least 5 hours in aquatic environments likely accelerating outbreaks in crowded regions with poor sanitation (22).

There are few differences between the environmental strains of *V. cholerae*, which do not cause cholera, and the epidemic strains that do (23). Of the 200 serotypes only two, 01 and 0139 have been shown to cause cholera, and in order to do so they must first acquire pathogenicity factors by phage and/or horizontal gene transfer. Toxigenic *V. cholerae* has a pathogenicity island (VPI-1), which is not prevalent in non-toxigenic non-O1/non-O139 strains (24). This pathogenicity island encodes *toxT*, the master virulence regulator. It also encodes genes required for TCP, which is required for microcolony formation and intestinal colonization (25). The TCP also acts as a CT phage (CTXΦ) receptor, allowing these bacteria to acquire the CT locus (26, 27). One of the transcription factors required for *toxT* activation, TcpPH, is encoded on VPI-1, while the other, ToxRS, is not (24). ToxR is found in pandemic *V. cholerae* strains, as well as non-toxigenic strains, and other *Vibrio* species. In addition to co-activation of *toxT*, ToxR has the additional role of regulating expression of two outer membrane porins: *ompU* and *ompT* (28, 29).

Environmental signaling and induction of the virulence cascade:

Transition from the aquatic environment to the intestine is accompanied by dramatic shifts in salinity, temperature, nutrient availability, and other factors. These environmental stimuli feed into multiple steps in the activation cascade, resulting in ToxR and TcpP activation.

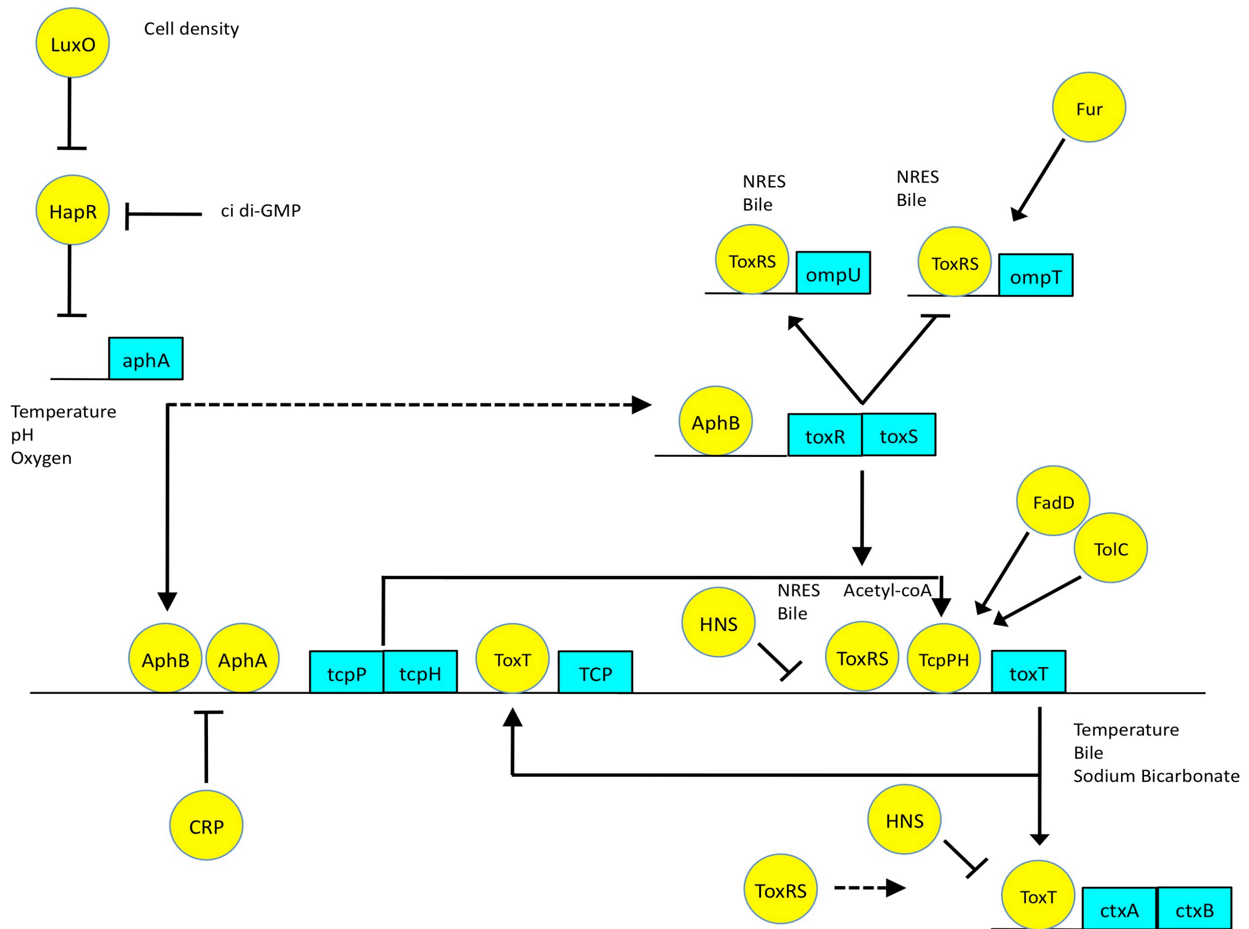


Fig 1.1 Virulence gene activation in *V. cholerae*. Environmental stimuli feed into the transcriptional activation cascade by activating transcription of *tcpP*, activating transcription of *toxT*, and regulating ToxT activity. Proteins in the activation cascade are designated by circles, and genes by rectangles. Direct activation of the virulence cascade is indicated by arrows, posited and unconfirmed activation pathways are designated with dashed arrows. Repression is designated by lines without arrowheads.

ToxR and TcpP, then activate *toxT* expression, which if the environmental conditions are correct, results in expression of TCP and CT (Fig 1-1). The ToxR regulon is often used to describe the transcriptional activation and repression cascades that occur as a result of activation of ToxR and TcpP.

Virulence gene expression is tied closely to several endogenous systems in *V. cholerae* through quorum sensing. Cell density of *V. cholerae* and other gram-negative bacteria is monitored by two autoinducers, AI-1 and AI-2, which feed into the LuxO system (30). Quorum sensing induces expression of LuxO, which represses HapR (30)(Fig. 1-1). HapR is a repressor of AphA, the master quorum-sensing regulator at low cell density (30, 31). Additionally, cyclic di-GMP the secondary messenger that regulates biofilm production, links into the quorum sensing machinery in *V. cholerae* (Fig. 1-1). The exact role of cyclic di-GMP is unclear because it both increases expression of AphA and represses virulence gene expression (32, 33). However, most studies agree that cyclic di-GMP likely ties into the quorum sensing machinery through repression of HapR (32). In the absence of HapR, due to low cell density and high di-cyclic-GMP levels, AphA is activated (33). AphA is a winged-helix transcription factor, which enhances activation of TcpPH expression by the LasR-type transcription factor AphB (34-37)(Fig. 1-1). AphB is most active at low pH (34), and low oxygen levels (38, 39). Additionally, transcription of *tcpP* is induced by decreased temperature in vitro (37). In addition to AphA and AphB binding sites on the *tcpP* promoter, there is an overlapping catabolite repression protein (CRP) binding site, indicating that cAMP and CRP may down-regulate *tcpP* expression (40, 41)(Fig 1-1). PhoBR is also able to bind to the *tcpP* promoter, thereby increasing expression in response to low phosphate levels (42). Although ToxRS has generally been believed to be constitutively expressed (43), expression of ToxR may also be activated by AphB (44). ToxR

and TcpP are co-transcribed with periplasmic co-activators designated ToxS and TcpH, respectively (6, 45-47).

The activities of ToxR and TcpP are also regulated by environmental stimuli. ToxR transcriptional activation activity, and possibly *toxR* transcription, is induced in media that contains the amino acids asparagine, arginine, glutamic acid, and serine (NRES) (48, 49). It is also likely that central metabolism is linked into regulation of virulence gene activation through acetyl CoA and ToxR, however the mechanism behind this remains elusive (50). ToxR activity is often investigated by monitoring expression of *ompU* and *ompT* since ToxR directly regulates these promoters, while it is a co-activator of *toxT*. ToxR induces expression of *ompU* through direct activation of this locus (28). ToxR also represses expression of *ompT*, which is induced by iron utilizing the Fur system (29, 51). Bile can increase activity of ToxR as can be seen by an increase in *ompU* expression and a decrease in *ompT* expression (52). Because OmpT increases *V. cholerae* bile sensitivity, the presence of bile results in increased bile resistance by increasing *ompU* expression and *ompT* repression (53). TolC, an outer membrane protein, is required for repression of either TcpP or ToxR activity under non-inducing laboratory conditions such as minimal media lacking NRES or LB with additional salts (54). Additionally, FadD, a long-chain fatty acyl coenzyme A ligase, is required for proper localization of TcpP to the membrane, and therefore *toxT* activation (55). ToxS and TcpH are co-transcribed with ToxR and TcpP, respectively, and can influence the ability of ToxR and TcpP to activate transcription by promoting dimerization and enhancing stability (45, 47).

Upon induction of TcpPH and ToxRS, these transcription factors bind to the *toxT* promoter, relieving repression by the histone-like nucleoid structuring protein H-NS, and activating *toxT* transcription (56). ToxT is bound to the lipid cis-palmitoleic acid when inactive,

indicating a critical role for bile in down regulating activity of this protein (57). Additionally, in classical strains, ToxT activity is higher at 30°C than 37°C indicating that activity of this protein is influenced by temperature (58). In both El Tor and classical strains the activity of ToxT is up-regulated in the presence of sodium bicarbonate (59). Expression of *toxT* is further activated by induction of an autoactivation loop in which read-through transcription from ToxT activation of the TCP locus results in increased activation of *toxT* (60, 61). The genes for accessory colonization factors (*acfA-D*), which are required for colonization in the infant mouse model, are downstream of *toxT* and part of the ToxR regulon (61, 62). ToxT is generally referred to as the master virulence regulator because, once active, it is able to directly activate expression of the genes required for CT, TCP, and other virulence factors (5). CT production is repressed by H-NS binding overlapping the ToxT-binding site, and must be relieved in order for transcription of *ctxAB* (63, 64). Although ToxT is accepted as the primary transcriptional activator of *ctxAB*, ToxR is also able to activate this locus under certain conditions (8, 65, 66).

One of the major environmental factors that *V. cholerae* encounters when it enters the small intestines is bile. Several studies have found that bile activates the virulence gene cascade, while others find bile has an inhibitory effect. Part of the reason for this may be due to the complex chemistry of bile. Bile contains conjugated acids, unconjugated acids, pigments, inorganic salts, cholesterol, and phospholipids (67). In classical strains grown under inducing conditions, addition of 0.2-0.4% bile reduced transcription of *ctxAB* resulting in a 97% decrease in CT secretion (68). A decrease in both CT and TCP expression in a strain containing plasmid-expressed *toxT* was also observed indicating that ToxT activity is reduced in the presence of bile (58). Fractions of bile containing unsaturated fatty acids including arachindonic, linoleic, and oleic acids were also found to decrease CT expression (67). However, bile acids can up-regulate

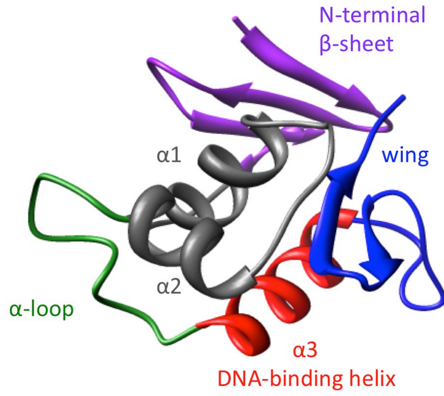
ToxR activity, thereby possibly increasing expression of *toxT* (52, 69). Bile can also increase dimerization and therefore transcription activation activity of TcpP (70). It is likely that bile may increase or decrease virulence factor expression in *V. cholerae* depending on composition and concentration, allowing for differential expression of virulence gene depending on the location of the bacterium in the intestine.

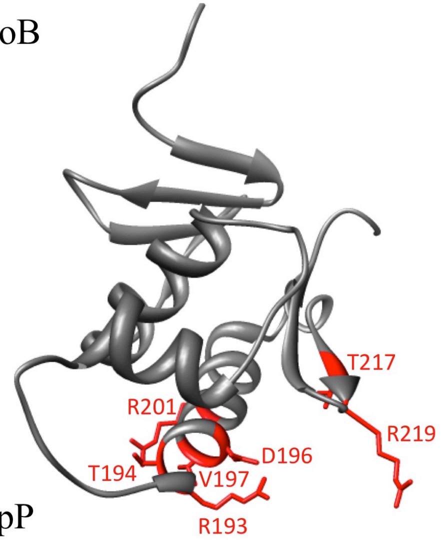
In the laboratory, virulence gene expression in classical strains is induced by decreasing the temperature to 30°C and the pH of the media to 6.5. Although the laboratory conditions differ from the inducing conditions found in the intestines, it is understandable why they work. Decreasing the pH of the media would likely increase AphB activity and therefore transcription of TcpPH. Additionally, growth in LB media would provide the necessary NRES amino acids for full ToxR activity. This results in increased induction of *toxT* expression, which is compounded by the increase in ToxT activity at this lower temperature. To induce expression of virulence factors in El Tor strains, they must be grown in AKI conditions (71). Under AKI conditions, cultures are grown without aeration for 4 hours and then switched to aerated conditions. During the non-aerated growth period, oxygen levels are reduced, theoretically increasing AphB activity and therefore *tcpPH* expression. Differences in virulence gene induction between El Tor and classical strains has been linked to a single nucleotide differences in the *tcpPH* promoter, leading to differences in expression of *tcpPH*, resulting in these two strains responding differently to environmental stimulation (43, 72, 73).

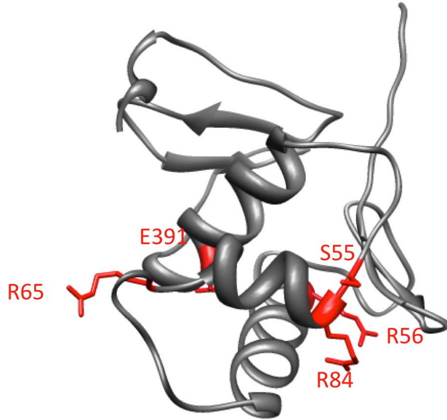
Structure and function of ToxR and TcpP:

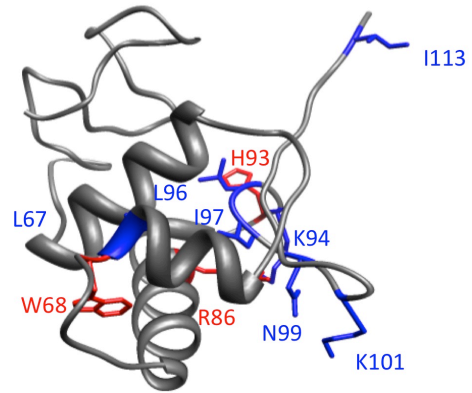
ToxR and TcpP are both transmembrane proteins with cytoplasmic domains homologous to the winged helix-turn-helix (w-HTH) family of transcription factors. The majority of w-HTH

transcription factors are part of a two-component regulatory system in which phosphorylation of an N-terminal regulatory domain of the response regulator protein results in transcriptional activation by the w-HTH domain. ToxR and TcpP do not have an N-terminal regulatory domain, but instead have a C-terminal periplasmic domain, with no known homology. The periplasmic domain of TcpP regulates stability by interaction with TcpH. Upon switching to non-inducing conditions, TcpP is proteolytically degraded in a controlled stepwise fashion, starting with the periplasmic domain, resulting in down-regulation of virulence gene expression (47, 74). The periplasmic domain of ToxR is involved in dimerization, but it is not required for transcriptional activation. Truncations of ToxR containing only the cytoplasmic domain are able to activate transcription of *ompU*. Additionally, truncations of ToxR missing the periplasmic domain, but leaving the cytoplasmic and transmembrane domains intact are able to activate transcription of both *ompU* and *toxT* (75). Under certain conditions, ToxR is also able to directly activate expression of *ctxAB*, and this function is dependent upon the periplasmic domain (76). Additionally, replacement of the periplasmic domain with alkaline phosphatase, or other periplasmic proteins, does not dramatically reduce *toxT* activation, making its function unclear (8, 77, 78). Homology of the w-HTH domain allows us to extrapolate structural and functional information about ToxR and TcpP cytoplasmic domains based on studies of other w-HTH proteins, particularly *E. coli* OmpR (Fig. 1-2A) and PhoB (Fig. 1-2B) which have been well characterized. Although, the overall homology of w-HTH proteins is low, 20-65%, many residues and the basic structure are conserved throughout this family (79). When residues of ToxR corresponding to conserved critical w-HTH residues were mutated, several of the resulting ToxR mutants were no longer able to bind DNA (Fig 1-2C). In particular, E39K, R56L/K, and R84K mutations disrupted DNA binding and transcriptional activation (13). These residues









E

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                                      β-sheet                                      α1
ToxR  MSHI--GT--------------KFI-LA-E-K-FTFDPLSNTLIDKEDS-E-EII------R-LGSNESRILWLLAQRPN   51
TcpP  ---M--GY--------------VRVIYQFPDN-LWWNECSNQVYYA-Q--D--PMKPERLIGTPSIMQAKLLKILCEYHP   55
PhoB  ----MAVE--------------EVIE-M-QG--LSLDPTSHRVMA--G-EE--PL------E-MGPTEFKLLHFFMTHPE   46
OmpR  --------RQANELPGAPSQEEAVI-A--F-GKFKLNLGTREMFR-----EDEP-M-----P-LTSGEFAVLKALVSHPR   56

          α2                      α3 DNA-binding                 wing
ToxR  EVISRNDLHDFV------WREQGFEVDDS--SLTQAISTLRKMLKDSTKS----PQYVKTVPKRGYQLIAR-----VETV  114
TcpP  SPCPNDQIIKAL------WPH--GFISSE--SLTQAIKRTRDFLNDE--H----KTLIENVKLQGYRINII-QVIV     114
PhoB  RVYSREQLLNHV------WGT-NVYVEDR--TVDVHIRRLRKALEP----GGHDR-MVQTVRGTGYRFSTRF         104
OmpR  EPLSRDKLMNLARGREYSA----M-----ERSIDVQISRLRRMVEEDPAH----PRYIQTVWGLGYVFVPD-----GSKA  118
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Fig 1.2. Structural comparison of w-HTH family members. A) The different regions of the w-HTH domain including the N-terminal β-sheet (purple), the α-loop (green), the DNA binding helix (red), and the C-terminal wing (blue) are highlighted on the OmpR crystal structure (1). B) The w-HTH domain of PhoB is shown based on the NMR structure (3). Residues that make base-specific contact with DNA nucleotides based on the NMR and crystal structures of PhoB bound to DNA are highlighted in red (3, 4). Models of the w-HTH domain of ToxR (C) and TcpP (D), based on homology with other w-HTH domains, were created using I-TASSER (9). C) ToxR residues that are conserved with OmpR/PhoB and required for DNA binding are shown in red (13). D) TcpP residues required for DNA (red) and DNA binding/ToxR-TcpP interaction (blue) highlight the role of the wing of TcpP in both ToxR-TcpP interaction and DNA binding (15, 16). E) Structural alignment of ToxR, TcpP, PhoB and OmpR was performed in Chimera.

correspond to PhoB residues that make contact with the DNA backbone (4). Two other residues tested that do not correspond to PhoB DNA binding residues, S55A/T and R65L were not required for promoter binding or transcriptional activation (13).

The w-HTH domain is comprised of an N-terminal β-sheet, 3 α-helixes with a loop between α2 and α3, and a C-terminal wing comprised of a β-stranded hairpin (Fig 1-2A). The N-terminal β-sheet is involved in maintaining the hydrophobic core as well as dimerization (4, 79-81). This β-sheet may also interact with the N-terminal regulatory domain, thereby governing activity of the protein (4). The first and second α-helices are involved in proper positioning of the DNA binding helix (α3), which is critical both for proper folding of the protein and for DNA recognition (79). The third α-helix is the DNA-binding helix, which sits in the major groove of the DNA and interacts directly with the DNA bases, allowing for sequence recognition (3, 4). Between the second and third α helix is the α-loop. This α-loop sits to one side of the DNA and is proposed to interact directly with RNA polymerase (RNAP). The α-loop can activate transcription through interaction with a variety of RNAP subunits including PhoB interaction with σ70 or OmpR interaction with α-CTD (79). The wing is the most C-terminal feature of the w-HTH domain. The wing inserts into the minor groove of the DNA, usually oriented towards the basal promoter element (3, 4). In addition to DNA binding, the wing can be involved in homodimerization of w-HTH domains (4).

DNA binding of w-HTH proteins is primarily mediated through the DNA-binding helix (α3) and the wing, although other regions of the protein also make contact with DNA. Multiple residues of the DNA-binding helix and wing of PhoB interact with the DNA backbone through hydrogen bonding, salt bridges, and Van der Waals interactions (3, 4). Additionally, residues in the α1 and α2 helixes and the α-loop interact with the DNA backbone, further stabilizing the

interaction between the protein and DNA (3, 4). Seven residues of the DNA-binding helix and five residues of the wing of PhoB make contact with the DNA. However, the majority of these contacts are with the backbone of DNA, and only 2-4 residues of the PhoB DNA binding helix and one to two residues of the wing interact directly with the bases (3, 4). Although the PhoB recognition sequence is 11 base pairs (bp), only three to four of these nucleotides are involved in base-specific contacts (3, 4), indicating that the positioning of the DNA-binding helix and the wing may provide additional sequence specificity. The wing of TcpP appears to be particularly critical for DNA binding, as mutations in several residues abolish DNA binding (Fig 1.2 D)(15, 16).

The dimerization of w-HTH proteins as well as the orientation of the wing relative to the DNA-binding helix results in a distinct DNA binding pattern. w-HTH proteins recognize binding sites consisting of two direct repeats of nucleotides oriented on the same face of the DNA helix. Between these conserved nucleotides is an A/T rich spacer that allows both recognition sites to sit on the same face of the DNA. For example, the TcpP-binding site consists of two TcpP-binding boxes with the consensus sequence TGTAA separated by an A/T rich 6 nucleotide spacer (82). This A/T richness provides flexibility to the DNA to allow for insertion of the wing into the minor groove. Binding by a w-HTH protein can induce a bend into the DNA due to the structural constraints of inserting the DNA-binding helix and the wing into the major and minor grooves (4). There are often multiple sets of these binding sites on the promoter, allowing the w-HTH transcription factors either work cooperatively or antagonistically. For example there are 3 pairs of Pho boxes, which are recognized by PhoB in *V. cholerae*. When PhoB binds to the most promoter proximal and promoter distal binding sites transcription is activated. Whereas, binding of PhoB to the middle site is inhibitory (83). On the *ompU* promoter, ToxR has multiple binding

sites from -238 to -24, again demonstrating the cooperative nature of w-HTH proteins binding to the promoter (28). Cooperative binding between two different w-HTH transcription factors is required to activate the *toxT* promoter. On the *toxT* promoter, TcpP binds to a promoter proximal region from -53 to -38 relative to the transcription start site (82) and ToxR binds further upstream (from -100 to -68) (16), but binding of both transcription factors is required for full transcriptional activation (16).

One important feature for activation of w-HTH proteins is dimerization. In w-HTH proteins with an N-terminal regulatory domain, such as OmpR and PhoB, the regulatory domain often initiates and regulates dimerization. In many cases, this domain may influence both whether dimerization occurs and the orientation of dimerization (84, 85). However, many w-HTH domains can still dimerize strongly when the regulatory domain is deleted (4, 81, 86). The w-HTH domain of OmpR can also bind DNA as a monomer, and will subsequently dimerize weakly on the promoter in different orientations depending on the orientation of the recognition sequences (87). When w-HTH proteins are analyzed bound to DNA, they are commonly found in a head-to-tail orientation with the C-terminal wing of the promoter distal w-HTH domain interacting with the N-terminal β-sheet of the promoter proximal w-HTH. PhoB dimers crystallized on DNA are oriented in this head-to -tail fashion (4). Additionally, when orientation of OmpR on the promoter was analyzed by copper phenanthroline cleavage, OmpR appeared to be oriented in similar head-to-tail dimers on the promoter (88). However, crosslinking studies indicate that w-HTH proteins can also form functional head-to-head dimers with the dimerization interface along the N-terminal β-sheets. Crosslinking between several residues in the N-terminal β-sheet of OmpR was enhanced in the presence of DNA, and the resulting dimer was able to activate transcription indicating that this head-to-head orientation is functional (81). In

Mycobacterium tuberculosis, PhoP has its greatest DNA-binding affinity when the proteins are dimerized such that the N-terminal β-sheets of the w-HTH domain interact in a head-to-head mechanism despite binding to a direct repeat (86). It is also possible for w-HTH proteins to dimerize through their C-terminal wings orienting the dimeric w-HTH proteins tail-to-tail, although this mechanism appears to be less common. HSF, from the yeast *Kluyveromyces lactis*, appears to function in a novel mechanism in which the C-terminal wing does not participate in DNA binding, but instead is involved in dimerization in a tail-to-tail orientation (89). Dimerization, although critical for activity of many w-HTH proteins, may be dependent on a variety of factors including, but not limited to, binding site orientation, mediation by regulatory domains, and the structure of the w-HTH domain. Interaction of ToxR and TcpP as both heterodimers and homodimers has been observed and is likely important for activity (15, 90), but the orientation of these dimers and the interaction faces involved is still unknown.

Neither ToxR nor TcpP have the N-terminal regulatory domain, which can regulate dimerization, but they still interact (15). Evidence that ToxR acts as a dimer comes from the demonstration that ToxR can dimerize the N-terminal DNA-binding domain of λ-repressor (90). The periplasmic domain, in conjunction with ToxS enhances dimerization of the w-HTH domain (45). ToxR activity and dimerization is maintained when the periplasmic C-terminus is replaced with a dimeric protein such as PhoA (77, 91). In some systems replacement of the periplasmic domain with a monomeric protein (MalE or Bla) does not reduce activity or dimerization of ToxR (77, 78). Based on this evidence it is believed that ToxR operates as a dimer and the periplasmic domain enhances, but is not required for, dimerization. Dimerization of ToxR is so robust that it can be used to test dimerization of other proteins (92). ToxR and TcpP also have been shown to interact by crosslinking (15) indicating that ToxR and TcpP form either

heterodimers or oligomers. Residues in the wing of TcpP are particularly critical for ToxR-TcpP interaction (15) indicating that ToxR and TcpP likely interact either in a head-to-tail orientation (like PhoB) or a tail-to-tail orientation (like HSF).

Models of transcriptional activation of *toxT*:

Although, both ToxR and TcpP can function as direct activators of transcription, ToxR is believed to play a co-activation role on the *toxT* promoter, assisting in *toxT* transcriptional activation by TcpP. The location of the TcpP binding site at -53 to -38 indicates it is likely the transcription factor that interacts with RNAP and the direct activator of *toxT* transcription (16, 82). Additionally, when TcpP is overexpressed from a plasmid, it is able to activate transcription of the *toxT* promoter in the absence of ToxR (6, 15). ToxR alone is unable to activate expression of *toxT*, even when overexpressed, despite being able to bind to the promoter in the absence of TcpP (5, 16). Because TcpP binds weakly to the *toxT* promoter, it has been hypothesized that ToxR acts as a co-activator by enhancing TcpP recruitment to the promoter via ToxR-TcpP protein-protein interaction.

One model for ToxR co-activation of *toxT* is the "hand-holding" model (Fig 1-3A). In this model ToxR and TcpP interact on the promoter, stabilizing the complex on the DNA. The main evidence for this model is that ToxR and TcpP interact, and that this interaction can compensate for TcpP DNA binding defects. Specifically, a TcpP mutant, TcpP-H93L, in the wing of TcpP, results in no significant defect in ToxR-TcpP interaction, but a severe defect in DNA binding (15, 16). Over-expressed TcpP-H93L is no longer able to activate *toxT* transcription in the absence of ToxR, However, the presence of ToxR rescues this mutant, returning *toxT* activation to around 60% of wild-type (16). Several other mutations in the wing of

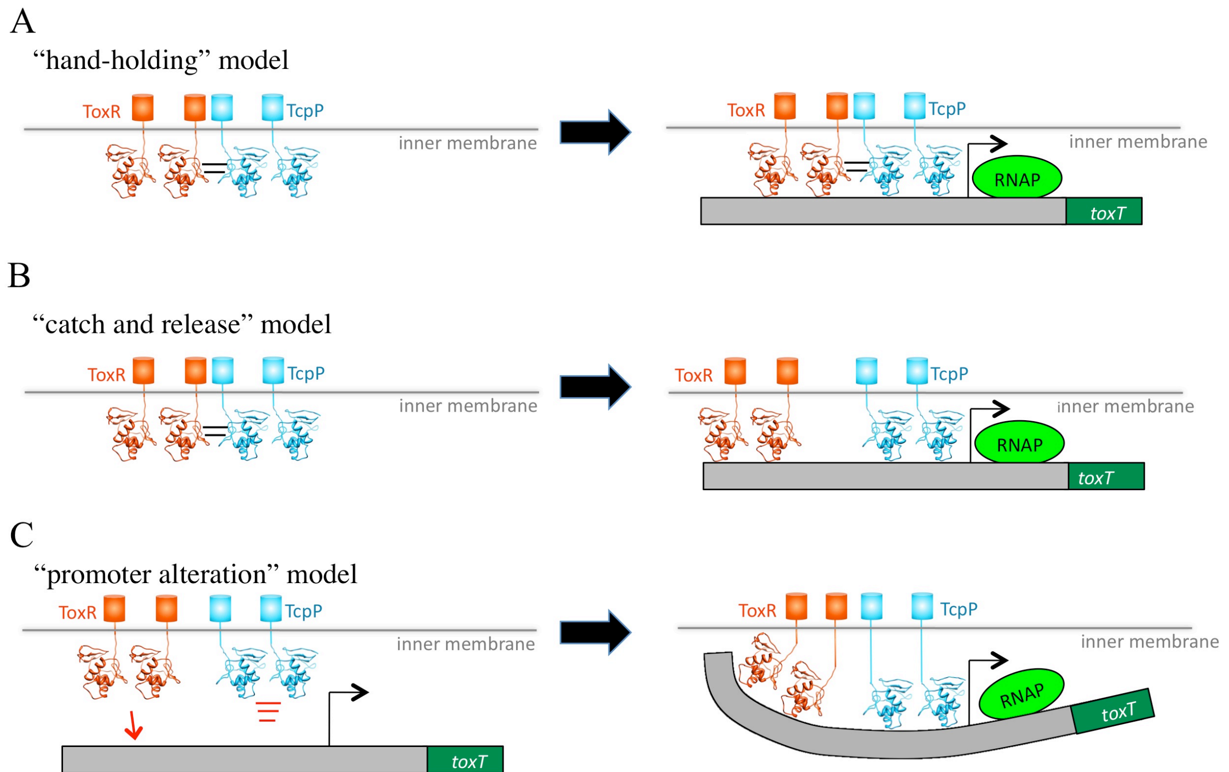

Fig 1.3. Models of ToxR co-activation of *toxT*. A) "Hand-holding model" in which ToxR-TcpP interaction holds TcpP in position on the *toxT* promoter allowing for transcriptional activation. B) "Catch and release model" in which ToxR-TcpP interaction recruits TcpP to the promoter, but upon DNA binding by ToxR, TcpP is released, allowing ToxR and TcpP bind to the promoter independently and at a distance from eachother. C) "Promoter modification model" in which ToxR enhances TcpP binding to the *toxT* promoter through relieving H-NS repression, bending the DNA to make the TcpP binding site more accessible, and bringing the *toxT* promoter to the membrane. In this model transcriptional activation of *toxT* is independent of ToxR-TcpP interaction.

TcpP resulted in a defect in both DNA binding and ToxR-TcpP interaction. None of these mutants were able to activate *toxT* transcription, even in the presence of ToxR, indicating a role for ToxR-TcpP interaction in rescuing TcpP DNA-binding mutants (15). If ToxR-TcpP interaction is critical for ToxR co-activation of *toxT*, a system can be envisioned where ToxR binds strongly to the *toxT* promoter, and due to the ToxR-TcpP interaction, ToxR brings TcpP to the promoter as well. An additional non-canonical ToxR-binding site is found between the ToxR and TcpP binding sites, indicating a possible ToxR-binding site directly adjacent to the TcpP binding site (16). Due to the strong interactions between ToxR and the promoter and ToxR and TcpP, TcpP is held in place on the promoter allowing for transcriptional activation.

A second model for ToxR dependent co-activation of the *toxT* promoter is the "catch and release" model (Fig 1-3B). This model, like the previous model, is dependent on ToxR-TcpP interaction to recruit TcpP to the promoter. However, in this model the ToxR-TcpP interaction is released upon DNA binding allowing ToxR and TcpP to bind to the promoter independently. One of the rationales behind this model is the potential involvement of the wing of ToxR and TcpP both in protein-protein interaction and DNA binding (15, 16, 45). Several residues in the wing of TcpP have been shown to be critical for both ToxR-TcpP interaction and DNA binding (15, 16), possibly indicating that ToxR-TcpP interaction and DNA binding do not occur simultaneously. Additionally, this model allows for more flexibility in the spacing of the ToxR and TcpP binding sites on the promoter since DNA footprinting analysis indicates that there is a space between the ToxR and TcpP binding sites (16). The "catch and release" model therefore combines the ability of ToxR-TcpP interaction to compensate for TcpP binding defects with the idea that the wing plays dual roles in DNA binding and transcriptional activation and the possibility that ToxR and TcpP are not directly adjacent to each other on the *toxT* promoter.

The third model for ToxR dependent co-activation is the "promoter alteration" model (Fig 1.3 C), which encompasses several mechanisms. In this model ToxR-TcpP interaction is not required for *toxT* activation; instead, the role of ToxR is to enhance TcpP binding to the promoter through changes in the promoter architecture. One way that ToxR may enhance TcpP binding is by relieving H-NS repression. H-NS binds to the *toxT* promoter and represses transcriptional activation (56, 63). In the absence of H-NS, endogenous TcpP is able to enhance transcriptional activation of *toxT*, even in the absence of ToxR (56). However, the presence of ToxR in the Δ*hns* strain further increases *toxT* expression, indicating that ToxR likely aids in activation in additional ways. A second way that ToxR could enhance activation is by bringing the promoter to the plasma membrane. ToxR membrane localization is required for transcriptional co-activation of *toxT*, but not for direct activation of *ompU* (75). One explanation for this it is that ToxR binds to the promoter, bringing it to the membrane, where TcpP is then able to bind. A third mechanism of ToxR-mediated promoter alteration that facilitates *toxT* activation is that when ToxR binds to the *toxT* promoter, it bends the DNA, making the TcpP binding site more accessible. w-HTH proteins often bend the promoter upon binding (4, 93). Furthermore, based on footprinting analysis, upon ToxR binding to the *toxT* promoter, a hypersensitivity site is exposed at the TcpP-binding site (16, 82). This hypersensitivity site indicates that the TcpP-binding site is more accessible when ToxR is bound to the promoter, presumably due to DNA bending. Together, these findings indicate that ToxR may prepare the *toxT* promoter in multiple ways, thereby enhancing TcpP-mediated activation of *toxT* transcription.

The main goal of my research is to expand our understanding of how *toxT* expression is co-activated by ToxR and TcpP. By testing these models, we can determine what the role of

protein-protein interactions has on *toxT* transcriptional activation and use this information to determine which of the 3 models best describes the system. It is also possible that ToxR co-activation of *toxT* is achieved by a combination of the “promoter alteration” model and either the “hand-holding” or the “catch and release” models. To address these models I will investigate the location of ToxR binding sites on the *toxT* promoter in order to better understand how ToxR binds to the promoter relative to TcpP. Additionally, I will examine critical residues in ToxR in order to better define the regions of ToxR required for direct activation (as with *ompU*) vs. co-activation (as with *toxT*) and how these residues affect DNA binding, transcriptional activation, and protein-protein interactions. As well as helping to understand how virulence factor production is induced in *V. cholerae*, this research may also provide insight into how w-HTH proteins cooperate to activate transcription in a variety of systems.

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Chapter 2

ToxR recognizes a direct repeat element in the *toxT*, *ompU, ompT* and *ctxA* promoters of *Vibrio cholerae* to regulate transcription

The majority of this work (except Fig. 2-6) was previously published as: Thomas J. Goss*, Sarah J. Morgan*, Emily L. French and Eric S. Krukonis. ToxR recognizes a direct repeat element in the *toxT*, *ompU, ompT* and *ctxA* promoters of *Vibrio cholerae* to regulate transcription. Infection and Immunity. March 2013. p884-895 * These authors contributed equally to this work and are considered co-first authors.

Note: With the assistance of Emily French I replicated the majority of the β-galactosidase assays in Figure 2-2 and 2-3. Additionally, I created all of the *ompU, ctxA, and ompT* promoter mutants, and with the assistance of Emily French and Eric Krukonis performed all of the β-galactosidase assays on these promoters (Fig. 2-7, 2-8, and 2-9). I also performed all of the mobility shift assays shown on the *toxT* promoter truncations (Fig. 2-5) and all of the *ompU, ctxA,* and *ompT* promoter mutants (Fig. 2-7, 2-8, and 2-9). I created and tested the *toxT* promoter spacing mutants (Fig. 2-6).

Summary

ToxR facilitates TcpP-mediated activation of the *toxT* promoter in *Vibrio cholerae,* initiating a regulatory cascade that culminates in cholera toxin secretion and toxin co-regulated pilus expression. ToxR binds a region from -104 to -68 of the *toxT* promoter, from which ToxR recruits TcpP to the TcpP-binding site from -53 to -38. To precisely define the ToxR-binding site within the *toxT* promoter, promoter derivatives with single base pair transversions spanning the ToxR-footprinted region were tested for transcription activation and DNA binding. Nine transversions between -96 to -83, reduced *toxT* promoter activity three-fold or greater, and all nine reduced the relative affinity of the *toxT* promoter for ToxR at least two-fold, indicating activation defects were largely due to reduced binding of ToxR to the *toxT* promoter. Nucleotides important for ToxR-dependent *toxT* activation revealed a consensus sequence of TNAAA-N_5-TNAAA extending from -96 to -83, also present in other ToxR-regulated promoters. When these consensus nucleotides were mutated in the *ompU*, *ompT* or *ctxA* promoters, ToxR-mediated regulation was disrupted. Thus, we have defined the core ToxR-binding site present in numerous ToxR-dependent promoters and we have precisely mapped the binding site for ToxR to a position three helical turns upstream of TcpP in the *toxT* promoter.

Introduction

The gastrointestinal disease cholera is primarily due to the secretion of cholera toxin (CT) by ingested *Vibrio cholerae* and is facilitated by TCP (the toxin co-regulated pilus)(10). The expression of CT and TCP, encoded by the *ctx and tcp* operons, are both positively and negatively regulated at the transcriptional level. Positive regulation of *ctx* and *tcp* requires ToxT (9, 11), the expression of which is initiated by the combined actions of ToxR and TcpP at the

toxT promoter (3, 4, 12, 13). While *toxR* expression is generally considered to be constitutive, *tcpP* expression is regulated by AphA, AphB, CRP and HapR according to environmental conditions (15-20). Moreover, TcpP is degraded under non-inducing conditions (23, 24). Thus, positive regulation of the transcription cascade culminating in CT secretion and TCP production is mediated by the sensing and integration of environmental signals by AphA, AphB and CRP at the *tcpPH* promoter and possible additional signals sensed by ToxR/ToxS and TcpP/TcpH. Furthermore, activity of the downstream regulator, ToxT, responds to the presence of bile and bicarbonate (25, 26) and ToxT itself is degraded in order to shut down virulence gene expression under non-inducing conditions (27). Negative regulation of CT and TCP expression is mediated by H-NS, which binds and represses the activities of the *ctxAB, tcpA* and *toxT* promoters (2, 28), and by the CRP-cAMP complex, which plays a role in HapR activation. HapR in turn represses *aphA* and *tcpPH* expression (18-20, 29).

TcpP and ToxR are inner membrane proteins with C-terminal periplasmic domains lacking homology to other proteins and N-terminal cytoplasmic domains with strong homology to the OmpR/PhoB family of winged helix-turn-helix transcriptional activators (31). The DNA-binding domains of OmpR/PhoB family proteins generally interact as dimers with direct repeat DNA sequences (32, 33), suggesting these domains dimerize in a head to tail configuration. We have recently shown TcpP also binds an RNA polymerase-proximal direct repeat element from -53 to -38 on the *toxT* promoter (8). However, the specific ToxR-binding site is undefined.

toxT expression requires that membrane-localized ToxR be co-expressed with TcpP (4, 12, 13, 21, 36), and we hypothesize that ToxR recruits TcpP to what appears to be a weak TcpP-binding site (relative to ToxR binding affinity, (4, 37)). Once recruited to the *toxT* promoter, TcpP activates *tox*T transcription (7). The ability of ToxR to facilitate TcpP-mediated *toxT*

activation requires that ToxR bind a poorly defined DNA-binding site containing sequences from an inverted repeat element that lies upstream of the TcpP-binding site (Fig. 2-1A, (3, 4)). ToxR-dependent recruitment of TcpP to the promoter may increase the local concentration of TcpP, facilitating TcpP binding to its weak binding site. This could occur while maintaining a ToxR/TcpP interaction, or ToxR may release TcpP upon DNA binding to allow TcpP to bind its adjacent binding site. Finally, it is possible that although ToxR and TcpP can establish a protein-protein interaction (7, 37), the main role of ToxR is to simply recruit the *toxT* promoter to the membrane where membrane-localized TcpP has easier access to its *toxT* promoter-binding site.

Although there are a large number of genes comprising the ToxR regulon (39), only a select few are known to be directly regulated by ToxR. In addition to facilitating the TcpP-dependent activation of the *toxT* promoter, ToxR directly activates the *ompU* promoter and represses the *ompT* promoter (35, 38). Furthermore, when overexpressed, ToxR can directly activate the *ctxA* promoter (40)), although under physiological conditions the *ctxA* promoter is activated by ToxT (6, 9, 11, 28, 41). The binding sites for ToxR at the *ompU, ompT, ctxA* and *toxT* promoters have been defined by DNAse I footprinting (4, 35, 38), however comparisons of these footprinted regions has not identified a clear consensus ToxR-binding sequence found at all ToxR-footprinted promoters.

At the *toxT* promoter, the ToxR footprint spans the region from -104 to -68, partially overlapping an inverted repeat sequence (Fig. 2-1A, black arrows, (3) (4)). Plasmid-born *lacZ* fusion and mobility shift studies using *toxT* promoter deletion derivatives indicate that at least some sequences important for ToxR-binding and ToxR-dependent promoter activation lie between –114 and –73 (3). Subsequently, a screen for *toxT* promoter mutants defective in ToxR-dependent activation identified single nucleotide substitutions in the *toxT* promoter at positions

–86 and –84 in the upstream half of the inverted repeat sequence that reduced both ToxR-dependent promoter activation and the affinity of the *toxT* promoter for ToxR (3). Moreover, substitutions at positions -67 and -65 within the downstream half of the inverted repeat that are complementary to the -86 and -84 substitutions in the upstream half of the inverted repeat had little affect on ToxR-dependent fusion activity (3). These results suggest that the inverted repeat sequence does not represent a symmetrical binding site for ToxR at the *toxT* promoter. Thus, some other sequence motif containing nucleotides -86 and -84 is likely to strongly influence ToxR binding or ToxR-dependent recruitment of TcpP to the *toxT* promoter.

In this report, systematic transversion mutagenesis of the ToxR-footprinted region of the *toxT* promoter was used to identify nucleotides that were critical for promoter activation. These studies defined the sequence TNAAA-N_5-TNAAA from -96 to -83 as the ToxR-binding site in the *toxT* promoter. Transversions altering these critical nucleotides reduced the affinity of the promoter for ToxR and defined a minimal region of the *toxT* promoter that was essential for ToxR-dependent *toxT* activation. Furthermore, mutation of this repeat element in the *ompU*, *ompT* and *ctxA* promoters resulted in loss of ToxR-responsiveness by those promoters as well.

Results

Specific mutations in the ToxR-binding region of *toxT* disrupt ToxR-dependent promoter activation. The ToxR-binding site within the *toxT* promoter has been defined previously by DNAse I footprinting analysis as extending from -104 to -68 (4), however the specific nucleotides within the ToxR-protected region important for *toxT* activation have not been systematically determined. To identify these nucleotides, a collection of *toxT* promoter derivatives with transversions at each base pair in the region from -100 to -57 were constructed

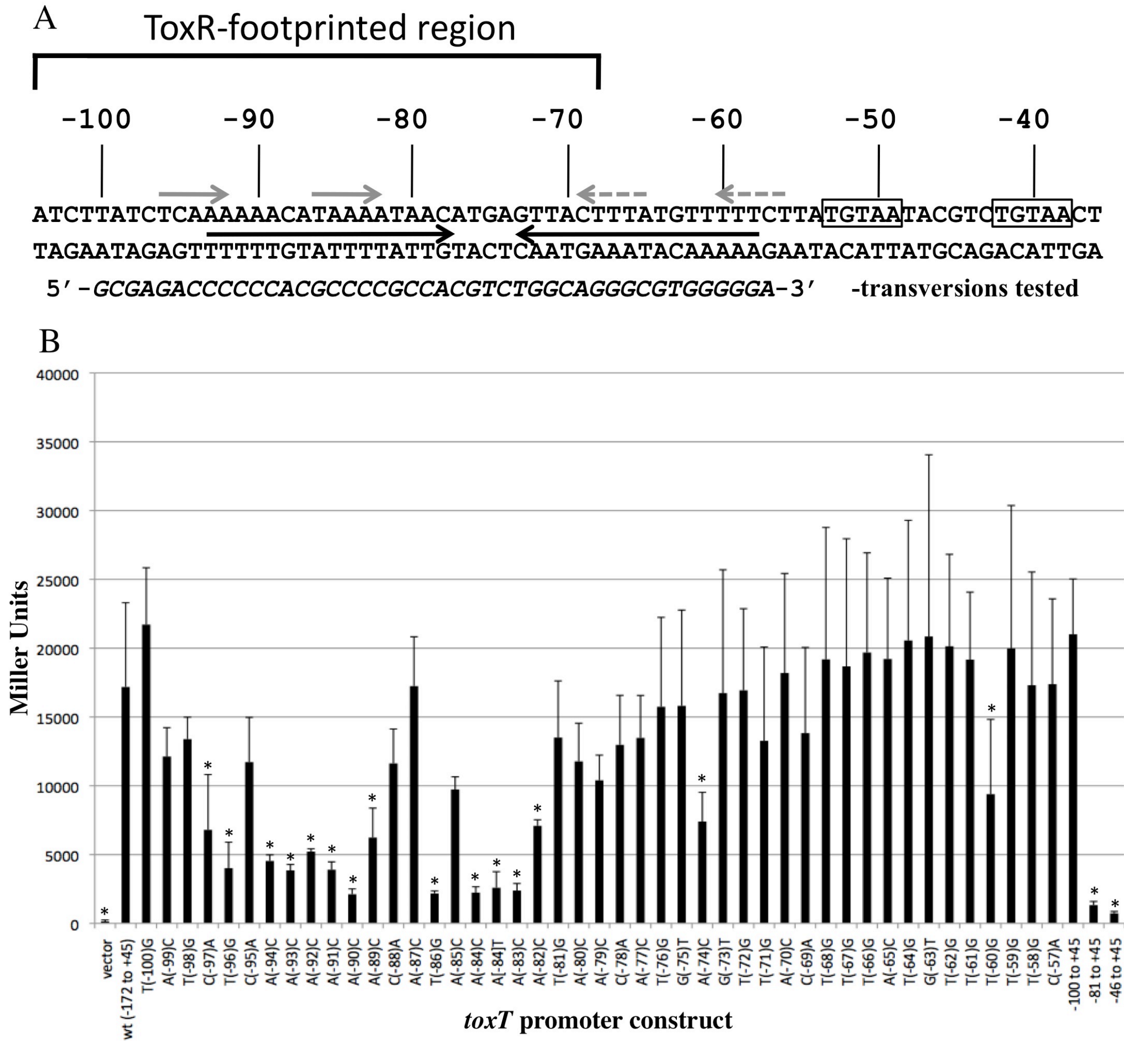


Figure 2-1. DNA sequence of the *V. cholerae* classical strain O395 promoter-proximal region of the *toxT* promoter and ToxR-dependent activation of single base pair substitutions. (A) Nucleotides are numbered relative to the *toxT* transcription start site (3). The region of ToxR-dependent DNAse I protection is indicated above the DNA sequence (4). The solid gray arrows above the sequence indicated the position of the 5’-TNAAA-N_5-TNAAA-3’ direct repeat motif important for ToxR binding. An inverted repeat sequence (3, 5) is indicated by the black convergent arrows between -93 and -58. A promoter-proximal degenerate ToxR-binding site is indicated by dashed gray arrows from -69 to -56. The boxed nucleotides indicate the pentameric direct repeat motif recognized by TcpP (8). Single nucleotide substitutions generated within the *toxT* promoter region from –100 to -57 are indicated on the bottom line in *italics*. (B) Effects of ToxR-binding site mutations on *toxT-lacZ* activity in wild-type *V. cholerae* strain O395. Strains carrying a plasmid-born wild type *toxT-lacZ* fusion (-172 to +45), single base pair substitution *toxT* promoter mutants, promoter deletion derivatives, or empty vector (promoterless *lacZ* vector, pTG24) were assessed for β-galactosidase activity. The positions of substitutions and endpoints are indicated relative to the *toxT* transcription start site. Error bars represent the standard deviation for each data set. * $p < 0.005$ as assessed using the students' t-test, n=6 or more measurements.

(Fig. 2-1A). Transversions were generated using the *toxT* promoter region from -172 to +45 fused to a promoterless *lacZ* reporter gene (8, 34). In O395 (*V. cholerae* classical strain), 13 transversions reduced *toxT* promoter activity greater than two-fold, and 12 of 13 mutations affect nucleotides in the region from -97 to -82 (Fig. 2-1B). Likewise, a previously identified A[–84]T substitution (42) also dramatically reduced fusion activity (Fig. 2-1B). Of the transversions in the region from –81 to –57, only that at A(-74)C reduced the activity of the fusion greater than twofold (Fig. 2-1B). Thus, nucleotides important for *toxT-lacZ* fusion activity were clustered within the promoter-distal portion of the ToxR-footprinted region while nucleotides in the promoter-proximal portion of the footprint contribute little to promoter activity.

Transversions that reduced *toxT* promoter activity most dramatically identified the nucleotide sequence 5'-CTNAAAAAANNTNAAA-3' (-97 to -82) as critical for ToxR-dependent *toxT* activation. Within this sequence is a direct repeat motif of (5'-TNAAA-N_5-TNAAA-3') composed of two half-sites that are centered one turn of the DNA helix apart. These features are consistent with the notion that two ToxR monomers bind in a head to tail configuration to two 5'-TNAAA-3' half-sites. Thus, the motif 5'-TNAAA-N_5-TNAAA- may represent a minimally defined ToxR-binding site.

***toxT* promoter transversion mutations do not affect ToxR-independent *toxT* activation by overexpressed TcpP.** To rule out the possibility that transversion-dependent changes in *toxT* expression are due to defects in TcpP interaction with the *toxT* promoter, the wild type *toxT-lacZ* fusion and mutant derivatives in the region from -100 to -80 were moved into an O395 Δ*toxR*Δ*tcpP*/pEK41 background (EK459/pEK41) to assess the effects on ToxR-independent *toxT* activation in response to TcpP overexpression (pEK41 encodes an HSV epitope-tagged version

of TcpP in vector pMMB207, (4)). Previous studies have shown overexpressed TcpP can efficiently activate the *toxT* promoter, even in the absence of ToxR (4, 12).

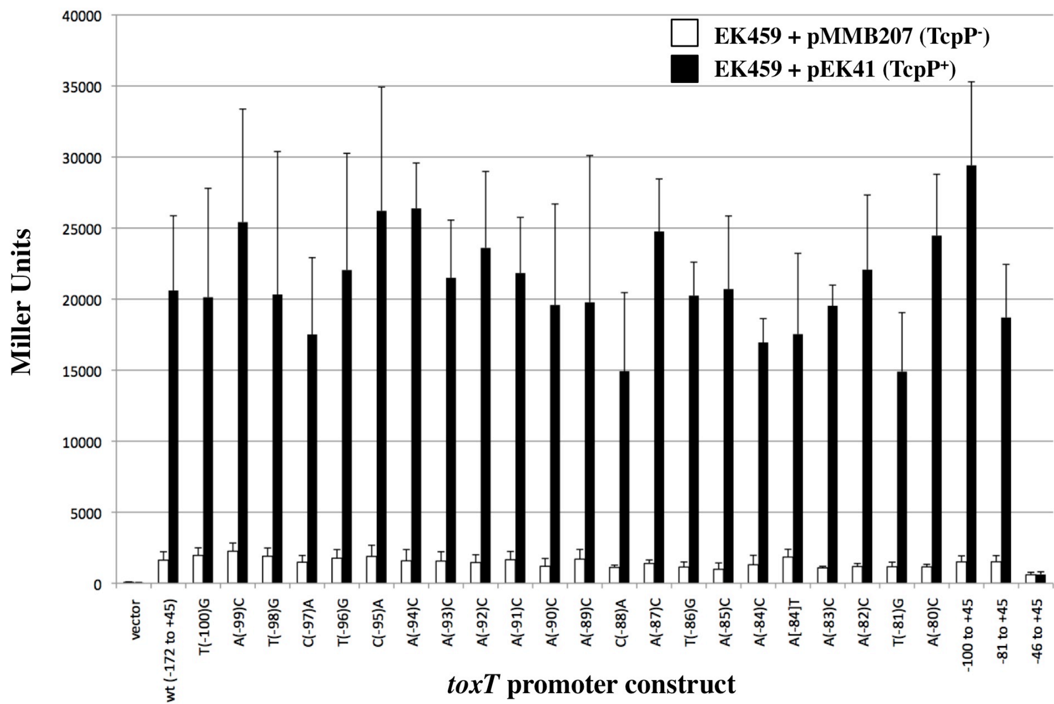
In the EK459/pEK41 background, all 22 transversion mutants tested had less than a 30% decrease in TcpP-mediated *toxT* activation (Fig. 2-2A, black bars). More importantly, none of the tranversions in the TNAAA-N_5-TNAAA putative ToxR-binding site had more than a 20% decrease in *toxT* activation (Fig. 2-2A). Thus, the effects of these promoter mutations on *toxT* activation are most likely due to defects in ToxR-dependent *toxT* activation.

Consistent with the interpretation that the TNAAA-N_5-TNAAA direct repeat element responds to ToxR, introduction of the *toxT* promoter mutants into a wild type O395 strain ($ToxR^+$) overexpressing TcpP (+pEK41) results in a strain with 50% higher levels of β-galactosidase expression (~30,000 Miller units, Fig. 2-2B), but this level drops to the level of activation mediated by overexpressed TcpP alone, when mutations in the TNAAA-N_5-TNAAA repeat element are encountered (Fig. 2-2B). Thus, the maximal level of *toxT* activation afforded by ToxR and overexpressed TcpP are not achieved when the ToxR-binding site is mutated.

In an EK459/pMMB207 background (O395 Δ*toxR*Δ*tcpP* + empty vector), the transversions did not dramatically alter the basal activity of the *toxT-lacZ* fusion (Fig. 2-2A, white bars).

Mutations in the putative ToxR binding-site of *toxT* disrupt ToxR/*toxT* interactions. To determine whether mutations in the TNAAA-N_5-TNAAA putative ToxR-binding site disrupt ToxR binding to the *toxT* promoter, electrophoretic mobility shift assays were performed. ^{32}P-labeled *toxT* promoter targets were mixed with increasing concentrations of ToxR-containing *V. cholerae* membranes or negative control membranes lacking ToxR.

A



B

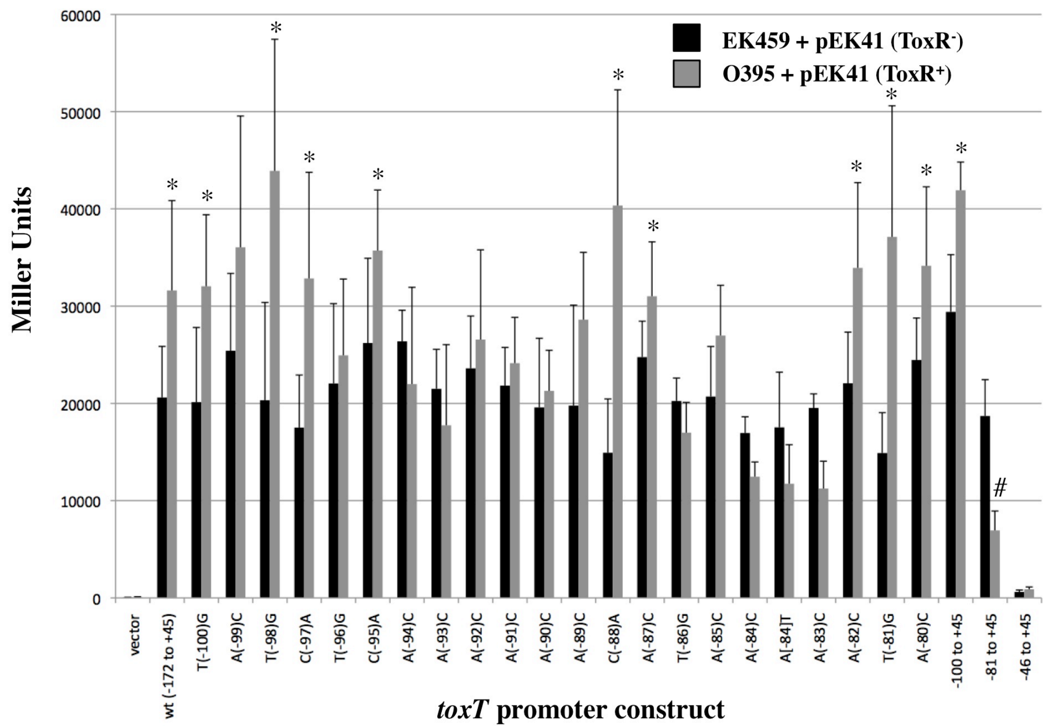


Figure 2-2. ToxR-binding site mutations do not affect *toxT* activation by overexpressed TcpP. (A) *toxT-lacZ* fusions with *toxT* promoter transversions from -100 to -80 were tested in a strain lacking ToxR (EK459 = O395 Δ*toxR*Δ*tcpP*), but overexpressing TcpP from plasmid pEK41. Strains were grown for 4 hrs at 30° pH=6.5 in the presence of 1 mM IPTG and β-galactosidase activities were determined for strains carrying either a promoterless *lacZ* fusion vector (vector), or its derivatives carrying either the wild type *toxT-lacZ* fusion (wt) or single base pair substitutions. Black bars represent a Δ*toxR*Δ*tcpP* background carrying the TcpP overexpression plasmid, pEK41. White bars represent a Δ*toxR*Δ*tcpP* background carrying the empty vector expression plasmid, pMMB207. (B) Enhanced activation by co-expression of ToxR and overexpressed TcpP is lost when mutations in the ToxR-binding site are present. *toxT-lacZ* activation was measured in the Δ*toxR*Δ*tcpP* strain EK459 harboring the TcpP-expressing vector pEK41 (black bars, same data as in Fig. 2-2A) or wild-type O395 (ToxR+) harboring pEK41 (gray bars). Error bars represent the standard deviation. * $p \leq 0.05$, $ToxR^+$ strains are significantly higher than $ToxR^-$ strains. # $p < 0.0001$, the $ToxR^+$ strain is significantly lower than the $ToxR^-$ strain. All assessed using the students' t-test. n=6 or more measurements.

In the presence of 0.77 mg/ml ToxR-containing membranes, approximately half of the input wild-type *toxT* promoter probe was shifted (Fig. 2-3, Panel D, lanes 1 and 25), while probes bearing mutations in the putative ToxR-binding site (TNAAA-N_5-TNAAA, from -96 to -83) were shifted with 10-30% efficiency (Fig. 2-3, Panel D, lanes 2 to 23). Thus, mutation of the putative ToxR-binding site led to a defect in ToxR binding, confirming the identity of the ToxR-binding site. Experiments with increasing concentrations of ToxR-containing membranes (Fig. 2-3, Panels B to H) were used to determine the concentration leading to an ~50% shift for each *toxT* promoter mutant probe (Table 2-1 and Fig. 2-3). *toxT* promoter probes bearing transversions in the ToxR-binding site, required two to five-fold more ToxR protein to reach 50% shifting (Table 2-1). Comparison of transversion-dependent effects on relative affinity and *toxT-lacZ* fusion activation indicates that reductions in relative affinity correlate well with reductions in promoter activation. The fact that the C(-95)A mutation at the N position of TNAAA consensus, had no significant defect in transcription (Fig. 2-1B) or ToxR binding (Table 2-1, Fig. 2-3), supports the conclusion that this nucleotide position is not recognized by ToxR.

Finally, further evidence that the TNAAA-N_5-TNAAA sequence from -96 to -82 represents the ToxR-binding site within the *toxT* promoter is that a double-stranded oligonucleotide from that region can compete with the full-length *toxT* promoter for ToxR-mediated gel shifting activity and mutations within the TNAAA-N_5-TNAAA consensus binding site within these oligonucleotides disrupts inhibition activity (data not shown).

In the presence of 4.4 mg/ml negative control membranes (lacking ToxR), less than 50% of the target promoters were shifted (Fig. 2-4), while a few targets shifted greater than 50% in the presence of 5.6 mg/ml negative control membranes (Fig. 2-3, panel I, Fig. 2-4), indicating that at

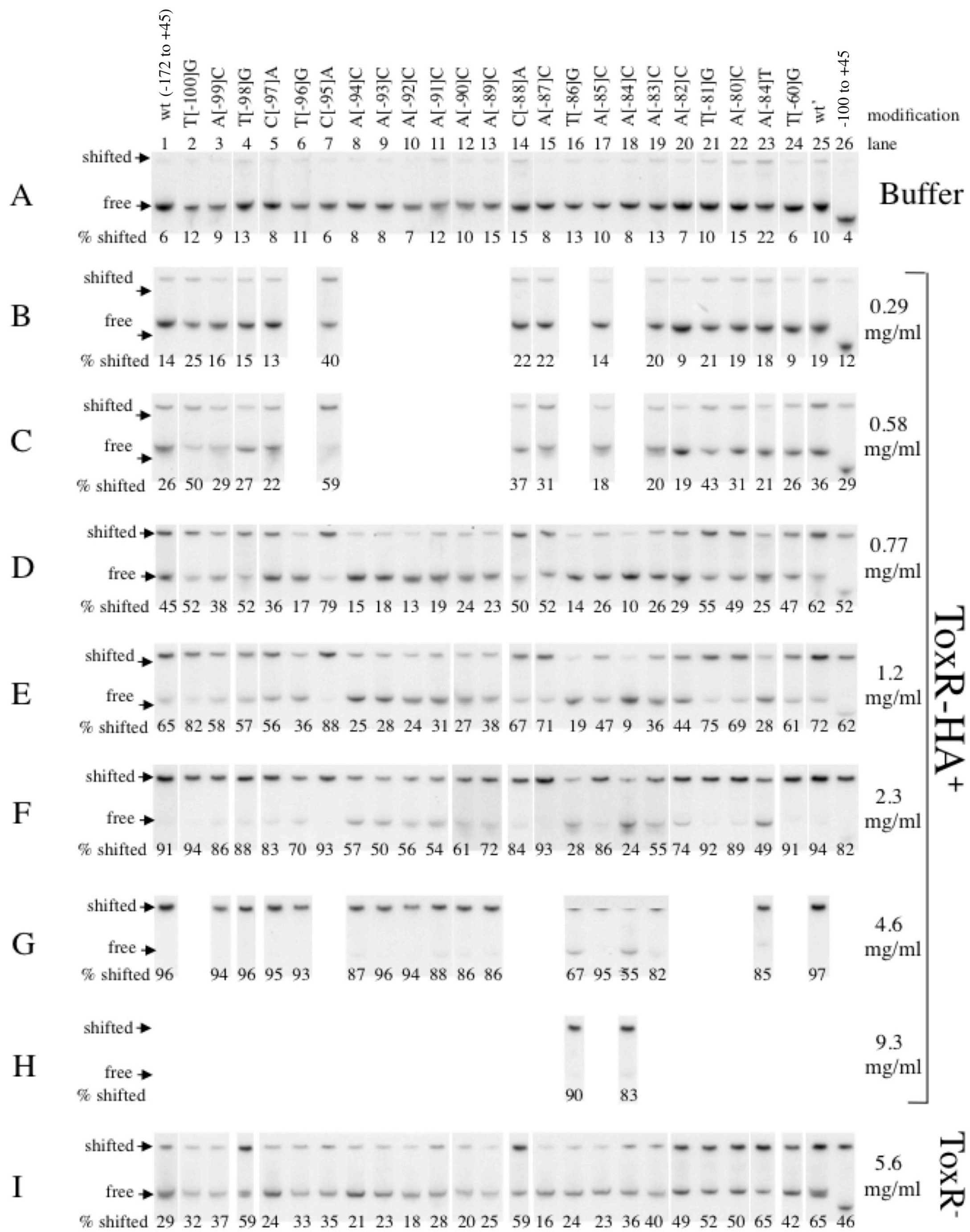

wt (-172 to +45)
T[-100]G
A[-99]C
T[-98]G
C[-97]A
T[-96]G
C[-95]A
A[-94]C
A[-93]C
A[-92]C
A[-91]C
A[-90]C
A[-89]C
C[-88]A
A[-87]C
T[-86]G
A[-85]C
A[-84]C
A[-83]C
A[-82]C
T[-81]G
A[-80]C
A[-84]T
T[-60]G
wt'
-100 to +45
modification
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26
lane
A
shifted
free
% shifted 6 12 9 13 8 11 6 8 8 7 12 10 15 15 8 13 10 8 13 7 10 15 22 6 10 4
Buffer
B
% shifted 14 25 16 15 13 40 22 22 14 20 9 21 19 18 9 19 12
0.29 mg/ml
C
% shifted 26 50 29 27 22 59 37 31 18 20 19 43 31 21 26 36 29
0.58 mg/ml
D
% shifted 45 52 38 52 36 17 79 15 18 13 19 24 23 50 52 14 26 10 26 29 55 49 25 47 62 52
0.77 mg/ml
E
% shifted 65 82 58 57 56 36 88 25 28 24 31 27 38 67 71 19 47 9 36 44 75 69 28 61 72 62
1.2 mg/ml
F
% shifted 91 94 86 88 83 70 93 57 50 56 54 61 72 84 93 28 86 24 55 74 92 89 49 91 94 82
2.3 mg/ml
G
% shifted 96 94 96 95 93 87 96 94 88 86 86 67 95 55 82 85 97
4.6 mg/ml
H
% shifted 90 83
9.3 mg/ml
ToxR-HA+
I
% shifted 29 32 37 59 24 33 35 21 23 18 28 20 25 59 16 24 23 36 40 49 52 50 65 42 65 46
5.6 mg/ml
ToxR-

Figure 2-3. Mutations in the putative ToxR-binding site disrupt ToxR binding to the toxT promoter. For toxT promoter electrophoretic mobility shift assays, the 32P-end-labeled toxT promoter targets used for most panels carry the wild type promoter (-172 to +45, lanes 1 and 25), single-transversion derivatives with mutations at positions -100 to -80 (lanes 2 to 22, respectively), -60 (lane 24), the A[–84]T substitution (lane 23) or the -100 to +45 deletion derivative (lane 26) are indicated at the top of the figure. For lanes 1 to 26, the DNA-binding solutions containing end-labeled DNA targets were mixed with membrane buffer only (Panel A), ToxR-containing membranes at either 0.29 mg/ml (Panel B), 0.58 mg/ml (Panel C), 0.77 mg/ml (Panel D), 1.2 mg/ml (Panel E), 2.3 mg/ml (Panel F), 4.6 mg/ml (Panel G), 9.3 mg/ml (Panel H) or the negative control ToxR-negative membrane preparation at 5.6 mg/ml (Panel I) as indicated on the right side of the figure. The positions of free and shifted end-labeled DNA target migration through the gel are indicated on the left side of the figure. DNA bound by membrane-localized ToxR is retained in the well of the gel. The % shifted values given below the free target band for each sample indicate the percentage of shifted signal, relative to the sum of free and shifted signals, as quantified by densitometry.

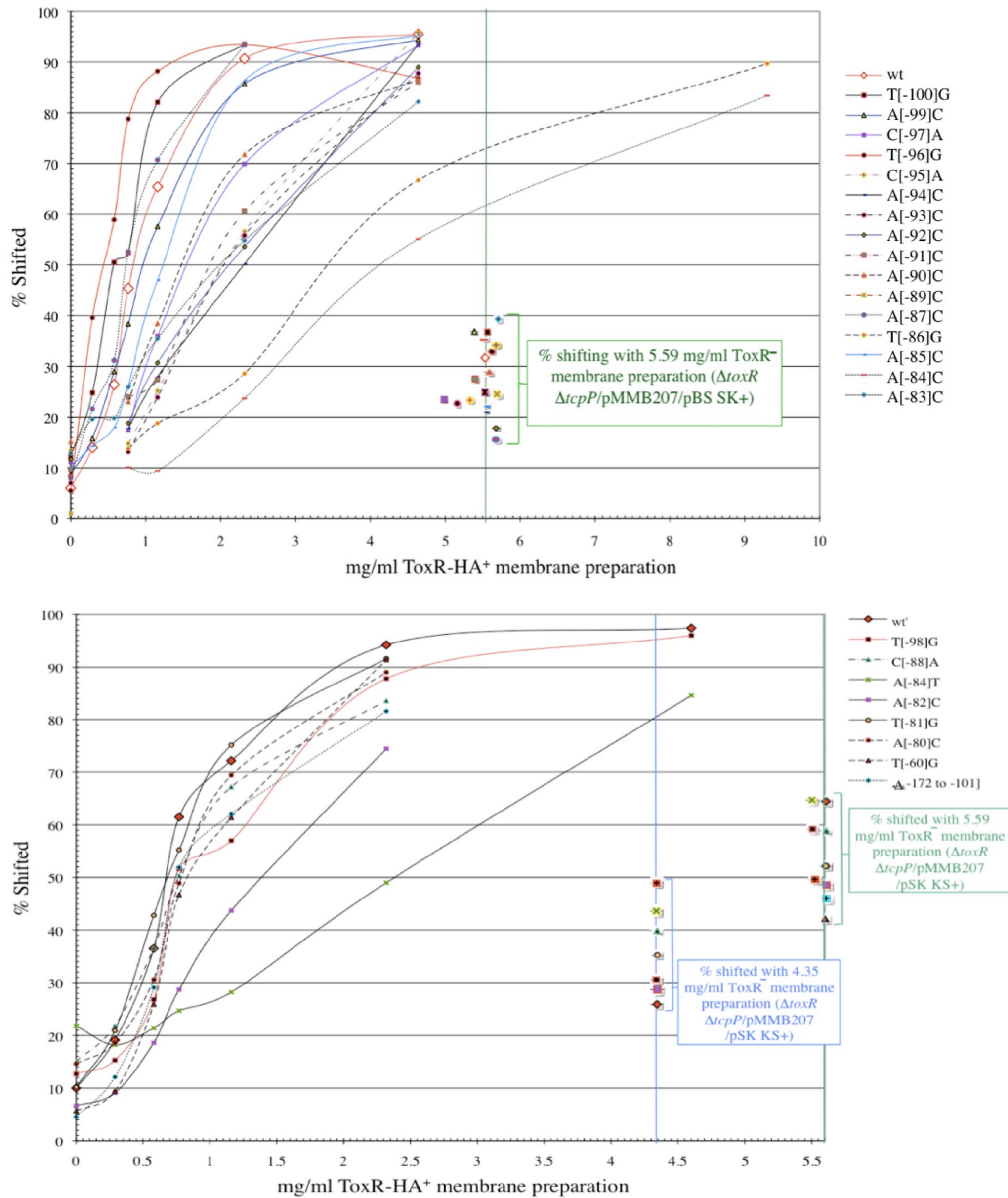


Figure 2-4: Assessment of 50% gel shifting activity of various toxT promoter mutants. The % of target shifted, as presented in Fig. 2-3, was plotted as a function of ToxR+ (TG129) membrane protein concentration. The binding curves were used to determine the concentrations of ToxR-containing membrane required for a 50% shift of each target. Data are split between two different binding curves for clarity, as having too many overlapping binding curves obscured the data.

Table 2-1. Relative affinities of *toxT* promoter mutants for membranes containing ($ToxR^+$) or lacking ($ToxR^-$) ToxR.

Target[a]	mg/ml protein required for a 50% shift		fold increase relative to wild-type
	$ToxR^+$	$ToxR^-$	
wild type (wt) (-172 to +45)	0.76	> 5.6	1.0
T[-100]G	0.57	> 5.6	0.8
A[-99]C	0.96	> 5.6	1.3
T[-98]G	0.74	> 4.2	1.0
C[-97]A	0.99	> 5.6	1.3
T[-96]G	**1.55**	**> 5.6**	***2.0***
C[–95]A	0.44	> 5.6	0.6
A[-94]C	**2.01**	**> 5.6**	***2.6***
A[-93]C	**2.33**	**> 5.6**	***3.1***
A[-92]C	**2.02**	**> 5.6**	***2.7***
A[-91]C	2.12	> 5.6	2.8
A[-90]C	1.83	> 5.6	2.4
A[-89]C	1.48	> 5.6	1.9
C[-88]A	0.77	> 4.2	1.0
A[-87]C	0.74	> 5.6	1.0
T[-86]G	**3.42**	**> 5.6**	***4.5***
A[-85]C	1.23	> 5.6	1.6
A[-84]C	**4.12**	**> 5.6**	***5.4***
A[-83]C	**1.98**	**> 5.6**	***2.6***
A[-82]C	**1.39**	**> 5.6**	***1.8***
T[-81]G	0.67	> 4.2	0.9
A[-80]C	0.74	> 4.2	1.0
T[-60]G	0.83	> 5.6	1.1
A[-84]T	***2.39***	***> 4.2***	***3.1***
toxT -100 to +45	0.74	> 5.6	1.0

[a] Numbers represent the position of the promoter mutation or the endpoints of deletions, relative to the *toxT* transcription start site. Italicized and bold nucleotides indicate the position of the ToxR-binding site direct repeat TNAAA-N_5-TNAAA (-96 to -82).

high membrane concentrations, one begins to detect increased background binding to toxT promoter probes.

The region from -82 to -68 of the *toxT* promoter, while containing a partially-conserved ToxR binding site, does not contribute to *toxT* activation. Now that we had identified the ToxR-binding site in the *toxT* promoter, we recognized that the *toxT* promoter also contains an imperfect ToxR-binding site (ANAAA-N_4-TNAAG) from -56 to -69 on the opposite strand from our recently defined ToxR-binding site (from -96 to -83). Thus, we sought to determine whether this imperfect ToxR binding site (Fig. 2-1A, dashed gray arrow) supported any detectable ToxR binding or ToxR-dependent *toxT* activation.

In O395, the activity of the wild type fusion was not altered by deletion of *toxT* promoter sequences upstream of -100 (Fig. 2-1B), indicating that the region from -172 to -101 does not significantly contribute to *toxT* promoter activation. In contrast, the deletions removing sequences upstream of -81 (or -47) reduced fusion activity by about ten-fold (Fig. 2-11B), indicating that the region from -100 to -82 strongly contributes to *toxT* promoter activity, as expected since this region contains the ToxR-binding site TNAAA-N_5-TNAAA. Previous studies by Higgins *et al* also demonstrated that while *toxT* promoter truncations lacking sequences from -172 to -114 maintained wild-type levels of activation, deleting the region from -114 to -73 resulted in a *toxT* promoter with just 10% activation (3). A *toxT* promoter fragment from -73 to +45 was also not bound by ToxR (3).

Since there is an imperfect ToxR-binding site from -69 to -56 of the *toxT* promoter, we assessed whether that region of the promoter has the potential for ToxR-dependent activation. As both our results with the -81 to +45 *toxT-lacZ* reporter construct and the -73 to +45 reporter

construct described previously (3) showed ToxR could not activate these promoter fragments, we hypothesized that the imperfect ToxR repeat from -69 to -56 of the *tox*T promoter may have a low affinity ToxR-binding site. Thus, we tested the ability of overexpressed ToxR to restore activation to the -81 to +45 *toxT-lacZ* reporter plasmid. Even overexpression of *toxR* (from pVJ21, (30)) was unable to restore activation to this promoter, as it showed β-galactosidase levels only slightly above O395 expressing the empty vector (Fig. 2-5A). This low level of ToxR responsiveness is similar to the negative control -46 to +45 reporter construct, which lacks the imperfect ToxR-binding site (Fig. 2-5A). Overexpressed ToxR in the Δ*toxR* strain EK307 was able to activate the full-length *toxT* promoter construct from -172 to +45 (Fig. 2-53A). Gel-shift analysis also indicated that ToxR is largely unable to bind this imperfect repeat element as a *toxT* promoter fragment from -81 to +45 showed nearly undetectable ToxR binding (Fig. 2-5B).

These data indicate ToxR binds the imperfect ToxR-binding site from -69 to -56 in the *toxT* promoter poorly and that this specific DNA sequence does not contribute to ToxR-dependent *toxT* activation. This conclusion is also supported by the fact that the ToxR-footprinted region of the *toxT* promoter only extends to -68(4).

Spacing of ToxR DNA binding site relative to TcpP DNA binding site is not critical for *toxT* activation. To further investigate the importance of positioning of ToxR relative to TcpP on the *toxT* promoter, constructs were made in which the ToxR binding site was moved either 2 helical-turns closer or 2 helical-turns further from the TcpP DNA binding site. Activation of both of these promoters with altered spacing still required both ToxR and TcpP (Fig. 2-6). Moving the ToxR binding site so that it is adjacent to the TcpP binding site or so that it is an additional two helical turns from the TcpP binding site resulted in around a 25% decrease in transcriptional

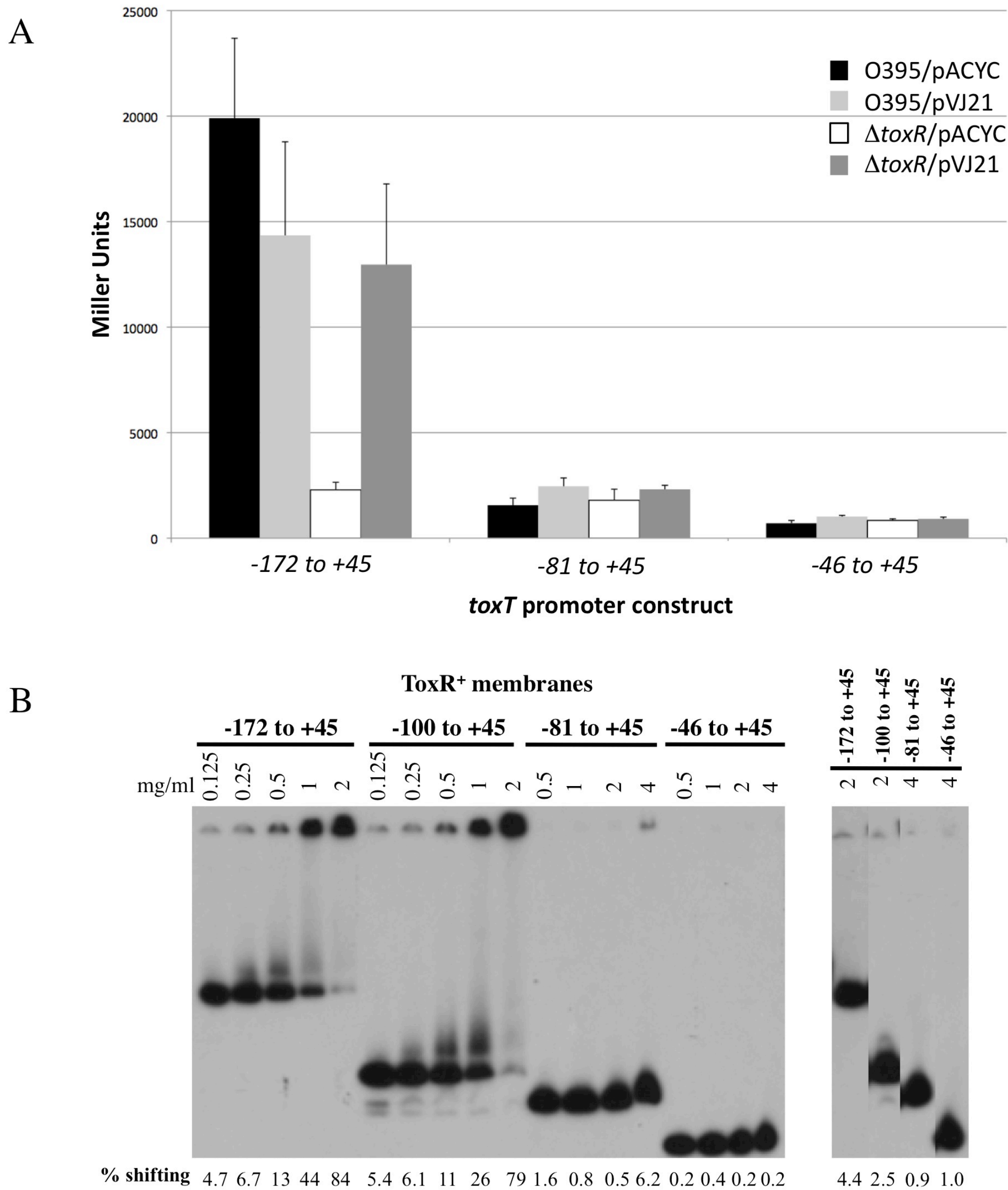


Figure 2-5. ToxR fails to bind or activate a *toxT-lacZ* derivative containing the degenerate ToxR-binding site from -69 to -56. A) *toxT* promoter derivatives driving *lacZ* expression were tested for activation in wild-type *V. cholerae* (O395) or the *toxR* mutant strain EK307 with or without overexpression of ToxRS from plasmid pVJ21. n=6 B) Electrophoretic mobility shift analysis of full length (-172 to +45), -100 to +45, -81 to +45 and -46 to +45 *toxT* derivatives with increasing concentrations of ToxR-containing membranes shows the degenerate ToxR-binding site from -69 to -56 has weak ToxR-binding capacity. Negative control gel shifting with membranes lacking ToxR (ToxR^{-}) were also tested and showed minimal background. DNA bound by membrane-localized ToxR is retained in the well of the gel. % shifting by membranes is indicated under each lane as determined by ImageJ.

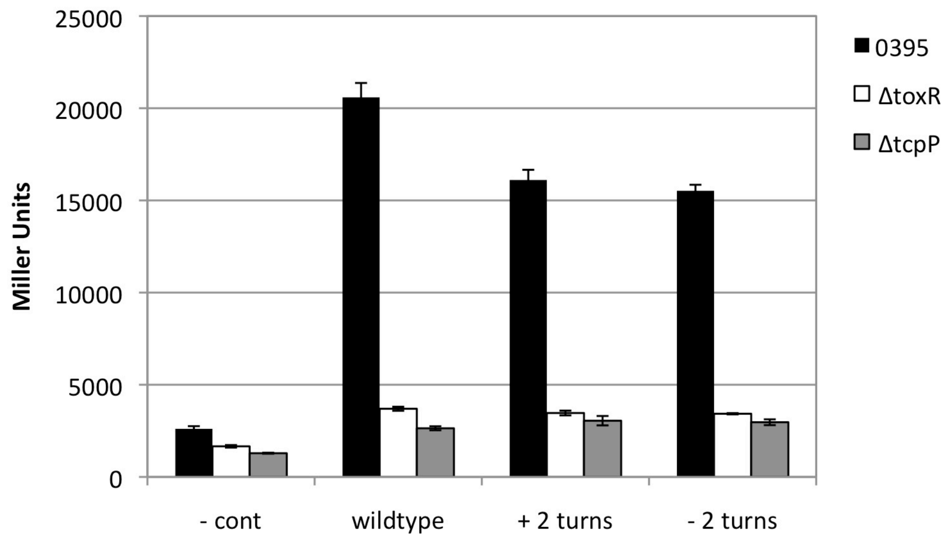


Figure 2-6. ToxR binding site location is not critical for transcriptional activation of *toxT*. The ToxR binding site was moved two helical-turns either upstream or downstream of the original binding site, placing it either adjacent to or 5 helical-turns away from the TcpP-binding site. Transcriptional activation, as measured by β-galactosidase assay only occurred in the *V. cholerae* strain expressing both ToxR and TcpP (O395)(black bars). Movement of the ToxR binding site resulted in a slight decrease in transcriptional activation. Deletion of either ToxR or TcpP (EK307 and RY1, respectively) resulted in background levels of activation (grey and white bars, respectively) for all promoters tested.

activation of the promoter (Fig 2-6). Moving the ToxR and TcpP binding sites so that they are adjacent, theoretically allowing for ToxR-TcpP interaction on the promoter, does not appear to enhance or dramatically reduce activation. Furthermore, deletion of the degenerate ToxR binding site from -69 to -56 by moving of the ToxR binding site adjacent to the TcpP binding site does not adversely affect activation. There is a slight, but significant defect in transcriptional activation of the *toxT* promoter when the spacing between the ToxR binding site and the TcpP binding site is altered, indicating that the positioning of these two transcription factors relative to each other contributes to, but is not critical for, transcriptional activation.

The newly identified ToxR-binding site in the *toxT* promoter is also required for ToxR-mediated activation of *ompU* and *ctxA* and repression of *ompT*. In addition to facilitating TcpP-mediated activation of the *toxT* promoter, ToxR can directly activate the *ompU* promoter and repress the *ompT* promoter (35, 38). Furthermore, while *ctxA* activation is usually accomplished by ToxT (11, 28, 41), when ToxR is expressed at high levels it can directly activate the *ctxA* promoter (9, 40). Thus, we examined the promoter sequences of the *ompU*, *ompT* and *ctxA* genes for elements similar to the TNAAA-N_5-TNAAA sequence identified in the *toxT* promoter. In the *ompU* promoter, we identified a similar sequence, 5' TNAAA-N_5-TNAAT 3', located from -51 to -37 relative to the transcription start site (on the opposite strand from the ToxR binding site in the *toxT* promoter), a position appropriate for direct activation of the *ompU* promoter by ToxR (Fig. 2-7A).

Transversion mutations introduced at positions -50, -49, -47, -40, -39 and -37 (conserved nucleotides) all resulted in a >10-fold decrease in *ompU-lacZ* activation, with promoter proximal mutations at -40, -39 and -37 resulting in ~100-fold decreases in promoter activity (Fig. 2-7B).

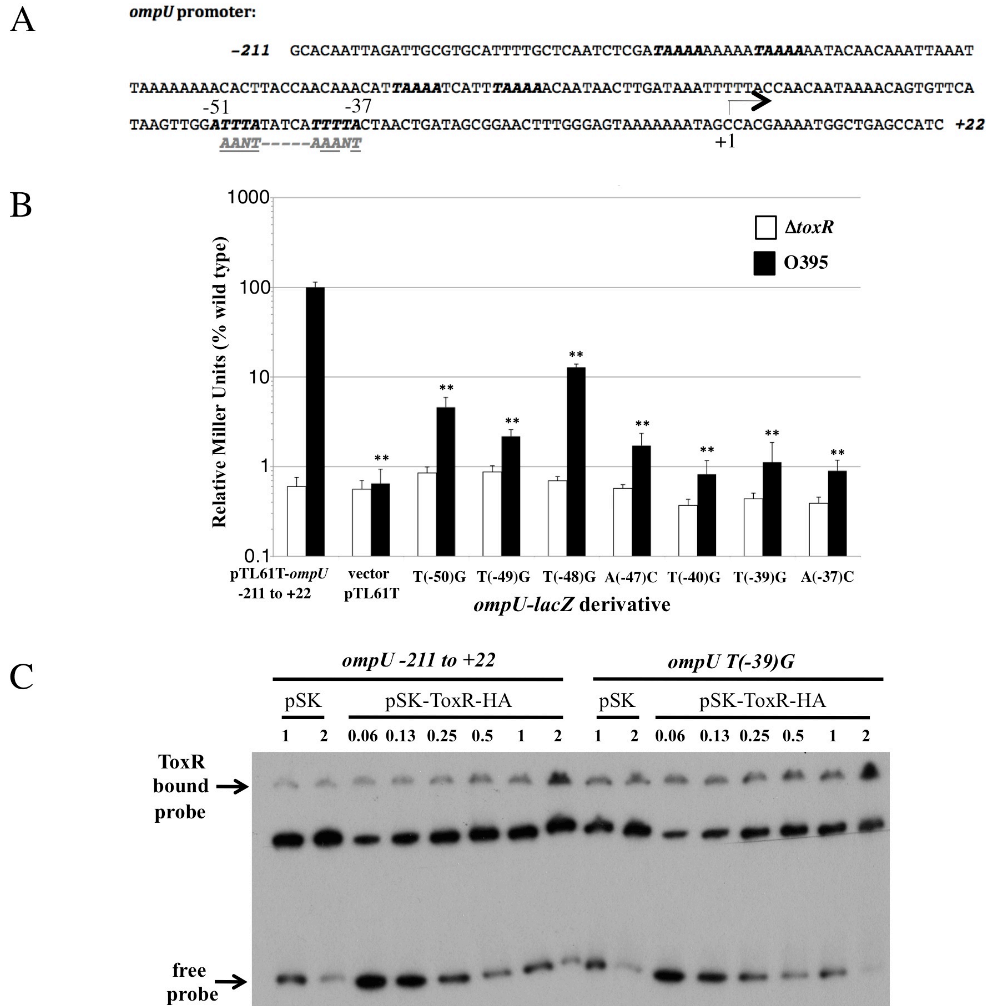


Figure 2-7. The ToxR consensus-binding site is required for ToxR-mediated activation of the *ompU*. A) Location of consensus ToxR-binding sites in the *ompU* promoters. Nucleotides comprising potential ToxR-binding sites are in bold, while the opposite strand sequences, matching the *toxT* promoter consensus ToxR-binding site, are shown in gray. Those nucleotides targeted for mutagenesis are highlighted in gray and underlined. B) Effects of transversion mutations on ToxR-mediated activation of the *ompU* promoter in wild-type *V. cholerae* or the *toxR* mutant strain, EK307. * $p<0.05$, ** $p<0.001$ relative to the wild-type promoter. # $p<0.001$ relative to the *ompT-lacZ* T(-47)G mutant. All assessed using the students' t-test, n=6 or more measurements. C) *V. cholerae* membranes containing (pSK-ToxR-HA) or lacking ToxR (pSK) were mixed at increasing concentrations with radioactively labeled probes from wild-type or mutated *ompU* promoters. Due to the fact that there are multiple ToxR-binding sites in the *ompU* promoter, no clear defect in DNA binding was observed with the mutated promoter probe.

These decreases were not due to disruption of the RNA polymerase binding site as activity of these promoters in the absence of ToxR was comparable to the wild-type *ompU-lacZ* promoter (Fig. 2-7B, white bars). Thus, the TNAAA-N_5-TNAA element in the *ompU* promoter contributed to ToxR-dependent activation as it did in the *toxT* promoter, confirming this as a minimal ToxR-responsive element of *V. cholerae*. Transversion mutation of the non-consensus nucleotide at position -48, T(-48)G also had an effect on *ompU* promoter activation, although it was the least defective (8-fold decrease) of all the mutations tested (Fig. 2-7B).

The *ctxA* promoter has an architecture made up of heptad repeats of TTTTGAT upstream of the basal promoter element. As such, it also contains a TNAAA repeat (on the opposite strand), but the spacing of this element does not provide the typical spacing, 10-11 base-pairs, corresponding to one turn of the DNA helix. We hypothesize this may explain why high levels of ToxR are required for activation of the *ctxA* promoter by ToxR. To assess ToxR-mediated *ctxA-lacZ* activation, we used a Δ*toxT* strain, VJ740 (9), overexpressing ToxRS from plasmid pVJ21 (30). When the most promoter-proximal ToxR-binding site in the *ctxA* promoter (-60 to -57, Fig. 2-8A) is mutated by transversion mutagenesis, *ctxA-lacZ* promoter activity is reduced three to five-fold (Fig. 2-8B), indicating this sequence in the *ctxA* promoter is ToxR-responsive, like in *toxT* and *ompU*. We also mutated the non-consensus nucleotide G(-58)C in the *ctxA* promoter and found it to have no effect on ToxR-mediated activation (Fig. 2-8B). Finally, as the *ctxA* promoter is typically directly activated by the ToxT protein, rather than ToxR (28, 41), we tested the effect of these promoter mutations on ToxT-mediated *ctxA-lacZ* activation in the wild-type strain, O395. The activation defects in O395 (ToxT-dependent) were inversely related to those in the Δ*toxT* mutant strain VJ740 overexpressing ToxR (EK3166, Fig. 2-8B). Thus, ToxR and ToxT have overlapping but non-identical binding sites in the *ctxA* promoter.

A

ctxA **promoter:**

−188 GCAGCGCAAGCGCTGTGGGTAGAAGTGAAACGGGGTTTACCGATAAAAACAGAAAATGATAAAAAAGGACTAAATAGT

-60 -57

ATAT***TTTGA***TT***TTTGA***TT***TTTGA***TT***TTTGA***TT***TTTGA***TT***TTTGA***TT***TTTGA***TTTCAAATAATACAAATTTATTTACTTATTTAATTG

AAANT--AAANT--AAANT--AAANT--AAANT--AAANT--AAANT

TTTTGATCAATTATTTTTCTGTTAAACAAAGGGAGCATTATATGGTAAAGATAATATTTGTGTTTTTTATTTTCTTATCATCA **+63**

+1

B

ToxR-dependent
ToxT-dependent
Relative Miller Units (% wild type)
160
140
120
100
80
60
40
20
0
pTL61T-*ctxA* -83 to +61
-21 to +61
T(-60)G
T(-59)G
G(-58)C
A(-57)C
ctxA-lacZ derivative

C

ctxA -83 to +61
ctxA T(-59)G
pSK
pSK-ToxR-HA
1 2 0.13 0.25 0.5 1 2
ToxR bound probe
free probe

Figure 2-8. The ToxR consensus-binding site is required for ToxR-mediated activation of the *ctxA* promoter. A) Location of consensus ToxR-binding sites in the *ctxA* promoter with nucleotides comprising potential ToxR-binding sites in bold, while the opposite strand sequences, matching the *toxT* promoter consensus ToxR-binding site, are shown in gray. Those nucleotides targeted for mutagenesis are highlighted in gray and underlined. B) Effect of mutations in the consensus ToxR-binding site within the promoter-proximal heptad repeat of the *ctxA* promoter. *ctxA-lacZ* expression was measured in a Δ*toxT* strain overexpressing ToxR (ToxR-dependent) or wild-type *V. cholerae* O395 (ToxT-dependent). * $p<0.05$, ** $p<0.001$ relative to the wild-type promoter. # $p<0.001$ relative to the *ompT-lacZ* T(-47)G mutant. All assessed using the students' t-test, n=6 or more measurements. C) *V. cholerae* membranes containing (pSK-ToxR-HA) or lacking ToxR (pSK) were mixed at increasing concentrations with radioactively labeled probes from wild-type or mutated *ctxA* promoter. Due to the fact that there are multiple ToxR-binding sites in the *ctxA* promoter, no clear defect in DNA binding was observed with the mutated promoter probe.

Finally, the *ompT* promoter, which is repressed by ToxR, also contains two consensus ToxR-binding sites, one from -78 to -66, the other from -47 to -33 (Fig. 2-9A). Since mutation of the promoter proximal ToxR-binding half-site would likely also affect RNAP binding, we mutated the promoter distal ToxR-binding half-site of the *ompT* promoter from -47 to -43 (Fig. 2-9A). Transversion mutation of nucleotides, -47, -46, -45 and -43 representing the TTTNA consensus binding site (opposite strand relative to *toxT*) resulted in loss of ToxR-mediated *ompT* repression, as *ompT* expression in the presence of ToxR increased 8 to 12-fold (Fig. 2-9B). Alternatively, mutation T(-44)G in the non-consensus nucleotide resulted in a <3-fold increase in *ompT* expression (Fig. 2-9B).

Thus, all promoters directly regulated by ToxR contain a consensus TTTNA-N_5-TTTNA ToxR-binding site (or near consensus) and mutation of that ToxR-binding site in each promoter leads to loss of ToxR responsiveness.

The *ompU*, *ctxA* and *ompT* promoters all contain multiple ToxR-binding sites (Fig. 2-7A, 2-8A, and 2-9A). Thus, while mutations of promoter-proximal ToxR-binding site nucleotides affected gene expression (Fig. 2-7B, 2-8B, and 2-9B), they did not affect ToxR binding to the promoter, as these mutations did not affect binding to the more promoter-distal ToxR-binding sites (2-7C, 2-8C, and 2-9C).

Discussion

The purpose of this study was to define nucleotides within the ToxR-binding site of the *toxT* promoter that influence ToxR-dependent *toxT* promoter activation. Using plasmid-based *toxT-lacZ* fusion vectors, nine transversions in the region of -96 to -83 reduced *toxT* promoter activity three-fold or greater, with those at -90, -86, -84 and -83 reducing this activity more than six-fold

A

***ompT* promoter:**

-323 AGAAATCTAGATCTTGTTCACAAATAAAAACTGAACCTTCAGAGACTTAAATTTATTTTGTTTTAT

AAAAAATATATAGCTCCAAATCCTAGGTTTGATTTTTATTAATATCCTAATTTCGCCATTTAAATGTTAATTTTTGGAGTTTTATTC

TGGTTTTCTGTCGGGTTGTAATCCTGTGATTTCGTCTGTTTTTGTTAAGAACTAAGCCGAAAAAAATCATGAAATAAATGTAATTTA

-78 -66 -47 -33

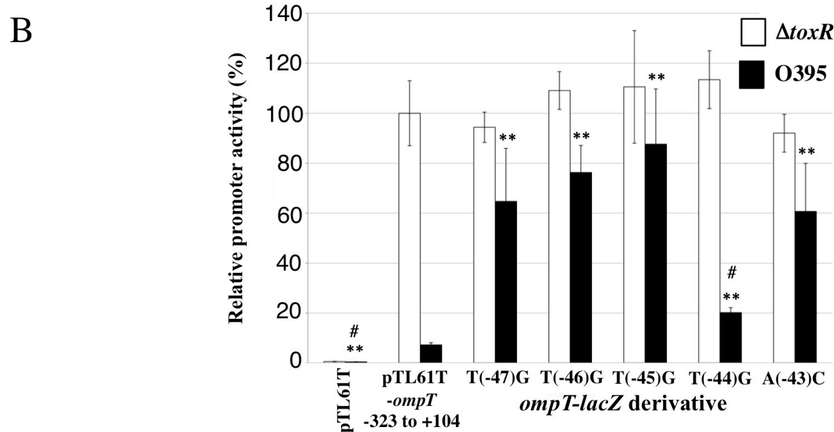
TTGAA***TTTTA***AGG***TTTTA***TGTTTTTTTCTTTGTTTT***TTTTA***TGGTA***TTTGA***CATGCAGAATATTCTTTTGCAATCTTAACCACGAAT

AAAAT---AAAAT ***AAAAT-----AAANT*** +1

CTAATGGCGGTGGTGACCAAACAAAGAGTTGGAAAACCACCTTCAAAAAACAGGGAACCGGACAAGGAATTCCCATATTAAGAAAAG

GCAGTGGATTAAC ***+108***

B



C

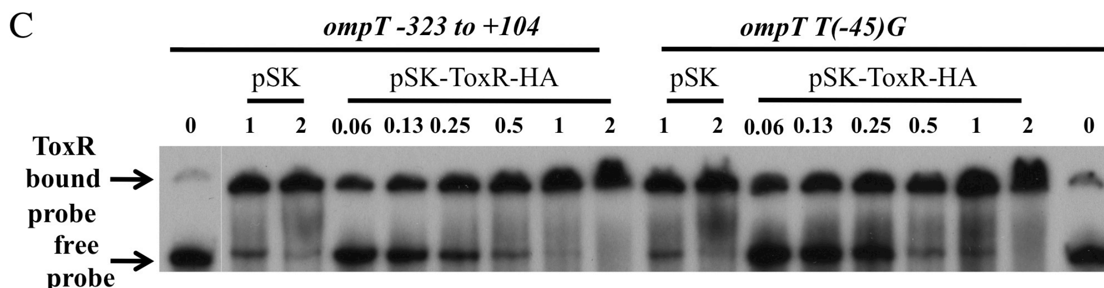


Figure 2-9. The ToxR consensus-binding site is required for ToxR-mediated repression of the *ompT* promoter. A) Location of consensus ToxR-binding sites in the *ompT* promoter. Nucleotides comprising potential ToxR-binding sites are in bold, while the opposite strand sequences, matching the *toxT* promoter consensus ToxR-binding site, are shown in gray. Those nucleotides targeted for mutagenesis are highlighted in gray and underlined. B) Effects of *ompT* transversion mutations on ToxR-mediated repression of the *ompT* promoter in wild-type *V. cholerae* or the *toxR* mutant strain, EK307. * $p<0.05$, ** $p<0.001$ relative to the wild-type promoter. # $p<0.001$ relative to the *ompT-lacZ* T(-47)G mutant. All assessed using the students' t-test, n=6 or more measurements. C) *V. cholerae* membranes containing (pSK-ToxR-HA) or lacking ToxR (pSK) were mixed at increasing concentrations with radioactively labeled probes from wild-type or *ompT* promoter. Due to the fact that there are multiple ToxR-binding sites in the *ompT* promoter, no clear defect in DNA binding was observed with the mutated promoter probe.

(Fig. 2-1B). Transversions that altered promoter activity three-fold or greater were located within the ToxR-footprinted region (-104 to -68, (4)), and led to the identification of a TNAAA-N_5-TNAAA consensus ToxR-binding site. Nucleotides within the second pentameric repeat from -86 to -82 may represent the more critical ToxR recognition site as mutations in three of four conserved nucleotides resulted in a greater than six-fold decrease in transcription activity whereas none of the mutations in the ToxR recognition site from -96 to -92 had such strong effects on *toxT* activation (Fig. 2-1B).

Substitutions at -86 and -84, which were found to strongly affect ToxR-mediated *toxT* promoter activation, were previously identified in a screen for the loss of ToxR-mediated *toxT* promoter activation (Fig. 2-1B, (42)). Furthermore, transversion mutations at these two nucleotides resulted in the greatest reduction in ToxR binding affinity (Table 2-1 and Fig. 2-3 and 2-4). Substitutions at -67 and -65 (the complementary nucleotides of -86 and -84 in an inverted repeat within the *toxT* promoter) had little influence on ToxR-mediated promoter activation both in this report and a previous report (Fig. 2-1B, (42)). Thus, substitutions occupying symmetrical positions with respect to that inverted repeat within the *toxT* promoter have differential effects on *toxT* activation, demonstrating that nucleotides critical to ToxR-mediated *toxT* promoter activation are not defined by the inverted repeat, but rather by the TNAAA-N_5-TNAAA direct repeat element overlapping the upstream half of the inverted repeat (Fig. 2-1A). As read-through transcription is known to occur from the upstream *tcpA* promoter, transversions within the region from -100 to -60 can alter the sequence of the inverted repeat within the mRNA initiated from the *tcpA* promoter and may influence transcription attenuation in the *tcpF-toxT* intergenic region in the context of a chromosomally-located *toxT* promoter, imposing an additional layer of control on *toxT* transcription levels (6, 42).

Based on ToxR-mediated DNA mobility shift experiments in this study, several transversions within the -96 and -83 region reduced the relative affinity of the *toxT* promoter for ToxR at least two-fold, with those at -86 and -84 reducing this affinity more than four-fold, again supporting the hypothesis that the -86 to -82 ToxR recognition site is more critical for ToxR interaction and *toxT* activation (Table 2-1 and Fig. 2-3 and 2-4). It is notable that several adenosine nucleotides in the N_5 spacer region were also required for efficient activation (Fig. 2-1B) and two, A[-91C] and A[-90]C reduced ToxR binding affinities more than two-told (Table 2-1). Thus, the N_5 spacer region also contributes to ToxR binding, possibly through wing domain/DNA interactions (32). This leads us to propose a modified (asymmetric) ToxR binding site on the *toxT* promoter of TNAAAAA-N_3-TNAAA. Alternatively, as poly-A tracts have been shown to induce bends in the DNA helix (43), it is possible that A to C transversions within the linker region alters the spatial orientation of the two ToxR-binding half-sites, indirectly altering its interactions with the ToxR molecules. Thus, the motif 5'-TNAAA-N_5-TNAAA-3' represents a minimally defined ToxR-binding site, with nucleotides between the two half-sites providing structural information or potentially, direct interactions with ToxR.

Transcription activation assays on truncated *tox*T promoter fragments demonstrated that the ToxR-binding site from -96 to -82 is required for binding and *toxT* activation, and that deletion of this region from our -81 to +45 promoter derivative or the previously described -73 to +45 derivative results in a promoter with greatly reduced transcription activation (Fig. 2-1B and Fig. 2-5A, (3)). Gel shift analysis with the -81 to +45 *toxT* promoter construct also demonstrated nearly undetectable levels of binding by ToxR (Fig. 2-5B), in agreement with previous studies using a truncated promoter from -73 to +45 (3). Thus, the promoter-proximal degenerate ToxR-binding site from -69 to -56 with two substitutions and altered spacing between the repeats

(ANAAA-N_4-TNAAG, hashed gray arrows in Fig. 2-1A) is unable to support efficient ToxR binding or *toxT* promoter activation. One surprising finding with the -81 to +45 *toxT* promoter construct lacking the ToxR-binding site was that activation by overexpressed TcpP was dramatically impeded if ToxR was co-expressed along with this promoter truncation (Fig. 2-2B). Since ToxR binds poorly to this promoter fragment (Fig. 2-5B), we propose this loss of activation is due to a previously established ToxR-TcpP interaction (7, 37) and diversion of TcpP away from the *toxT* promoter (perhaps towards the *ompU* and *ompT* promoters) by ToxR. Alternatively, the weak ToxR-binding activity of the -81 to +45 *toxT* promoter fragment observed in Fig. 2-5B may be sufficient to allow ToxR binding inside bacterial cells and this binding may interfere with TcpP binding to its binding site from -53 to -38. According to this second hypothesis, binding of ToxR to its consensus ToxR-binding site from -96 to -82, would displace the weakly bound ToxR from the -81 to +45 region. This would be similar to PhoB repression of the *phoBR* promoter and de-repression by PhoB binding to a neighboring upstream PhoB binding site (44).

The ToxR binding site from -96 to -82 is 3 helical-turns upstream of the TcpP binding site from -53 to -38. This places ToxR and TcpP on approximately the same face of the DNA. This would place one face of the *toxT* promoter facing the membrane where it can be bound by both ToxR and TcpP, which are both transmembrane transcription factors. The orientation of these two binding sites on the same face could allow ToxR to bind to the *toxT* promoter, recruiting it to the membrane in the proper orientation for TcpP binding. The degenerate ToxR binding site from -69 to -56 is approximately 1/2 of a helical-turn from the TcpP binding site and therefore opposite face of the DNA. This would make it difficult for this site to be bound by ToxR simultaneously with TcpP or with ToxR bound at the -96 to -83 site. The positioning of the ToxR

binding site three helical-turns upstream of the TcpP binding site could make it difficult for the DNA binding domains of ToxR and TcpP proteins to interact while these proteins are bound to the promoter. The ToxR binding site can be moved adjacent to the TcpP binding site (two helical-turns downstream) or two helical turns further upstream without disrupting transcriptional activation (Fig 2-6). If the ToxR and TcpP DNA binding domains were interacting while ToxR and TcpP were bound to the *toxT* promoter, movement of these sites relative to each other would likely disrupt that interaction. Therefore, it is likely that although the DNA binding domains of ToxR and TcpP do interact (7, 37), they do not interact when these proteins are bound to the promoter. Although the exact spacing of these two binding sites is not critical, a slight (25%) defect in transcriptional activation of the *toxT* promoter is observed when the ToxR binding site is moved (Fig. 2-6). This could be due to disruption of the DNAse I hypersensitivity site that is revealed when ToxR binds the *toxT* promoter (4).

The motif 5'-TNAAA-N_5-TNAAA-3', or its complement (5'-TTTNA-N_5-TTTNA-3'), occurs three times in the ToxR-footprinted region of the *V. cholerae ompU* promoter, (35), twice in the ToxR-footprinted region of the *V. cholerae ompT* promoter (38), and within a heptad repeat element (TTTTGAT) in the *ctxA* promoter (45) (Fig. 2-7A, 2-8A, and 2-9A). Mutation of the promoter-proximal ToxR-binding site in both the *ompU* and *ctxA* promoters dramatically reduced ToxR-dependent activation (Fig. 2-7B and 2-8B), and similar mutation in the *ompT* promoter prevented ToxR-mediated repression of the *ompT* expression (Fig. 2-9B). These results provide more evidence that we have identified the consensus ToxR-binding site that controls numerous ToxR-regulated promoters in *V. cholerae*. A recent study by Dittmer *et al* using different point mutations in the *ctxA* promoter indicated some nucleotides within the TNAAA ToxR consensus-binding site may also contribute to ToxT binding (46). Differences in our

results regarding nucleotides required for ToxT responsiveness of the *ctxA* promoter may reflect differences in the specific mutations tested, the way in which the cells were grown prior to assaying *ctxA-lacZ* expression or other factors.

These studies provide us with a working model of *toxT* promoter activation that involves the binding of two ToxR molecules to the region from -96 to -83, allowing ToxR to displace H-NS (Fig. 2-10A, (2)) and recruit two molecules of TcpP to bind the region from -53 to -38 (8). Whether ToxR releases TcpP upon DNA binding so TcpP can engage its binding site 30 nucleotides closer to the RNA polymerase binding site (“catch and release” model, Fig. 2-10C) or ToxR and TcpP maintain interaction while bound to the *toxT* promoter ("hand-holding" model, Fig. 2-10B), remains to be determined. The argument against the "hand-holding" model is that the ToxR-binding site is three helical turns of the DNA upstream of TcpP, a distance that would require dramatic DNA-bending to maintain this protein-protein interaction. Furthermore, the ToxR-binding site can be moved an additional two helical turns upstream from the TcpP-binding site and maintain strong ToxR and TcpP-dependent *toxT* activation (Fig. 2-6).

In an alternative activation model, ToxR binding to the *toxT* promoter may alter the promoter architecture such that TcpP binding is facilitated, even without any direct contact between ToxR and TcpP ("promoter alteration" model, Fig. 2-10D). This could be due to ToxR removing the repressor H-NS from the *toxT* promoter and/or ToxR inducing DNA bending that allows TcpP better access to its DNA binding site (Fig. 2-10D). Although, removal of H-NS alone does not account for full *toxT* activation as in an H-NS mutant, *toxT* is expressed to just 20% of the level expressed under ToxR and TcpP-induced conditions (2). Evidence supporting the "promoter alteration" model comes from the fact that when ToxR binds the *toxT* promoter, a DNAse I hypersensitivity site is revealed overlapping the TcpP-binding site (4, 8). This suggests ToxR

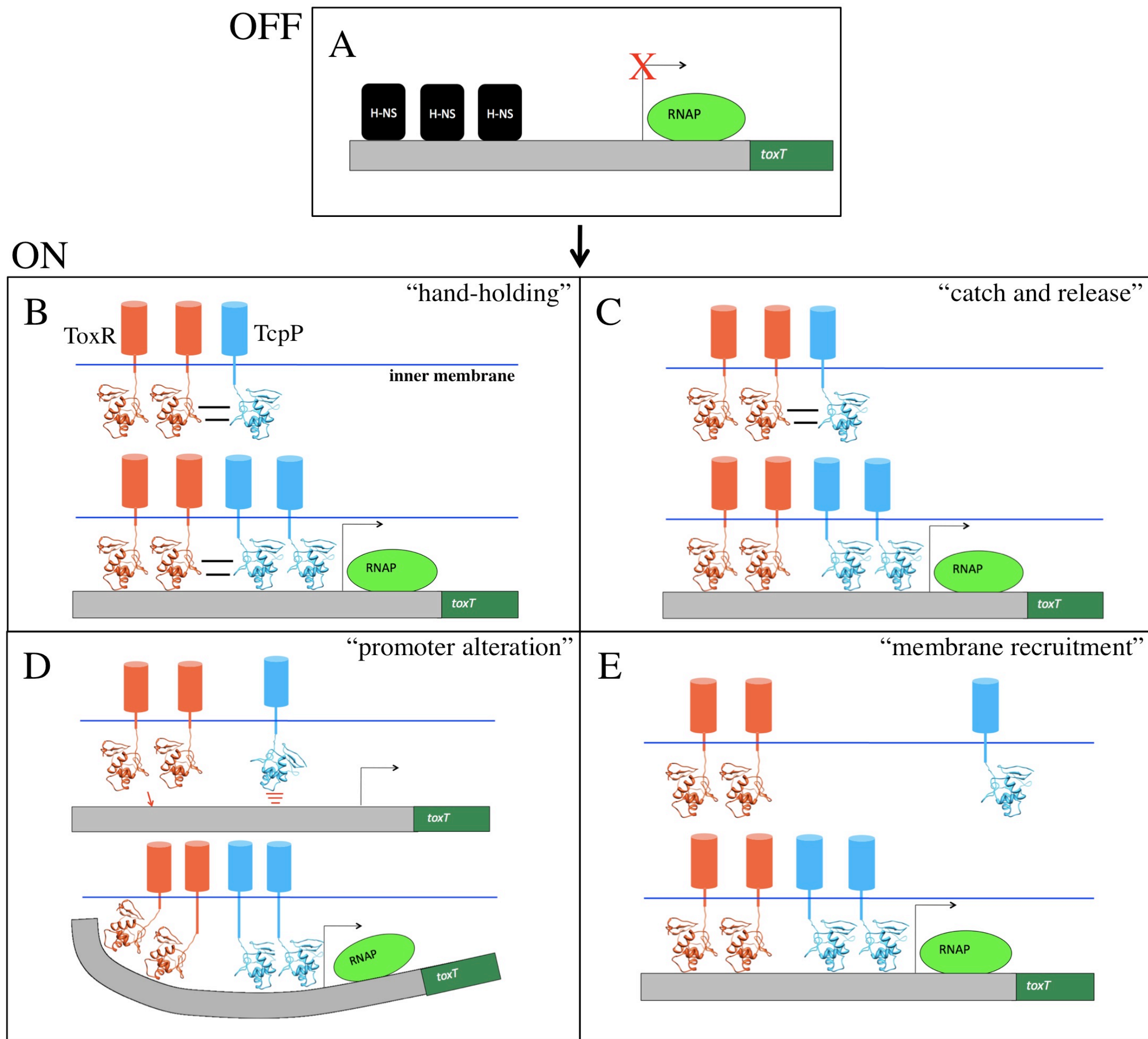


Figure 2-10. Models for the role of ToxR in TcpP-mediated *toxT* activation. A) As previously described, the *toxT* promoter in repressed by H-NS (2). B) In the "hand-holding" model ToxR and TcpP interact in the inner membrane of *V. cholerae* as previously described (7), then ToxR escorts TcpP to the *toxT* promoter where ToxR relieves H-NS repression and maintains interaction with TcpP while TcpP stimulates transcription. C) In the "catch and release" model, ToxR also interacts with TcpP and recruits TcpP to the *toxT* promoter, but upon DNA-binding by ToxR, H-NS is displaced and ToxR releases TcpP so TcpP can bind the TcpP-binding site 30 nucleotides downstream of the ToxR-binding site (8). D) In the "promoter alteration" model interaction between ToxR and TcpP is not required for *toxT* activation, rather ToxR binding to the *toxT* promoter displaces H-NS and alters the *toxT* promoter architecture such that a normally weak TcpP-binding site is altered in some way to facilitate enhanced TcpP binding, thus allowing TcpP-mediated activation of the *toxT* promoter. E) In the "membrane recruitment" model, again interaction between ToxR and TcpP is not required, but the role of ToxR is to simply recruit the *toxT* promoter to the membrane where TcpP has easier assess to its DNA-binding site. This model takes into account the fact that TcpP binding to the *toxT* promoter requires higher concentrations of *V. cholerae* membranes than ToxR binding (4) and the fact that membrane localization was previously shown to be required for ToxR to facilitate TcpP-mediated *toxT* activation (21).

binding results in DNA bending or unwinding that might allow TcpP better access to its *toxT* promoter-binding site. However, this role alone cannot be sufficient for promoting TcpP-mediated activation, as a soluble form of ToxR that binds the same DNA-binding site, does not facilitate TcpP-mediated *toxT* activation (21). Finally, it is possible that the main role of ToxR is to recruit the *toxT* promoter to a membrane-proximal location where TcpP can more efficiently interact with its relatively weak DNA-binding site ("membrane recruitment" model, Fig. 2-10E, (4, 8)). According to this model, ToxR should be able to facilitate TcpP-mediated *toxT* activation from a considerable distance (so long as it still displaces H-NS binding), a model to be tested in the future. Most likely, aspects from several of these models contribute to how ToxR facilitates activation of the *toxT* promoter including: membrane recruitment, H-NS displacement, alterations to the promoter architecture and possibly ToxR-TcpP interaction.

This study defines a minimal ToxR-responsive site, TNAAA-N_5-TNAAA, in the *toxT*, *ompU*, *ompT* and *ctxA* promoters. Based on the direct repeat nature of this ToxR-binding site, we hypothesize two ToxR molecules bind this repeat element in a head-to-tail fashion, consistent with the structure determined for the *E. coli* PhoB-DNA co-crystal (32). The fact that the ToxR-binding site in the *toxT* promoter is in the opposite orientation from the promoter-proximal ToxR-binding sites of other ToxR-regulated promoters (*ompU*, *ctxA* and *ompT*; Fig. 2-1A, 2-7A, 2-7A, and 2-8A) suggests ToxR favors this inverted orientation when playing a supporting role in TcpP-mediated *toxT* activation.

By defining the ToxR-binding site we can compare the recognition sequences for a number of OmpR/PhoB family regulators in *V. cholerae* including, ToxR, TcpP and PhoB. All three proteins have very similar recognition sequences TTTNA-N_5-TTTNA (ToxR), TGTAA-N_6-TGTAA (TcpP, (8)) and TGTCA-N_6-TGTCA (PhoB, (44)). This raises the question of how *V.*

cholerae avoids cross-talk among these closely related binding sites and what determines sequence specific recognition of DNA within each protein. Previous studies on winged-HTH proteins suggest that rather than differences in residues in the α3 DNA recognition helix, sequence specificity may be dictated by the preceding α2 helix and loop domain which influence the positioning of the α3 helix relative to the rest of the molecule (47, 48)). Future experiments will test whether this hypothesis holds true for ToxR and TcpP in *V. cholerae* as well.

Materials and Methods

Bacterial strains and plasmids. All *V. cholerae* used in this study are derived from O1 serotype Classical biotype strain O395 (1). *V. cholerae*, *E. coli* and plasmids used in this study are listed in Table 2-2. Strains were routinely grown in LB medium containing 10 g/L NaCl at 37°C or Vc LB containing 5 g/L NaCl. Unless otherwise indicated, antibiotics were used at the following concentrations: streptomycin 100 μg/ml; ampicillin 100 μg/ml; chloramphenicol 25 μg/ml; and kanamycin 30 μg/ml.

DNA manipulations. Cloning procedures and transformation of *E. coli* strains were carried out using standard protocols (49). pTG24-based fusion plasmids were transferred to *V. cholerae* by electroporation (2.2 kV) using an *E. coli* Pulsor (Bio-Rad) and pMMB207-based plasmids were transferred to *V. cholerae* by triparental mating using mobilization plasmid pRK2013 (22).

Generation of promoter mutants. The wild type *toxT* promoter, $toxT_{pro}$, was amplified using purified *V. cholerae* strain O395 chromosomal DNA as template, the *toxTpro* -172 *Bam*HI and *toxTpro* +45 *Eco*RI primers (Table 2-3) and the Expand High Fidelity PCR system (Roche). The amplified DNA fragment was gel purified, digested with *Eco*RI and *Bam*HI, and ligated into *Eco*RI/*Bam*HI-digested pBluescript SK(+), generating pTG3. Nucleotide

Table 2-2. Strains and plasmids

Name	Description	Source
V. cholerae		
O395	Wild type, Str^R	(1)
RY6	(O395 *toxTΔpro*)	(6)
EK307	O395 Δ*toxR*	(4)
EK459	O395 Δ*toxR* Δ*tcpP*	(4)
TG128	EK459/pBluescript SK(+)/pMMB207	This study
TG129	EK459/pSK-*toxR*-HA/pMMB207	This study
EK3124	O395 + pTL61T	
EK3122	O395 + pTL61T-*ompU*(-211 to +22)	
EK3139	EK307 + pTL61T	
EK3137	EK307 + pTL61T-*ompU*(-211 to +22)	
VJ740	O395 $toxT_{\Delta HTH}$	(9)
EK3165	VJ740 + pACYC184-TetS (Cm^R)	This study
EK3166	VJ740 + pVJ21 (Cm^R)	This study
EK3177	O395 + pTL61T-*ctxA* (-83 to +63)	This study
EK3178	O395 + pTL61T-*ctxA* (-21 to +63)	This study
EK3183	EK3165 + pTL61T-*ctxA* (-83 to +63)	This study
EK3184	EK3165 + pTL61T-*ctxA* (-21 to +63)	This study
EK3189	EK3166 + pTL61T-*ctxA* (-83 to +63)	This study
EK3190	EK3166 + pTL61T-*ctxA* (-21 to +63)	This study
SM1544	O395 + pTL61T-*ompT* (-323 to +104)	This study
SM1550	EK307 + pTL61T-*ompT* (-323 to +104)	This study
E. coli		
DH5α	*supE44 ΔlacU169(F80lacZDM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Laboratory collection
Plasmids		
pBluescript SK(+)	Ap^R, high copy number cloning vector	Stratagene
pMMB207	IPTG-inducible expression vector, Cm^R, *lacI*^{q}*, tacP*	(14)
pEK41	pMMB207-*tcpP*-HSV	(4)
pRK2013	ColE1::RK2 *tra*$^+$ *ori*$^-$, Km^R	(22)
pSK-*toxR*-HA	pBluescript SK(+)::*toxR*-HA	(8)
pTG3	pBluescript SK(+)::*toxT* promoter (–172 to +45) as an *Eco*RI-*Bam*HI fragment	This study
pTG24	Ap^R, promoterless *lacZ* fusion vector derived from pTL61T	(8)
pTG25	pTG24:: *toxT* promoter (–172 to +45) from pTG3 as an *Nru*I-*Sal*I fragment	This study
pTG212	pTG24:: *toxT* promoter (-100 to +45)	This study

Name	**Description**	**Source**
pTG228	pTG24:: toxT promoter (-81 to +45)	This study
pVJ21	pACYC184-*toxRS*	(30)
pACYC184-Tet^S	pACYC184 cut with *Bam*HI, Klenow filled and religated to disrupt tetracycline resistance	This study
pTL61T	promoter-less *lacZ* fusion vector	(34)
pTL61T-*ompU* (-211 to +22)	*lacZ* fusion vector containing the *ompU* promoter from -211 to +22	(35)
pTL61T-*ctxA* (-83 to +63)	*lacZ* fusion vector containing the *ctxA* promoter from (-83 to +63)	(28)
pTL61T-*ctxA* (-21 to +63)	*lacZ* fusion vector containing the *ctxA* promoter from (-21 to +63)	(28)
pTL61T-*ompT* (-323 to +104)	*lacZ* fusion vector containing the *ompT* promoter from (-323 to +104)	(38)

Table 2-3: Oligonucleotides used in this study

primer name	purpose	sequence 5' to 3'
toxT D-100 forw	mutagenesis	cgggatcctatctcaaaaaacataaaataacatgagttac
toxT D-82 forw	mutagenesis	cgggatcctaacatgagttactttatgtttttcttatg
toxT D-47 forw	mutagenesis	gcgcggatcccgtctgtaacttgttcttatgtctg
toxT -57 forw	mutagenesis	catgagttactttatgtttttattatgtaatacgtctgtaact
toxT -57 rev	mutagenesis	agttacagacgtattacataataaaaacataaagtaactcatgttat
toxT -58 forw	mutagenesis	acatgagttactttatgttttgcttatgtaatacgtctgtaac
toxT -58 rev	mutagenesis	gttacagacgtattacataagcaaaacataaagtaactcatgttatt
toxT -59 forw	mutagenesis	aacatgagttactttatgtttctcttatgtaatacgtctgtaa
toxT -59 rev	mutagenesis	ttacagacgtattacataagagaaacataaagtaactcatgttattttatgttttttgag
toxT -60 forw	mutagenesis	gagttactttatgttgttcttatgtaatacg
toxT -60 rev	mutagenesis	cgtattacataagaacaacataaagtaactc
toxT -61 forw	mutagenesis	tgagttactttatgtgtttcttatgtaatac
toxT -61 rev	mutagenesis	gtattacataagaaacacataaagtaactca
toxT -62 forw	mutagenesis	atgagttactttatggttttcttatgtaata
toxT -62 rev	mutagenesis	tattacataagaaaaccataaagtaactcat
toxT -63 forw	mutagenesis	catgagttactttatttttttcttatgtaat
toxT -63 rev	mutagenesis	attacataagaaaaaaataaagtaactcatg
toxT -64 forw	mutagenesis	acatgagttactttaggtttttcttatgtaa
toxT -64 rev	mutagenesis	ttacataagaaaaacctaaagtaactcatgt
toxT -65 forw	mutagenesis	aacatgagttactttctgtttttcttatgta
toxT -65 rev	mutagenesis	tacataagaaaaacagaaagtaactcatgtt
toxT -66 forw	mutagenesis	taacatgagttacttgatgtttttcttatgt
toxT -66 rev	mutagenesis	acataagaaaaacatcaagtaactcatgtta
toxT -67 forw	mutagenesis	ataacatgagttactgtatgtttttcttatg
toxT -67 rev	mutagenesis	cataagaaaaacatacagtaactcatgttat
toxT -68 forw	mutagenesis	aataacatgagttacgttatgtttttcttat
toxT -68 rev	mutagenesis	ataagaaaaacataacgtaactcatgttatt
toxT -69 forw	mutagenesis	aaataacatgagttaatttatgtttttctta
toxT -69 rev	mutagenesis	taagaaaaacataaattaactcatgttattt
toxT -70 forw	mutagenesis	aaaataacatgagttcctttatgtttttctt
toxT -70 rev	mutagenesis	aagaaaaacataaaggaactcatgttatttt
toxT -71 forw	mutagenesis	taaaataacatgagtgactttatgtttttct
toxT -71 rev	mutagenesis	agaaaaacataaagtcactcatgttatttta
toxT -72 forw	mutagenesis	ataaaataacatgaggtactttatgtttttc
toxT -72 rev	mutagenesis	gaaaaacataaagtacctcatgttattttat
toxT -73 forw	mutagenesis	cataaaataacatgatttactttatgttttt
toxT -73 rev	mutagenesis	aaaaacataaagtaaatcatgttattttatg
toxT -74 forw	mutagenesis	acataaaataacatgcgttactttatgtttt
toxT -74 rev	mutagenesis	aaaacataaagtaacgcatgttattttatgt
toxT -75 forw	mutagenesis	aacataaaataacattagttactttatgttt

primer name	**purpose**	**sequence 5' to 3'**
toxT -75 rev	mutagenesis	aaacataaagtaactaatgttattttatgtt
toxT -76 forw	mutagenesis	aaacataaaataacaggagttactttatgtt
toxT -76 rev	mutagenesis	aacataaagtaactcctgttattttatgttt
toxT -77 forw	mutagenesis	aaaacataaaataacctgagttactttatgt
toxT -77 rev	mutagenesis	acataaagtaactcaggttattttatgtttt
toxT -78 forw	mutagenesis	aaaaacataaaataaaatgagttactttatg
toxT -78 rev	mutagenesis	cataaagtaactcattttattttatgttttt
toxT -79 forw	mutagenesis	aaaaaacataaaataccatgagttactttat
toxT -79 rev	mutagenesis	ataaagtaactcatggtattttatgtttttt
toxT -80 forw	mutagenesis	caaaaaacataaaatcacatgagttacttta
toxT -80 rev	mutagenesis	taaagtaactcatgtgattttatgttttttg
toxT -81 forw	mutagenesis	atctcaaaaaacataaaagaacatgagttactttatg
toxT -81 rev	mutagenesis	cataaagtaactcatgttcttttatgttttttgagat
toxT -82 forw	mutagenesis	tatctcaaaaaacataaactaacatgagttactttat
toxT -82 rev	mutagenesis	ataaagtaactcatgttagtttatgttttttgagata
toxT -83 forw	mutagenesis	ttatctcaaaaaacataacataacatgagttacttta
toxT -83 rev	mutagenesis	taaagtaactcatgttatgttatgttttttgagataa
toxT -84 forw	mutagenesis	atctcaaaaaacatacaataacatgagttac
toxT -84 rev	mutagenesis	gtaactcatgttattgtatgttttttgagat
toxT A[-84]T forw	mutagenesis	atctcaaaaaacatataataacatgagttac
toxT A[-84]T rev	mutagenesis	gtaactcatgttattatatgttttttgagat
toxT -85 forw	mutagenesis	tcttatctcaaaaaacatcaaataacatgagttactt
toxT -85 rev	mutagenesis	aagtaactcatgttatttgatgttttttgagataaga
toxT -86 forw	mutagenesis	atcttatctcaaaaaacagaaaataacatgagttact
toxT -86 rev	mutagenesis	agtaactcatgttattttctgttttttgagataagat
toxT -87 forw	mutagenesis	tatcttatctcaaaaaacctaaaataacatgagttac
toxT -87 rev	mutagenesis	gtaactcatgttattttaggttttttgagataagata
toxT -88 forw	mutagenesis	ttatcttatctcaaAaaaaAtaaaataacaTGAgtta
toxT -88 rev	mutagenesis	taactcatgttattttatttttttgagataagataa
toxT -89 forw	mutagenesis	gttatcttatctcaaAaaccataaaataacaTGAgtt
toxT -89 rev	mutagenesis	aactcatgttattttatggtttttgagataagataac
toxT -90 forw	mutagenesis	tgttatcttatctcaaAaCacataaaataacaTGagt
toxT -90 rev	mutagenesis	actcatgttattttatgtGttttgagataagataaca
toxT -91 forw	mutagenesis	ctgttatcttatctcaaACaacataaaataacaTgag
toxT -91 rev	mutagenesis	ctcatgttattttatgttGtttgagataagataacag
toxT -92 forw	mutagenesis	gctgttatcttatctcaaCaaacataaaataacatga
toxT -92 rev	mutagenesis	tcatgttattttatgtttGttgagataagataacagc
toxT -93 forw	mutagenesis	ggctgttatcttatctcacaaaacataaaataacatg
toxT -93 rev	mutagenesis	catgttattttatgttttgtgagataagataacagcc
toxT -94 forw	mutagenesis	Tggctgttatcttatctccaaaaacataaaataacat
toxT -94 rev	mutagenesis	Atgttattttatgtttttggagataagataacagcca
toxT -95 forw	mutagenesis	gctgttatcttatctaaaaaaacataaaata
toxT -95 rev	mutagenesis	tgttattttatgtttttttagataagataacagccacgaatgtggc

primer name	**purpose**	**sequence 5' to 3'**
toxT -96 forw	mutagenesis	cgtggctgttatcttatcgcaaaaaacataaaataac
toxT -96 rev	mutagenesis	gttattttatgttttttgcgataagataacagccacg
toxT -97 forw	mutagenesis	tggctgttatcttatatcaaaaaacataaaa
toxT -97 rev	mutagenesis	ttttatgttttttgatataagataacagcca
toxT -98 forw	mutagenesis	ttcgtggctgttatcttagctcaaaaaacataaaata
toxT -98 rev	mutagenesis	tattttatgttttttgagctaagataacagccacgaa
toxT -99 forw	mutagenesis	attcgtggctgttatcttctctcaaaaaacataaaat
toxT -99 rev	mutagenesis	attttatgttttttgagagaagataacagccacgaat
toxT -100 forw	mutagenesis	cattcgtggctgttatctgatctcaaaaaacataaaa
toxT -100 rev	mutagenesis	ttttatgttttttgagatcagataacagccacgaatg
*toxT*p -172 BamHI	cloning primer	gcgcggatccgtatagcaaagcatattcagagaac
*toxT*p +45 EcoRI	cloning primer	gcgcgaattcaaataaacgcagagagccatcc
ToxR bind site -105 to -70 TOP	Competitor oligo	tatcttatctcaaaaaacataaaataacatgagtta
ToxR bind site -105 to -70 BOT	Competitor oligo	taactcatgttattttatgttttttgagataagata
ToxR bind site -100 to -75 TOP	Competitor oligo	tatctcaaaaaacataaaataacatg
ToxR bind site -100 to -75 BOT	Competitor oligo	catgttattttatgttttttgagata
toxT + 2 turns TOP	mutagenesis	atgagttactttatgagataacagccacattcgtggtttttcttatgtaat
toxT + 2 turns BOTTOM	mutagenesis	attacataagaaaaaccacgaatgtggctgttatctcataaagtaactcat
toxT – 2 turns TOP	mutagenesis	aaaacataaaataactatgtaatacgtctg
toxT -2 turns BOTTOM	mutagenesis	cagacgtattacatagttattttatgtttt
ompU T(-50)G TOP	mutagenesis	gtgttcataagttggagttatatcattttactaac
ompU T(-50G BOTTOM	mutagenesis	gttagtaaaatgatataactccaacttatgaacac
ompU T(-49)G TOP	mutagenesis	gtgttcataagttggatgtatatcattttactaac
ompU T(-49)G BOTTOM	mutagenesis	gttagtaaaatgatatacatccaacttatgaacac
ompU T(-48)C TOP	mutagenesis	gttcataagttggattcatatcattttactaac
ompU T(-48)C BOTTOM	mutagenesis	gttagtaaaatgatatgaatccaacttatgaac
ompU A(-47)C TOP	mutagenesis	gttcataagttggatttctatcattttactaac
ompU A(-47)C BOTTOM	mutagenesis	gttagtaaaatgatagaaatccaacttatgaac
ompU T(-40)G TOP	mutagenesis	gttggatttatatcatgttactaactgatagcgg
ompU T(-40)G BOTTOM	mutagenesis	ccgctatcagttagtaacatgatataaatccaac
ompU T(-39)G TOP	mutagenesis	gttggatttatatcattgtactaactgatagcgg
ompU T(-39)G BOTTOM	mutagenesis	ccgctatcagttagtacaatgatataaatccaac

primer name	purpose	sequence 5' to 3'
ompU A(-37)C TOP	mutagenesis	ggatttatatcattttcctaactgatagcggaac
ompU A(-37)C BOTTOM	mutagenesis	gttccgctatcagttaggaaaatgatataaatcc
ctxA T(-60)G TOP	mutagenesis	gatttttgatttGtgatttcaaataatacaaatttatttac
ctxA T(-60)G BOTTOM	mutagenesis	gtaaataaatttgtattatttgaaatcaCaaatcaaaaatc
ctxA T(-59)G TOP	mutagenesis	gatttttgattttGgatttcaaataatacaaatttatttac
ctxA T(-59)G BOTTOM	mutagenesis	gtaaataaatttgtattatttgaaatcCaaaatcaaaaatc
ctxA G(-58)C TOP	mutagenesis	gatttttgatttttCatttcaaataatacaaatttatttac
ctxA G(-58)C BOTTOM	mutagenesis	gtaaataaatttgtattatttgaaatGaaaaatcaaaaatc
ctxA A(-57)C TOP	mutagenesis	gatttttgatttttgCtttcaaataatacaaatttatttac
ctxA A(-57)C BOTTOM	mutagenesis	gtaaataaatttgtattatttgaaaGcaaaaatcaaaaatc
ompT T(-47)G TOP	mutagenesis	gtttttttctttgttttgtttatggtatttgacatg
ompT T(-47)G BOTTOM	mutagenesis	catgtcaaataccataaacaaaacaaagaaaaaaac
ompT T(-46)G TOP	mutagenesis	gtttttttctttgtttttgttatggtatttgacatg
ompT T(-46)G BOTTOM	mutagenesis	catgtcaaataccataacaaaaacaaagaaaaaaac
ompT T(-45)G TOP	mutagenesis	gtttttttctttgttttttgtatggtatttgacatg
ompT T(-45)G BOTTOM	mutagenesis	catgtcaaataccatacaaaaaacaaagaaaaaaac
ompT T(-44)G TOP	mutagenesis	gtttttttctttgtttttttgatggtatttgacatg
ompT T(-44)G BOTTOM	mutagenesis	catgtcaaataccatcaaaaaaacaaagaaaaaaac
ompT A(-43)C TOP	mutagenesis	gtttttttctttgttttttttctggtatttgacatg
ompT A(-43)C BOTTOM	mutagenesis	catgtcaaataccagaaaaaaaacaaagaaaaaaac

substitutions within the ToxR-binding region of the *toxT* promoter region were generated by a one-step process in which the entire plasmid is amplified using complementary mutagenic primers, pTG3 as template and Pfu Turbo DNA polymerase (Stratagene), followed by *Dpn*I cleavage for enrichment for PCR-amplified plasmids or the two-step SOEing PCR amplification technique (50) using complementary mutagenic primers, the exterior primers *toxTpro* -172 *Bam*HI and *toxTpro* +45 *Eco*RI primers (Table 2-3), pTG3 as template and Expand High Fidelity PCR System (Roche), followed by PCR product purification, digestion with *Eco*RI and *Bam*HI, and ligation into *Eco*RI/*Bam*HI-digested pBluescript SK(+). Deletion derivatives of $toxT_{pro}$ were generated using PCR amplification using pTG3 as template, the *toxTpro* +45 *Eco*RI primer and either the Δ-101 *Bam*HI, Δ-82 *Bam*HI or Δ-47 *Bam*HI primer (Table 2-3). The DNA sequences of all PCR-generated *V. cholerae* DNA fragments were determined at The University of Michigan Core sequencing facility to verify the mutations and confirm the absence of additional nucleotide changes. DNA fragments carrying the wild type, deleted and substituted *toxT* promoters were excised from pBluescript-based constructs as *Not*I/*Sal*I fragments and recloned promoters were excised from pBluescript-based constructs as *Not*I/*Sal*I fragments and recloned into *Not*I/*Sal*I-digested pTG24 (8), generating *lacZ* transcriptional fusions.

ompU promoter DNA from -211 to +22 relative to the transcription start site was PCR amplified in plasmid pBluescript SK(+)-*ompU* using mutagenic primer pairs listed in Table 2-3. Following *Dpn*I digestion and DH5α transformation, candidate *ompU* mutants were confirmed by sequencing prior to excision with *Eco*RI and *Bam*HI and ligation into the promoter-less *lacZ* vector pTL61T (34).

ctx-lacZ fusions published previously (28), were PCR amplified using mutagenic primers listed in Table 2-3, *Dpn*I digested and transformed into DH5α. Candidate *ctxA* promoter mutants were

confirmed by sequencing. Previously described *ompT-lacZ* fusions (38) were PCR amplified using mutagenic primers listed in Table 2-3, *Dpn*I digested and transformed into DH5α. Candidate *ompT* promoter mutants were confirmed by sequencing.

Measurement of *lacZ* fusion activity. Cultures of *toxT-lacZ* reporter strains carrying both pTG24 and pMMB207 or their derivatives were grown overnight in Luria-Bertani (LB) broth containing 5 g/L NaCl (Vc LB) at 30° C, diluted 1:50 in LB broth which had been adjusted to an initial pH of 6.5 and supplemented with chloramphenicol, ampicillin, streptomycin and 1mM isopropyl-β-D-thiogalactopyranoside (IPTG) where required. After incubation for 4 hr at 30°C (ToxR-inducing conditions), the OD_{600} of the cultures were determined and 5 to 100 µl samples were used in a standard β-galactosidase assay (51). For *ompU-lacZ* fusion constructs, β-galactosidase activity was measured on overnight cultures of *V. cholerae* grown at 30°C in Vc LB pH=7. For *ctxA-lacZ* and *ompT-lacZ* fusions, β-galactosidase assays were performed on overnight cultures grown at 30°C in Vc LB pH=6.5.

Mobility shift assays. DNA gel mobility shift assays were performed essentially as previously described (8) using membrane preparations obtained either from *V. cholerae* strains TG128 (ToxR-) and TG129 (ToxR+, O395 Δ*toxR*Δ*tcpP* expressing HA-tagged ToxR from the plasmid pSK-*toxR*-HA, Table 2-2) grown in Vc LB broth supplemented with 1 mM IPTG, streptomycin, chloramphenicol and ampicillin. Protein concentrations were determined using the Quick-Start Bradford Dye reagent (Bio-Rad). DNA fragments carrying the either the entire region from -172 to +45, relative to the *toxT* transcription start site, or upstream deletion derivatives thereof, were excised from pBluescript clones using *Nru*I and *Sal*I, gel purified and end-labeled by Klenow DNA polymerase (Invitrogen) in the presence of [α-^{32}P]-dCTP or [α-^{32}P]-dATP (MP Biomedicals) as previously described (8). Increasing amounts of membrane preparations were

mixed with the end-labeled DNA targets in a solution containing 10 mM Tris (pH 7.4), 1 mM EDTA, 5 mM NaCl, 50 mM KCl, 50 µg/ml BSA and 10 µg/ml sheared salmon sperm DNA. Binding reactions were performed at 30°C for 30 min and the free and membrane-associated DNA targets samples were separated by electrophoresis on a 6% polyacrylamide/TBE gel prerun with 5% thioglycolic acid as previously described (8). After electrophoresis, the gels were dried, the extents of DNA migration were recorded by autoradiography and in some cases the relative intensities of the recorded signals were determined using a Biospectrum image analyzer (UVP, LLC) or using ImageJ (http://rsbweb.nih.gov/ij/).

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Chapter 3

The two faces of ToxR: activator of *ompU*, co-regulator of *toxT* in *Vibrio cholerae*

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Notes: I replicated all of the β-galactosidase transcriptional activation assays shown (Fig. 3-1 and Tables 3-1, 3-2, and 3-3) for all of the ToxR mutants. I created all of the strains for the BACTH assay, validated the BACTH assay for ToxR-TcpP interaction, and performed all of the BACTH assays shown (Fig. 3-7). Additionally, I created the models shown in Figures 3-2 and 3-8.

Summary

ToxR of *Vibrio cholerae* directly activates the *ompU* promoter, but requires a second activator, TcpP to activate the *toxT* promoter. *ompU* encodes a porin, while *toxT* encodes the transcription factor, ToxT, that activates *V. cholerae* virulence genes including cholera toxin and the toxin co-regulated pilus. Using an *ompU-sacB* transcriptional fusion, *toxR* mutant alleles were identified that encode ToxR molecules defective for *ompU* promoter activation. Many *toxR* mutants

defective for *ompU* activation affected residues involved in DNA binding. Mutants defective for *ompU* activation were also tested for activation of the *toxT* promoter. ToxR-F69A and ToxR-V71A, both in the α-loop of ToxR, were preferentially defective for *ompU* activation, with ToxR-V71A nearly completely defective. Six mutants from the *ompU-sacB* selection showed more dramatic defects in *toxT* activation than *ompU* activation. All but one of the affected residues map to the wing domain of the winged-helix-turn-helix of ToxR. Some ToxR mutants preferentially affecting *toxT* activation had partial DNA-binding defects, and one mutant, ToxR-P101L, had altered interactions with TcpP. These data suggest that while certain residues in the α-loop of ToxR are utilized to activate the *ompU* promoter, the wing domain of ToxR contributes to both promoter binding and ToxR/TcpP interaction facilitating *toxT* activation.

Introduction

Vibrio cholerae is the causative agent of the diarrheal disease cholera, a disease estimated to affect 18 million people each year worldwide (11). *V. cholerae* lives in aquatic environments year-round, but during seasonal outbreaks the bacterium is ingested by humans in contaminated food or water. Following ingestion, *V. cholerae* senses the changes in environmental conditions and responds by activating a number of genes whose products are required for effective colonization and pathogenesis in human hosts. These include the genes encoding cholera toxin and the toxin co-regulated pilus among others (13-16). Activation of these virulence genes is dependent upon two inner membrane localized transcription factors ToxR and TcpP (17-20). Both ToxR and TcpP have C-terminal periplasmic domains with the potential to sense environmental signals as well as N-terminal DNA-binding and transcription activation domains similar to the winged helix-turn-helix (winged-HTH) family of transcription factors (22). ToxR

and TcpP stimulate virulence gene expression by combining to activate the *toxT* promoter (1, 19, 23). ToxT then activates various virulence genes directly (24-26).

DNAse I footprinting studies, *toxT* promoter mutations and analysis of a series of *tcpP* mutants have suggested that TcpP directly interacts with RNA polymerase at the *toxT* promoter while ToxR serves an accessory role from a position further upstream of the basal promoter elements (1, 5, 27). Furthermore it has been demonstrated that TcpP and ToxR physically interact and that this interaction may contribute to the activity of ToxR on the *toxT* promoter (5). In fact, a mutant derivative of TcpP that is deficient in *toxT* promoter binding (TcpP-H93L) is still functional for *toxT* activation if ToxR is co-expressed (1). TcpP binds a pentameric direct repeat element TGTAA-N_6-TGTAA from -53 to -38 relative to the start site of *toxT* transcription (27). Within this repeat element, the central nucleotide of both repeats is critical for TcpP-mediated *toxT* activation, even in the presence of ToxR (27). These data lead to a model in which ToxR binds a region upstream of the TcpP-binding site and facilitates *toxT* promoter recognition and activation by TcpP (27).

While both ToxR and TcpP are required for *toxT* activation (19, 28, 29), ToxR is able to regulate other promoters in a TcpP-independent fashion. One such promoter, *ompU*, controls expression of the outer membrane porin OmpU. ToxR directly activates *ompU* (30) and represses expression of the alternative porin, OmpT (31). Proper regulation of outer membrane protein synthesis is critical as expression of OmpT leads to greater sensitivity to bile and other related detergents (32).

Given that ToxR directly activates *ompU* yet plays an accessory role in *toxT* activation, we hypothesized that the molecular mechanism by which ToxR regulates these two promoters may be different and we might be able to isolate point mutations in *toxR* that affect activation of

ompU or *toxT* specifically. Identification of such mutants would strengthen the hypothesis that different regions of ToxR are more critical for one type of activation or the other, and based on homology to the winged-HTH family of transcription factors, the position of mutant substitutions may direct us to the function of certain domains of ToxR. Precedent for such differential activation of the *ompU* and *toxT* promoters comes from the fact that *toxT* expression requires that ToxR be membrane-localized, while *ompU* can be efficiently activated by a soluble form of ToxR (Crawford *et al*., 2003).

In this study we identified residues in the α-loop of ToxR, especially V71, as being critical for *ompU* promoter activation, while several ToxR mutants had preferential defects in *toxT* promoter activation, including six in the wing domain of ToxR.

Results

We hypothesized that since ToxR appears to function by different mechanisms at the *ompU* and *toxT* promoters, we might be able to isolate *toxR* mutants that affect *ompU* transcription, but not *toxT*. Identification of such mutants would indicate which domain(s) of ToxR is critical to directly activate transcription at the *ompU* promoter.

Isolation of toxR alleles defective for ompU activation. To isolate *toxR* mutants defective for *ompU* activation, an *ompU-sacB* transcriptional fusion strain was constructed in which an *ompU-sacB* fusion was inserted at the *lacZ* locus of the Δ*toxR* classical *V. cholerae* strain EK307 (EK406 derived from EK307; Table 3-4). The Δ*toxR ompU-sacB* selection strain is able to grow on 5% sucrose, however, upon introduction of a wild-type copy of *toxR* on a plasmid, this strain becomes sensitive to 5% sucrose. To isolate *toxR* mutants, three pools of mutagenized *toxR*

alleles were introduced into EK406 by electroporation. Transformants were plated on LB plates containing 5% sucrose and colonies capable of growing on 5% sucrose were isolated. The *toxR* encoding plasmids were sequenced, and selected mutants were studied further.

In the course of constructing the *toxR* allele for PCR mutagenesis, a C-terminal (periplasmic) HA-epitope tag was added to the *toxR* coding sequence to allow assessment of protein stability and translation with an anti-HA tag antibody (Materials and Methods).

Quantification of *ompU* activation defects. Once candidate sucrose-resistant *toxR* alleles were identified that were defective for *ompU* activation, their protein stability was assessed by anti-HA epitope tag Western blot analysis (data not shown) and alleles encoding full-length ToxR derivatives were introduced into a *V. cholerae* Δ*toxR ompU-lacZ* reporter strain (EK410) to quantify *ompU* activation defects. Like the *ompU-sacB* selection strain the *ompU-lacZ* reporter was inserted at the *lacZ* locus of *V. cholerae* (9). The various plasmid-encoded ToxR derivatives were tested for their ability to activate *ompU* as compared to wild-type ToxR or an empty vector control (pSK). Only mutants harboring a single amino acid substitution were studied further. If a sequenced *toxR* allele defective for *ompU* activation contained two or more mutations, the mutations were separated by allelic exchange of a restriction fragment into the parental plasmid pSK-*toxR*-HA or by site-directed mutation. Following these reconstructions, 13 mutants from the randomly mutagenized pools were studied. β-galactosidase assays were performed and Miller Units for wild-type ToxR were set at 100% and all mutant derivatives were expressed relative to 100%.

Of these 13 ToxR derivatives, seven showed little or no ability to activate the *ompU* promoter (≤3% wild type activity); ToxR-W64R, ToxR-V71A, ToxR-Q78R, ToxR-L83P, ToxR-

T99K, ToxR-T99R and ToxR-G104S, although ToxR-L83P was somewhat less stable than the other mutants (Fig. 3-1). The remaining six mutants retained 24-79% activity relative to wild-type ToxR.

Effect of ToxR substitutions on *toxT* activation. While the ToxR mutants were isolated based on their reduced activation of the *ompU* promoter, we were also interested in whether any mutants showed a differential effect on *toxT* activation. If the mechanism of *ompU* and *toxT* activation differs, we expected to find some mutants that were defective for *ompU* activation to be less affected for *toxT* activation.

To examine the effect of ToxR substitutions on *toxT* activation, the various plasmid-borne alleles were introduced into the Δ*toxR toxT-lacZ* reporter strain EK1072 (Table 3-4). Of the seven ToxR mutants that showed little or no *ompU-lacZ* expression, all but mutant ToxR-V71A showed little or no activity for *toxT-lacZ* expression as well (Fig. 3-1). While many of these mutants may be affected in their DNA-binding activity (see below), ToxR-V71A seemed to be particularly interesting since it showed only 3% activity on the *ompU* promoter, but maintained 15% activation of the *toxT* promoter (Fig. 3-1). This suggests the ToxR-V71A mutation affects a region of ToxR that is especially important for ToxR to directly activate the *ompU* promoter, but less critical when ToxR plays an accessory role with TcpP to activate the *toxT* promoter. Based on homology with other winged-HTH proteins, residue V71 of ToxR lies within the α-loop that is proposed to directly interact with RNA polymerase to activate transcription of target promoters (Fig 3-2A & B). Thus, some mutations in this region of ToxR would be predicted to affect the direct activation mechanism of ToxR at the *ompU* promoter more than the accessory role at the *toxT* promoter.

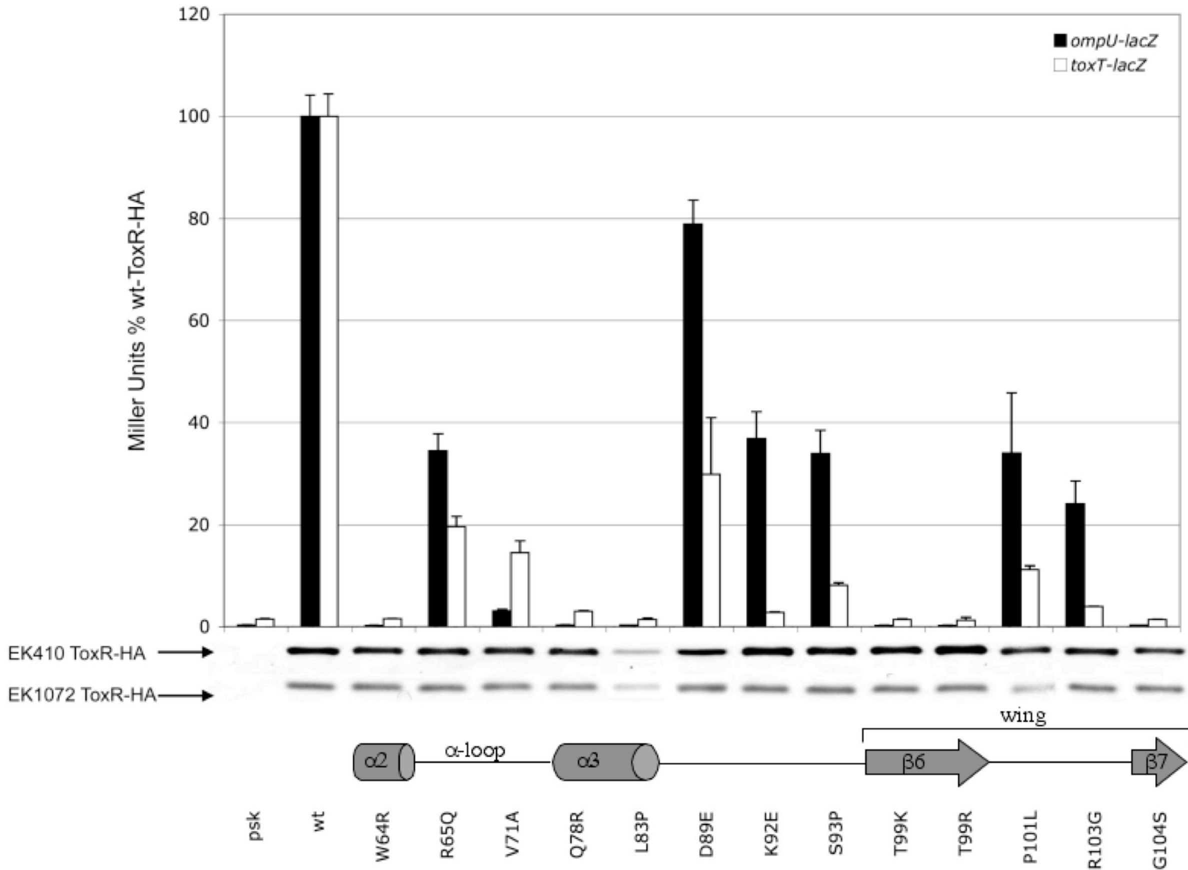


Figure 3-1. Activation of *ompU-lacZ* or *toxT-lacZ* chromosomal reporter constructs in *V. cholerae* by various ToxR mutant derivatives. Δ*toxR V. cholerae* strains were complemented with HA-tagged wild-type ToxR, the empty vector pSK Bluescript, or various ToxR mutant proteins. β-galactosidase activity was measured after a 3-4 hour induction with 100mM IPTG at 30°C. Miller units were expressed as percent, relative to activation seen with wild-type ToxR-HA. Data shown are from at least two experiments performed in triplicate. ToxR-HA from the same samples used in the β-galactosidase assay was detected with an anti-HA antibody to assess protein stability.

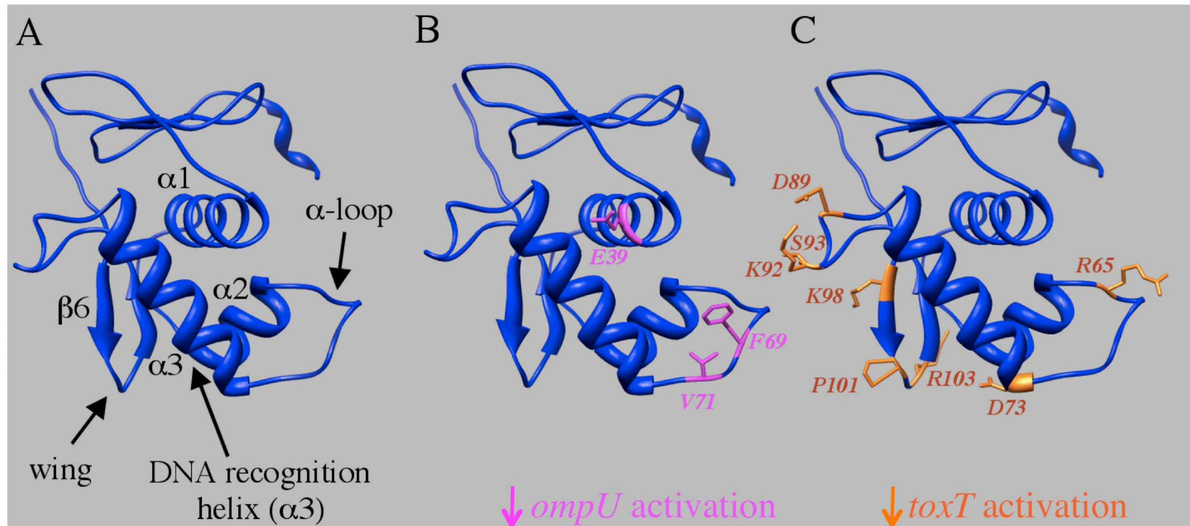


Figure 3-2. Modeling of the N-terminal DNA-binding/transactivation domain of ToxR (residues 1-114) using threading of the ToxR sequence onto structurally related winged-HTH family members. A) labeling of putative domains of interest within the ToxR transcription activation and DNA-binding domain, model assembled using I-TASSER (2-4) B) homology modeling of residues within ToxR that affect *ompU* promoter activation more dramatically than *toxT* promoter activation. C) homology modeling of residues within ToxR that affect *toxT* promoter activation more dramatically than *ompU* promoter activation.

In addition to identifying mutant ToxR-V71A that affects *ompU* activation more dramatically than *toxT*, six of the ToxR mutants isolated were more strongly attenuated for *toxT* activation than *ompU*. While this phenotype was not necessarily expected from our selection strategy, we were excited to identify such differential ToxR mutants. Again, such mutants suggest that activation of *ompU* and *toxT* by ToxR have different specific requirements within the transcription activator ToxR. Five of six mutants lie just N-terminal to or within the putative wing domain of the ToxR winged-HTH structure; ToxR-D89E, ToxR-K92E, ToxR-S93P, ToxR-P101L, and ToxR-R103G (Fig. 3-2A & C). The wing domain of winged-HTH proteins can perform different functions including DNA binding and protein-protein interaction, depending on the activator protein (5, 33-35).

When the ToxR sequence is threaded onto the structures of related winged-HTH activator proteins (Fig. 3-2A), five of the amino acid substitutions preferentially affecting *toxT* expression are predicted to lie on a distinct face of ToxR (Fig. 3-2C) from amino acids required for efficient *ompU* activation in the α-loop (Fig. 3-2B). We did identify one α-loop mutant, ToxR-R65Q, in the *ompU-sacB* screen that was preferentially defective for *toxT* activation, but it was the least preferential (<2-fold differential, Fig. 3-1 and 3-2C).

DNA binding activity of ToxR mutant derivatives. Six of the 13 isolated ToxR mutants were severely defective for activation of both the *ompU* and *toxT* promoters. These include, ToxR-W64R, ToxR-Q78R, ToxR-L83P, ToxR-T99K, ToxR-T99R and ToxR-G104S. One simple explanation for the defect in activation by these mutants is that they fail to recognize either promoter. In fact, two of the seven mutants lie within the putative DNA-recognition helix (α3, Fig. 3-1 & 3-2A) of ToxR (ToxR-Q78R and ToxR-L83P).

We first assessed binding to the *ompU* promoter. *V. cholerae* lacking endogenous copies of *toxR* and *tcpP* (strain EK459, Table 3-4) was transformed with the pSK-*toxR-HA* plasmid encoding each *toxR* mutant allele. Membranes were prepared from these strains (18) and used in a gel mobility shift assay with a radiolabeled fragment of the *ompU* promoter from -211 to +22 relative to the transcription start site. This fragment has been shown previously to contain ToxR-binding sites (30).

ompU promoter probes were mixed with 0.05 or 0.25mg/ml of total membrane proteins. While negative control membranes (pSK, vector alone) gave some shifting of the probe in the absence of ToxR (Fig. 3-3A, asterisk), ToxR-mediated binding could be distinguished because ToxR causes retention of the probe in the well (membranes are retained in the well in this assay, Fig. 3-3A). This experiment demonstrated that the six ToxR derivatives severely defective for both *ompU* and *toxT* activation were unable to bind the *ompU* promoter. These include ToxR-W64R, ToxR-Q78R, ToxR-L83P, ToxR-T99K, ToxR-T99R and ToxR-G104S. To confirm that sufficient amounts of ToxR were available to bind the *ompU* promoter, Western blot analysis was performed to determine the relative amount of each ToxR derivative present in each gel-shift reaction. Levels of all 14 ToxR proteins (including wild-type) were similar (Fig. 3-3A). As a negative control probe for these experiments we used a fragment of the *toxT* promoter lacking a ToxR-binding site (from -46 to +45).

Most ToxR derivatives that demonstrated an intermediate *ompU* activation defect (Fig. 3-1) showed either modest or no DNA-binding defect for the *ompU* promoter (Fig. 3-3A). While ToxR-R65Q, ToxR-V71A and ToxR-K92E were clearly not as efficient as wild-type ToxR for *ompU* promoter binding (even with ToxR-K92E being present at higher protein levels than wild-type ToxR), ToxR-P101L and ToxR-R103G were somewhat less obvious in their DNA-binding

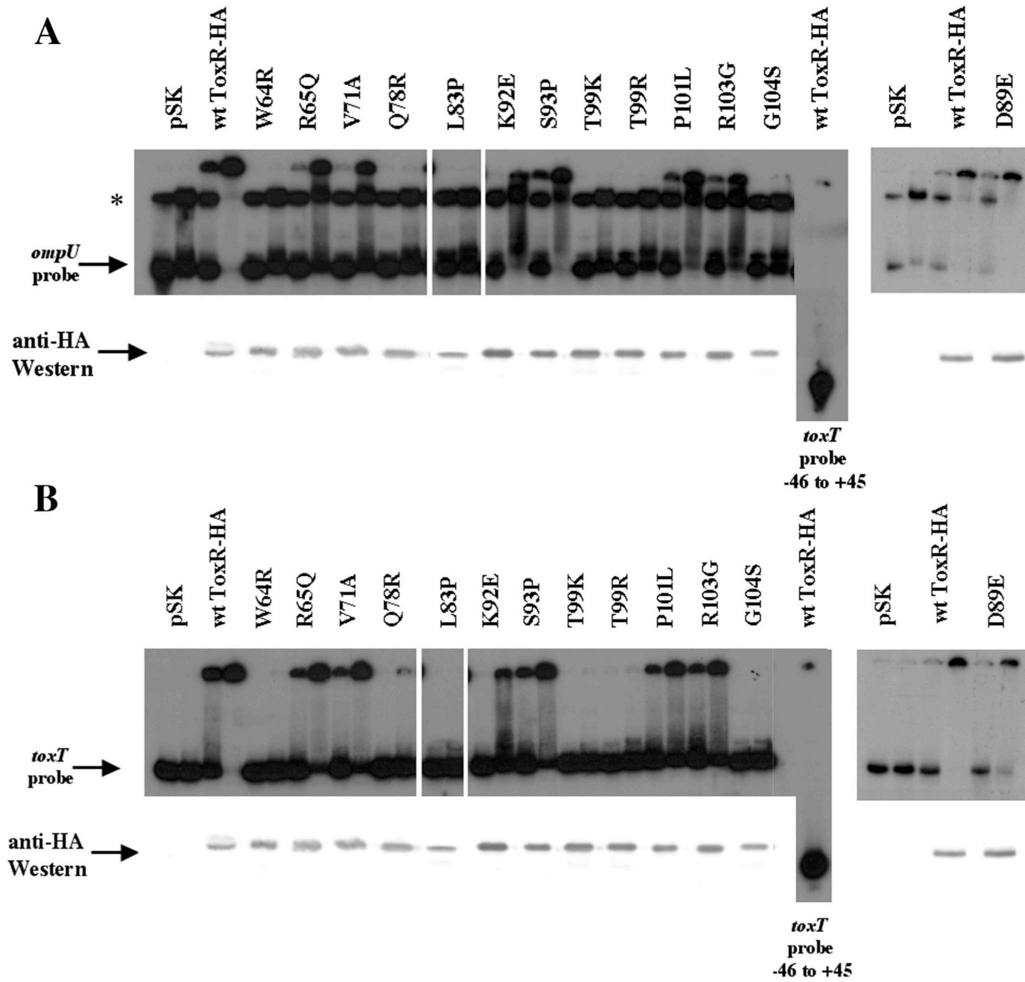


Figure 3-3. Gel-shift assays to assess promoter recognition by various ToxR mutant proteins identified in a random mutagenesis *ompU-sacB* selection strategy. *V. cholerae* membranes prepared from a Δ*toxR*Δ*tcpP V. cholerae* strain (EK459) expressing each mutant ToxR derivative (or wild-type ToxR-HA) were mixed at 0.05 and 0.25 mg/ml (two lanes for each sample) with radiolabeled *ompU* promoter DNA (A) or 0.1 and 0.5 mg/ml with *toxT* promoter DNA (B) prior to running samples in a non-denaturing PAGE.

defects. Finally, ToxR-D89E and ToxR-S93P bind the *ompU* promoter similar wild-type ToxR, although ToxR-S93P does not retain all of the *ompU* probe in the well (some smearing).

Since the ToxR derivatives with intermediate *ompU* activation defects all have differential effects on *toxT* activation (Fig. 3-1), we examined whether these ToxR derivatives have differential defects in *toxT* promoter binding as compared to the *ompU* promoter. A *toxT* promoter probe (from -172 to +45) containing the ToxR-binding site was radiolabeled and used in a gel-shift assay with the same membranes used for the *ompU* promoter gel-shift assays (Fig. 3-3B). The six mutants defective for both *ompU* and *toxT* activation (ToxR-W64R, ToxR-Q78R, ToxR-L83P, ToxR-T99K, ToxR-T99R and ToxR-G104S) were also defective for *toxT* promoter binding.

The two α-loop mutants, ToxR-R65Q and ToxR-V71A showed intermediate defects in *toxT* promoter binding, consistent with their ability to activate the *toxT* promoter to 15-20% of wild-type ToxR activity (Fig. 3-1).

The five ToxR mutants in the wing domain (or just N-terminal to the wing) had intermediate *toxT* promoter-binding defects (Fig. 3-3B). These wing and wing-proximal mutants appear slightly more defective for *toxT* than *ompU* promoter binding (Fig, 3-3A vs. 3-3B). However, the level of their *toxT* activation defect is not solely determined by their DNA-binding defect. Specifically, ToxR-P101L and ToxR-R103G have similar *toxT* promoter DNA-binding defects, yet ToxR-R103G is 3-fold more defective for *toxT* activation than ToxR-P101L. Thus, this region may perform a role in addition to DNA binding that affects *toxT* transcription activation. While ToxR-K92E maintains some (albeit modest) DNA-binding activity on the *ompU* and *toxT* promoters (Fig. 3-3), it has a much stronger defect in *toxT* activation than *ompU* activation (Fig. 3-1). This could reflect a critical role for residue K92 in *toxT* activation or the

fact that *toxT* promoter activation is more sensitive to ToxR mutations affecting DNA-binding affinity. Finally, mutant ToxR-D89E showed nearly wild-type binding to both the *ompU* and *toxT* promoters, with a very slight defect in *toxT* binding (Fig. 3-3). This mutant can only activate the *toxT* promoter to about 30% the level of wild-type ToxR, while *ompU* activation shows 79% of wild-type activity. This suggests this conservative amino acid substitution is particularly detrimental to ToxR/TcpP-mediated activation of the *toxT* promoter either due to a slight defect in *toxT* promoter binding or some other *toxT* activation function.

Characterization of previously identified ToxR mutant derivatives. Over the years a number of ToxR mutant derivatives have been constructed based on homology modeling of specific residues with the OmpR/PhoB of transcription activators (7) or isolated from a mutagenesis screen that affected the accessibility of ToxR to periplasmic proteases (10). Comparing activities of the various mutants has been somewhat complicated by the fact that authors focus on different promoters for assessing ToxR activity such as *ctx-lacZ* fusions (7, 10, 36-38), a promoter that is now thought to be directly activated by ToxT rather than ToxR in *V. cholerae* (25, 26, 28). In other cases, *ompU* promoter activity was based on the levels of OmpU or OmpT protein as measured in a semi-quantitative fashion on Coomassie gels. Thus, to standardize comparison of the activities of these various ToxR derivatives, we constructed several of these mutants in our ToxR-HA expression system and tested them for activation of our *ompU-lacZ* and *toxT-lacZ* reporters. In this way we could directly compare activation levels with β-galactosidase assays and determine protein levels by anti-HA Western blots.

Results for various constructed ToxR-HA mutants are presented in Table 3-1. The initial set of ToxR-HA mutants tested were based on studies by Ottemann *et. al.* (7) where several

Table 3-1: Activation phenotypes of ToxR derivatives from various mutagenesis studies

ToxR derivative	location	*ompU-lacZ*	*toxT-lacZ*
Controls			
ToxR-HA wild-type	-	100% ± 5.6	100% ± 6.9
pSK	-	0.3% ± 0.03	1.6% ± 0.1
Ottemann mutants			
ToxR-E39K-HA	α1	18.6% ± 1.2	104.5% ± 8.2
ToxR-S55A-HA	β5	94.6% ± 2.4	111.8% ± 3.3
ToxR-R56K-HA	α2	1.2% ± 0.2	2.0% ± 0.1
ToxR-R56L-HA	α2	0.3% ± 0.009	1.8% ± 0.1
ToxR-R65L-HA	α-loop	39.4% ± 0.9	32.3% ± 1.9
ToxR-R84K-HA	α3/DNA-binding domain	0.3% ± 0.03	1.9% ± 0.1
ToxR-R84L-HA	α3/DNA-binding domain	0.3% ± 0.03	1.8% ± 0.04
ToxS-blind mutants			
ToxR-K85E-HA	α3/DNA- binding domain	0.3% ± 0.02	2.0% ± 0.05
ToxR-D89N-HA	between α3 & wing domain	1.0% ± 0.02	2.4% ± 0.1
ToxR-T99M-HA	wing domain	0.4% ± 0.04	2.1% ± 0.1
***toxT-lacZ* white mutants**			
ToxR-R84C-HA	α3/DNA- binding domain	0.3% ± 0.02	2.0% ± 0.05
ToxR-D89Y-HA	between α3 & wing domain	0.5% ± 0.09	2.1% ± 0.2
ToxR-K98E-HA	wing domain	65.7% ± 6.8	25.8% ± 1.2

ToxR residues conserved in OmpR were targeted for mutagenesis. These mutants had been tested previously for their ability to activate a *ctx-lacZ* fusion in *E. coli*, to alter outer membrane protein profiles from OmpU to OmpT in *V. cholerae* and for the ability to direct cholera toxin production in *V. cholerae* (presumably via the TcpP-dependent activator, ToxT). One should note that since the studies by Ottemann *et. al.*, Pfau and Taylor mapped the N-terminus of the ToxR protein and it begins at the second ATG predicted start codon (39), thus we have renumbered the reconstructed ToxR-HA mutants to represent the position of the mutation.

Ottemann *et. al.* noted previously that ToxR-E39K (E51K, by their numbering system) failed to activate a *ctx-lacZ* fusion in *E. coli* and showed an altered OmpU/OmpT profile suggesting ToxR-E39K was defective for transcription activation of *ompU*, but maintained *ompT* repression indicating DNA-binding was unaffected. Our studies confirm that ToxR-E39K has only 19% activity at the *ompU* promoter, yet it still maintains wild-type levels (105%) activity at the *toxT* promoter. Our findings suggest this mutation affects the direct activation of *ompU*, while having no effect on TcpP-dependent activation of *toxT*. This further emphasizes the different mechanism used by ToxR for activation of these two promoters. DNA-binding studies with the ToxR-E39K mutant suggest it is partially defective for DNA binding (Fig. 3-4). Homology modeling of the ToxR-E39K mutant places it in the first a helix (α1) of the PhoB/OmpR-type DNA-binding and transcription activation domain (Fig. 3-2). This region does not directly bind DNA, but is a scaffolding helix that is packed against helix 3 (the DNA-recognition helix) and the more C-terminal wing domain (33, 40, 41). As such, the ToxR-E39K mutation may affect proper orientation of these two domains as well as the orientation of the transactivating α-loop. Alternatively, portions of the E39 side-chain may directly contact RNA polymerase when ToxR is bound to the *ompU* promoter.

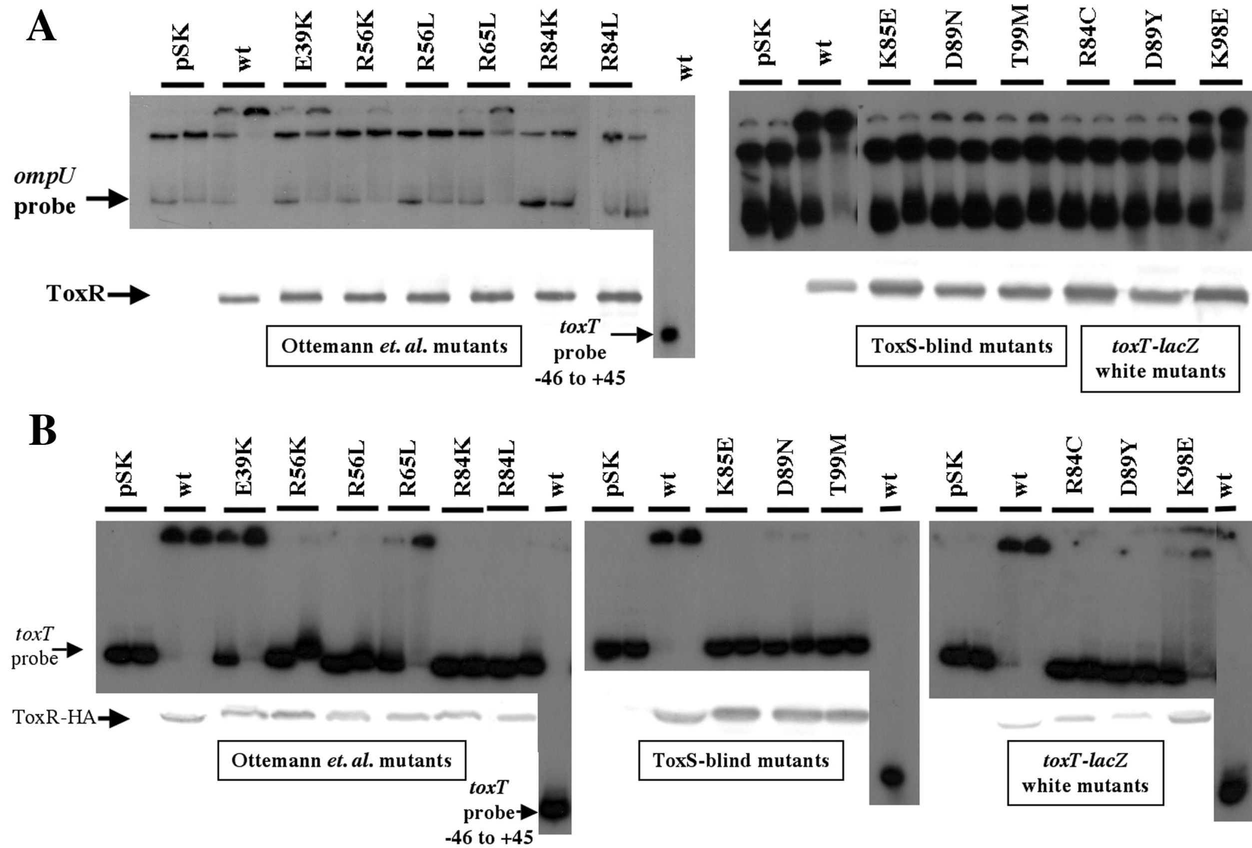


Figure 3-4. Gel-shift assays to assess promoter recognition by various ToxR mutant proteins identified previously (7, 10) or in a random mutagenesis *toxT-lacZ* blue-white screen (this study). *V. cholerae* membranes prepared from a Δ*toxR*Δ*tcpP V. cholerae* strain (EK459) expressing each mutant ToxR derivative (or wild-type ToxR-HA) were mixed at 0.05 and 0.25 mg/ml with radiolabeled *ompU* promoter DNA (A) or 0.1 and 0.5 mg/ml with *toxT* promoter DNA (B) prior to running samples in a non-denaturing PAGE.

Four *toxR* mutants that Ottemann *et. al.* showed to have little or no activity on the *ompU* or *ctx* promoter and led to almost no cholera toxin (CT) production (ToxR-R56K, ToxR-R56L, ToxR-R84K and ToxR-R84L; (7)) showed no activity at either the *ompU* or *toxT* promoters using our *lacZ* reporter strains (Table 3-1). DNA-binding assays demonstrated three out of four derivatives (ToxR-R56L, ToxR-R84K and ToxR-R84L) lack DNA-binding activity for both promoters even though the proteins were stably expressed in the membrane of *V. cholerae* (Figs. S1A & B). One mutant derivative, ToxR-R56K, had weakly detectable binding to the *ompU* and *toxT* promoters (Fig. 3-4). However, ToxR-R56K failed to activate either promoter. Thus, the level of DNA-binding observed with the ToxR-R56K mutant is insufficient for promoter activation.

The final mutant that we reconstructed from Ottemann's studies was ToxR-R65L which was shown previously to have a partial defect for *ompU* activation based on outer membrane protein profiles and was 2 to 3-fold decreased for CT production. We also found that ToxR-R65L was partially defective for both *ompU* and *toxT* activation with activation levels of 39% and 32% of wild-type, respectively (Table 3-1). This mutation lies within the putative α-loop and behaves similarly to our previously generated alanine mutant at this position (ToxR-R65A, Table 2) and mutant ToxR-R65Q from our random mutagenesis and sucrose selection (Fig. 3-1). ToxR-R65L (Table 3-1), ToxR-R65A (Table 3-2) and ToxR-R65Q (Fig. 3-3) had modest DNA-binding defects that may explain their partially impaired abilities to activate both the *ompU* and *toxT* promoters (Fig. 3-5, 3-4 and 3-1, respectively).

In addition to the mutants generated by Ottemann *et. al.* we also reconstructed three ToxR mutants that were originally isolated from a screen for ToxR derivatives that were susceptible to periplasmic cleavage (resulting in increased activity of an alkaline phosphatase

fusion domain) even in the presence of ToxS, that normally protects ToxR from degradation in *E. coli* (10). Such "ToxS-blind" alleles were mapped to the cytoplasmic domain of ToxR (10). All three ToxS-blind derivatives of ToxR tested (ToxR-K85E, ToxR-D89N and ToxR-T99M) were severely defective for *ompU* and *toxT* activation (Table 3-1). DNA-gel shift assays revealed that while the proteins are stably expressed, one ToxR derivative (ToxR-K85E) is completely defective for DNA binding activity and derivatives ToxR-D89N and ToxR-T99M maintain only weak binding to the *ompU* promoter (Fig. 3-4A) and negligible binding to the *toxT* promoter (Fig. 3-4B).

Characterization of three mutants isolated based on decreased activation of a toxT-lacZ fusion reporter. The last group of mutants generated in this study was isolated from a randomly mutagenized pool of pSK-*toxR-HA* alleles for their inability to active a *toxT-lacZ* fusion in *V. cholerae*. For these studies we used a blue/white screen to identify random ToxR mutant derivatives that were defective for activation of the *toxT* promoter. Three mutants were identified in an initial screen that affected *toxT* activation, ToxR-R84C, ToxR-D89Y and ToxR-K98E. ToxR-R84C and ToxR-D89Y were both completely defective for both *toxT* and *ompU* activation (Table 3-1), whereas ToxR-K98E maintained some activation of both promoters, yet showed a preferential defect (26% activation vs. 66%) for *toxT* activation as opposed to *ompU*. Like other mutants from our *ompU-sacB* selection that showed preferential *toxT* activation defects, ToxR-K98E affects a residue in the putative wing domain of ToxR (Fig. 3-2C).

Mutant derivatives ToxR-R84C and ToxR-D89Y were unable to bind either the *toxT* or *ompU* promoters (Fig. 3-4), thus explaining their inability to activate these promoters. Residue K98 clearly plays a role in DNA-binding (Fig. 3-4), as would be expected by its location in the

wing domain (33). However, based on its preferential defect in *toxT* activation as opposed to *ompU* activation, it may also play a role in TcpP-mediated activation of the *toxT* promoter.

The role of α-loop residues on ompU and toxT activation by ToxR. Since we found one ToxR residue essential for *ompU* expression in the α-loop of ToxR, ToxR-V71 (Fig. 3-1), we performed alanine-scanning mutagenesis of the entire the putative α-loop (R65-S75). Mutations were constructed in the pSK-*toxR-HA* vector and expressed in the either the *ompU-lacZ* (EK410) or *toxT-lacZ* (EK1072) reporter strains to assess transcription activation activity.

One additional mutant ToxR protein, ToxR-F69A, showed a >two-fold preferential defect for *ompU* expression. It activated *ompU-lacZ* to 35% of wild-type levels and *toxT-lacZ* to 92% of wild-type (Table 3-2). Three mutants, ToxR-R65A, ToxR-G68A and ToxR-S74A, showed decreases in both *ompU* and *toxT* expression, ToxR-G68A and ToxR-S74A being slightly more defective for *ompU* than *toxT* activation (Table 3-2). One mutant protein, ToxR-S75A, showed little or no activation of either *ompU* or *toxT*. This residue approaches the predicted DNA-binding helix of ToxR and disrupts the DNA-binding activity of ToxR (see below).

One alanine mutant in the α-loop, ToxR-D73A, showed a more dramatic defect on *toxT* (41% of wild-type) than on *ompU* (93% of wild-type) expression. When residue D73 of ToxR is modeled onto homologous winged-HTH transcription factor structures, ToxR residues F69 and V71 are oriented away from the α-loop in one direction, while residue D73 is predicted to be oriented in the opposite direction, toward residues P101 and R103 (Fig. 3-2C). Our random mutagenesis selection identified mutations in these latter two residues, ToxR-P101L and ToxR-R103G, that are also more dramatically affected for *toxT* expression than *ompU*. Thus, this face

Table 3-2. Activation phenotypes of ToxR α-loop mutants. Miller Units measured in strains EK410 (O395 Δ*toxR ompU-lacZ*) and EK1072 (O395 Δ*toxR toxT-lacZ*). Data are from one representative experiment performed in triplicate.

ToxR derivative	location	*ompU-lacZ*	*toxT-lacZ*
ToxR-HA wild-type	-	100.0% ± 5.6	100.0% ± 6.9
pSK (vector)	-	0.3% ± 0.03	1.6% ± 0.1
ToxR-R65A	α-loop	51.3% ± 2.9	43.1% ± 1.4
ToxR-E66A	α-loop	103.3% ± 4.6	106.4% ± 2.0
ToxR-Q67A	α-loop	101.4% ± 3.6	164.5% ± 3.9
ToxR-G68A	α-loop	12.0% ± 0.9	19.2% ± 2.3
ToxR-F69A	α-loop	35.2% ± 1.1	91.6% ± 3.0
ToxR-E70A	α-loop	79.5% ± 3.4	91.7% ± 3.1
ToxR-D72A	α-loop	102.8% ± 2.5	118.0% ± 9.5
ToxR-D73A	α-loop	92.9% ± 0.6	40.8% ± 15.3
ToxR-S74A	α-loop	47.4% ± 5.1	63.1% ± 6.7
ToxR-S75A	α-loop/DNA- binding domain	0.4% ± 0.02	4.5% ± 0.3

of the ToxR molecule appears to play a critical role in *toxT* activation in conjunction with TcpP, while V71 and F69 of the α-loop are more important for *ompU* activation by ToxR.

These alanine mutant studies also revealed that the preferential defect of ToxR-R65Q on *toxT* activation (Fig. 3-1) was largely side-chain dependent since the ToxR-R65A mutant had more similar defects in both *ompU* and *toxT* activation (albeit still slightly more defective for *toxT* activation, Table 3-2).

Promoter recognition by α-loop alanine mutants. Since some of our α-loop ToxR alanine mutants have activation defects, we assessed the DNA-binding activity of the various mutant proteins. Membranes were isolated from a *V. cholerae* Δ*toxR*Δ*tcpP* mutant (EK459) expressing various ToxR derivatives and the membrane preparations were used in gel-shift assays on the *ompU* (Fig. 3-5A) or *toxT* (Fig. 3-5B) promoter.

While wild-type ToxR shifted about 50-75% of the *ompU* probe at the lower membrane concentration (Fig. 3-5A), three mutant proteins partially defective for *ompU* activation (ToxR-R65A, ToxR-G68A, and ToxR-S74A; Table 3-2) had somewhat weaker binding to the *ompU* promoter. ToxR-S75A is unable to activate either the *ompU* or *toxT* promoter (Table 3-2) and is severely defective for DNA-binding (Fig. 3-5). Thus, it may define the first residue of the DNA-binding helix of ToxR. Finally, ToxR-F69A maintains wild-type levels of binding to the *ompU* promoter (Fig. 3-5A) and displays slightly reduced binding to the *toxT* promoter (Fig. 3-5B). However, ToxR-F69A is preferentially defective for *ompU* activation (Table 3-2). Thus, residue F69 (like V71 identified in our original random mutagenesis selection) appears to be particularly important for ToxR to directly activate RNAP at the *ompU* promoter.

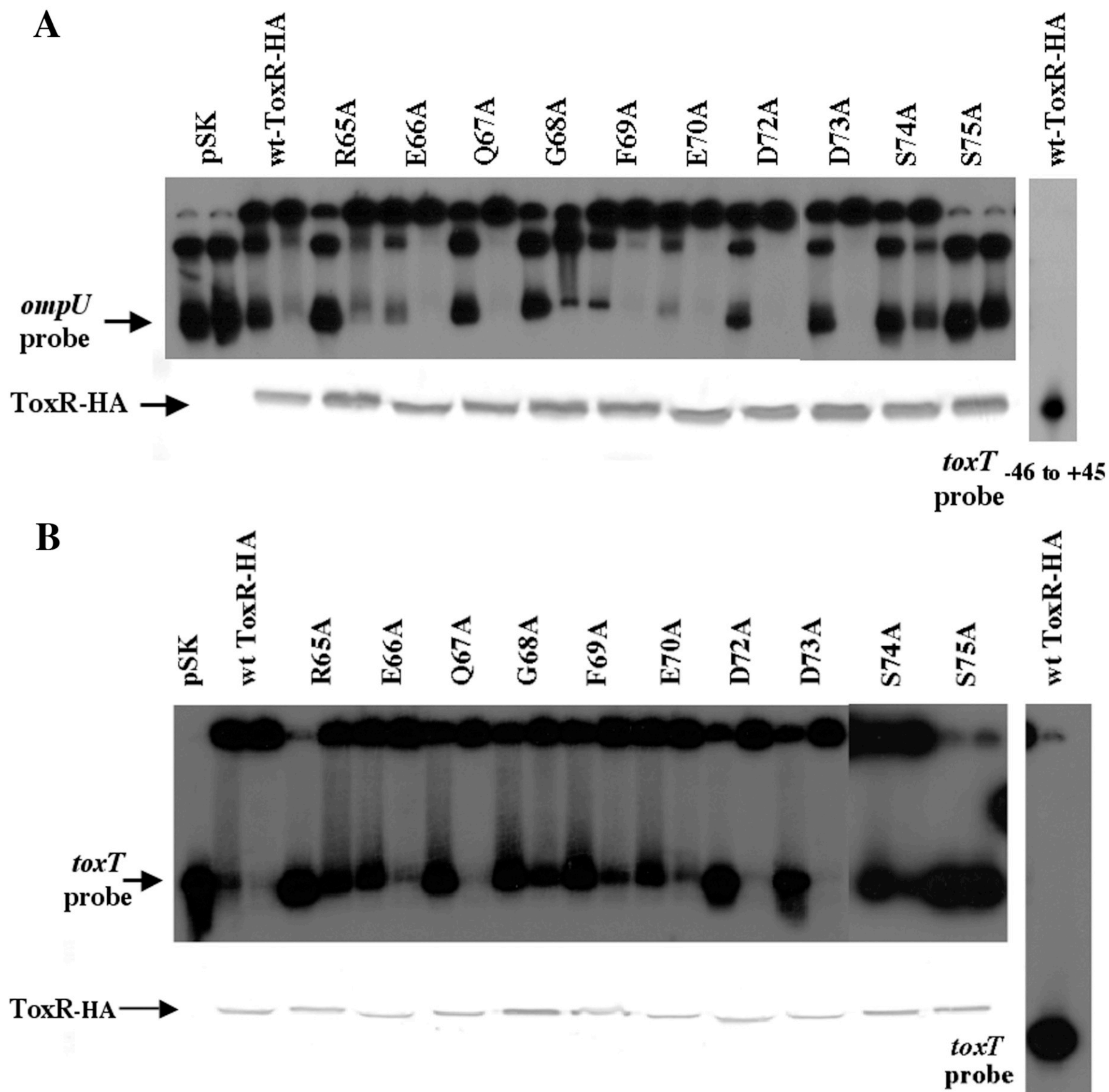


Figure 3-5. Gel-shift assays to assess promoter recognition by putative α-loop site-directed ToxR mutant proteins. *V. cholerae* membranes prepared from a Δ*toxR*Δ*tcpP V. cholerae* strain (EK459) expressing each mutant ToxR derivative (or wild-type ToxR-HA) were mixed at 0.05 and 0.25 mg/ml with radiolabeled *ompU* promoter DNA (A) or 0.2 and 1.0 mg/ml with *toxT* promoter DNA (B) prior to running samples in a non-denaturing PAGE.

Effect of specific ToxR wing residue substitutions on differential promoter activation. Since alanine scanning mutagenesis of the α-loop indicated some preferential defects were side-chain dependent (R65Q vs. R65A, Fig. 3-1 and Table 3-2), we determined whether the preferential *toxT* activation defects of certain ToxR wing mutants were due to the specific side-chain substitutions, each residue was mutated to alanine (ToxR-D89A, ToxR-K92A, ToxR-S93A, ToxR-K98A, ToxR-P101A and ToxR-R103A). These ToxR derivatives were then tested for *ompU* and *toxT* activation in *V. cholerae*. While the absolute levels of *ompU* and *toxT* activation changed in the alanine mutants relative to the original side-chain substitution mutants, five of six wing mutants tested maintained preferential defects in *toxT* activation; ToxR-K92A, ToxR-S93A ToxR-K98A, ToxR-P101A and ToxR-R103A (Table 3-3), indicating these *toxT*-specific defects were not side-chain dependent.

The ToxR-D89A mutant was completely defective for both *ompU* and *toxT* activation (Table 3-3) whereas the original conservative mutant, ToxR-D89E, maintained some activation of *ompU* and *toxT* (although it was preferentially *toxT*-defective, Fig. 3-1). All six alanine wing mutants were stably expressed similar to wild-type ToxR (data not shown).

Mutant ToxR-P101L in the putative wing domain of ToxR is deficient in ToxR-TcpP crosslinking. Given that ToxR appears to play a supporting role for activation of the *toxT* promoter by assisting direct activation by TcpP (5), we tested our various ToxR wing domain mutants that were preferentially affected for *toxT-lacZ* activation, for their ability to interact with TcpP in the membrane of *V. cholerae*. We used a crosslinking capture ELISA protocol described previously (5) for assessing the interaction of a number of ToxR mutant derivatives with wild type TcpP. For these experiments a *V. cholerae* Δ*tcpP* strain (RY1) was first modified to harbor each *toxR* allele of interest on the chromosome at the *toxR* locus. The resulting strains

Table 3-3. Affect of side chain substitutions in the wing domain on promoter activation. Miller Units measure in strains EK410 (O395 Δ*toxR ompU-lacZ*) and EK1072 (O395 Δ*toxR toxT-lacZ*). * Data same as that shown in Fig. 3-1. ** Data same as that shown in Table 3-1. Data are from at least two experiments performed in triplicate.

ToxR derivative	*ompU-lacZ* (% wild-type)	*toxT-lacZ* (% wild-type)
ToxR-HA wild type	100.0 ± 3.5	100.0 ± 5.0
pSK (vector)	0.3 ± 0.04	1.4 ± 0.07
ToxR-D89E-HA *	78.9 ± 4.7	29.8 ± 11.1
ToxR-D89A-HA	1.2 ± 0.23	1.7 ± 0.1
ToxR-K92E-HA *	36.9 ± 5.2	2.8 ± 0.1
ToxR-K92A-HA	73.8 ± 15.6	33.4 ± 3.9
ToxR-S93P-HA *	33.9 ± 4.6	8.1 ± 0.6
ToxR-S93A-HA	104.0 ± 6.9	21.4 ± 3.4
ToxR-K98E-HA **	37.3 ± 7.3	17.0 ± 0.5
ToxR-K98A-HA	74.1 ± 6.6	18.0 ± 1.0
ToxR-P101L-HA *	34.0 + 11.8	11.2 + 0.8
ToxR-P101A-HA	52.2 ± 16.0	10.3 ± 0.4
ToxR-R103G-HA *	24.0 + 4.5	3.9 + 0.1
ToxR-R103A-HA	49.3 ± 17.5	30.0 ± 4.1

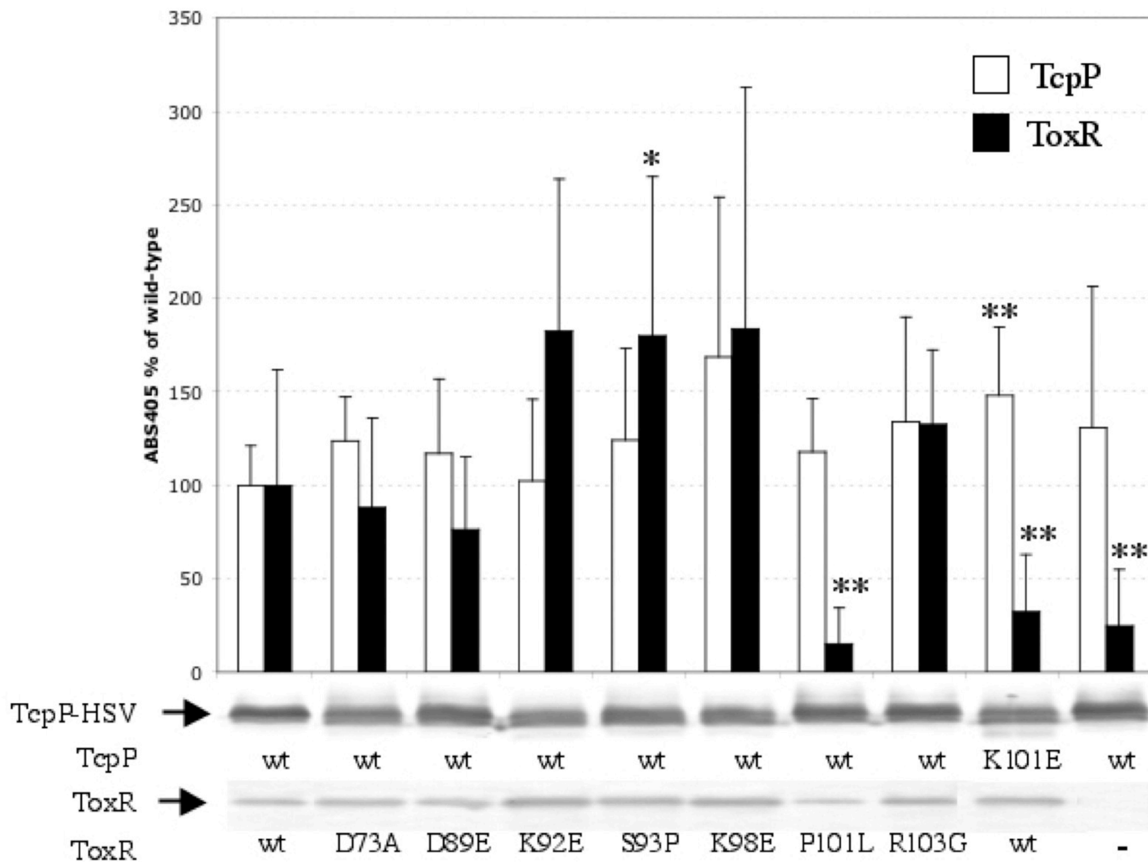


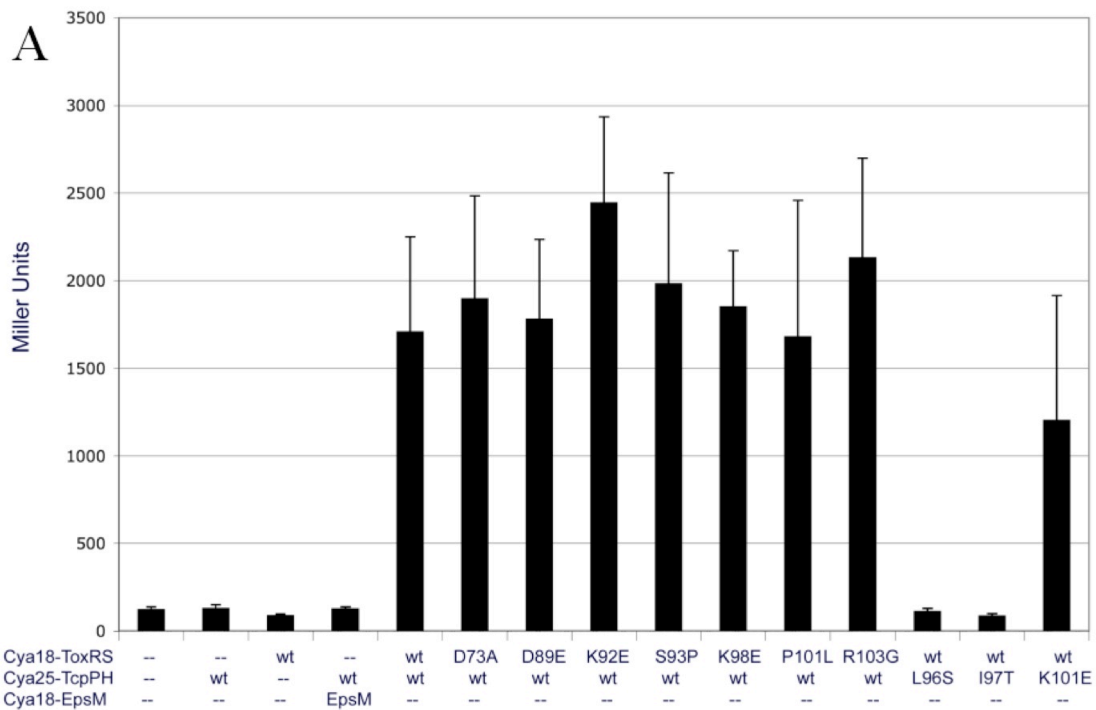
Figure 3-6. Assessment of TcpP protein interaction by various ToxR mutant proteins that preferentially affect *toxT* transcription. *V. cholerae* membranes from cells expressing each ToxR mutant derivative were assessed for their ability to interact with wild-type TcpP-HSV or TcpP-K101E. ToxR mutant proteins were expressed from the chromosomal *toxR* locus while TcpP-HSV was expressed from the IPTG-inducible plasmid pEK41 in a Δ*tcpP V. cholerae* background. TcpP-HSV was captured to 96-well microtiter plates using an anti-HSV monoclonal antibody. TcpP capture was assessed by probing the wells with an anti-TcpP polyclonal antibody (5, 6), while ToxR co-capture was assessed by probing parallel wells with an anti-ToxR polyclonal antibody. Levels of each ToxR mutant protein and TcpP-HSV were assessed by Western blot on the same protein extracts used for crosslinking. Statistical comparisons were made using the students' t-test and compare samples relative to a strain expressing wild-type ToxR and wild-type TcpP-HSV. * $p=0.05$, ** $p<0.02$

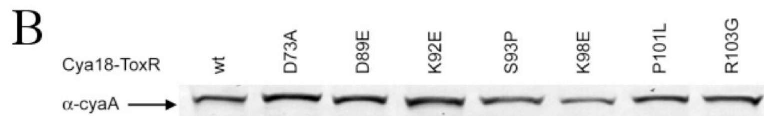
were transformed with an HSV epitope-tagged version of TcpP (encoded on pEK41, (1)). The interaction between ToxR and TcpP-HSV was assessed using *V. cholerae* membranes harvested after a six-hour IPTG induction step to allow for TcpP-HSV expression and membrane insertion.

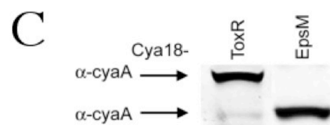
We tested the ability of seven ToxR mutant proteins with preferential defects in *toxT* activation to interact with wild-type TcpP: ToxR-D73A, ToxR-D89E, ToxR-K92E, ToxR-S93P, ToxR-K98E ToxR-P101L, and ToxR-R103G (six of which lie adjacent to or in the putative wing domain). As a negative control we assessed the levels of interaction between wild-type ToxR and a TcpP mutant protein, TcpP-K101E, shown previously to be defective for ToxR interaction (5).

Of the ToxR mutants tested, only ToxR-P101L (located at the tip of the wing domain) showed reduced levels of interaction with TcpP, with about 15% of wild-type levels of interaction (Fig. 3-6). The level of ToxR-P101L interaction with TcpP was as low as the previously characterized weak interaction between ToxR and TcpP-K101E, although ToxR-P101L was somewhat less stable than wild-type ToxR and this may contribute to the reduced level of ToxR/TcpP interaction (Fig. 3-6). One other mutant, ToxR-D89E, showed a trend towards decreased TcpP interaction, but the defect did not reach statistical significance (Fig. 3-6, $p=0.07$). All other ToxR mutants maintained wild-type (or in some cases increased) levels of TcpP interaction (Fig. 3-6), despite their reduced levels of *toxT-lacZ* activation (Fig. 3-1, Tables 3-1 & 3-2)

As the ToxR/TcpP capture assay is dependent upon lysine-mediated crosslinking, we established a second assay for ToxR/TcpP interaction, a membrane-bound ToxR and TcpP bacterial two-hybrid reporter system. This system is based on the reconstitution of a split adenylate cyclase enzyme of *Bordetella pertussis*, which upon ToxR/TcpP interaction brings the







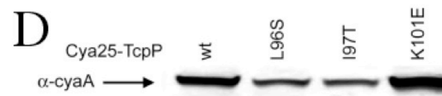


Figure 3-7. Assessment of ToxR/TcpP interaction by various ToxR and TcpP mutants using an adenylate cyclase-based membrane-anchored bacterial two-hybrid system. A) Interaction between wild-type TcpPH fused to the cya25 fragment of *B. pertussis* CyaA and ToxRS fused to the cya18 fragment of CyaA were assessed in strain BTH101. Interaction was determined by measuring β-galactosidase activity produced as a result of reconstitution of a functional adenylate cyclase enzyme. Known non-interacting mutants of TcpP were fused to cya25 as controls. As additional controls cya18-ToxR and cya25-TcpP alone were tested B) Western blot analysis of ToxR mutant derivatives fused to cya18. C) Western blot analysis of wild-type ToxR and EpsM fused to cya18. D) Western blot analysis of TcpP fused to cya25. All western blots were probed with rabbit anti-CyaA antibodies (Santa Cruz Biotechnology).

two Cya fragments together leading to cAMP generation and increased β-galactosidase production in *E. coli* (12, 42). Unlike the crosslinking assay described above, this assay does not rely upon primary amines for DSP-mediated crosslinking. Using the bacterial two-hybrid system we were unable to detect a significant defect in ToxR-P101L interaction with TcpP (Fig. 3-7), suggesting the defect in interaction in the crosslinking assay may be due to alterations in the orientation of lysines in the vicinity of P101.

These data indicate that ToxR-P101L maintains interaction with TcpP, but in an altered form as compared to wild-type ToxR. It should be noted that the bacterial two-hybrid assay is performed using plasmids expressing ToxR and TcpP in *E. coli* and the levels of ToxR-P101L may be higher than was achieved in *V. cholerae* when ToxR-P101L was expressed from its normal chromosomal locus. None of the other ToxR wing domain mutants tested had a defect in the bacterial two-hybrid assay (Fig. 3-7).

Two previously characterized TcpP mutants, TcpP-L96S and TcpP-I97T, that failed to interact with ToxR in the capture assay (5), were completely defective for ToxR/TcpP interaction in the bacterial two-hybrid assay (Fig. 3-7A), while a third TcpP mutant, TcpP-K101E, completely defective for ToxR crosslinking (Fig. 3-6, (5)), was only partially defective in the bacterial two-hybrid assay (Fig. 3-7A; $p=0.035$). This mutant may have lost some level of crosslinking in the capture assay (Fig. 3-6) due to substitution of the lysine, which carries a primary amine group. While TcpP-L96S and TcpP-I97T were less stable than wild-type TcpP in this system, it should be noted that some ToxR mutants (ToxR-S93P and ToxR-K98E) were similarly less stable and maintained robust interaction with TcpP (Fig. 3-7B & D).

Discussion

This manuscript set out to address the differential roles for ToxR at two distinct *V. cholerae* promoters, *ompU* and *toxT*. Using an *ompU-sacB* fusion-based selection strategy, we identified a number of residues in ToxR required for full activation of the *ompU* promoter (Fig. 3-1). While several of these mutations severely affected *ompU* promoter binding, those in the putative α-loop of ToxR (Fig. 3-2) had less dramatic DNA-binding defects (Fig. 3-3A). One particular α-loop mutant, ToxR-V71A, was nearly completely defective for *ompU* activation (3% of wild-type) while it maintained intermediate levels of *toxT* activation (15% of wild-type, Fig. 3-1). Thus, this residue in the α-loop may be particularly critical for engagement of the RNAP machinery at the *ompU* promoter. The fact that one other α-loop mutant, ToxR-R65Q, had an *ompU* promoter-binding defect similar to ToxR-V71A (Fig. 3-3A), yet maintained 35% *ompU* activation also supports the hypothesis that V71 has a specific role in ToxR-mediated *ompU* activation. The importance of residues in the α-loop in *ompU* activation was reiterated with the generation of site-directed α-loop mutations in which one additional α-loop alanine substitution, ToxR-F69A, led to a defect in *ompU* activation (Table 3-2), but had no effect on *ompU* promoter binding (Fig. 3-5A). Residue E39 in the neighboring helix α1 (Fig. 3-2A and B) also appears to contribute specifically to *ompU* activation (Table 3-1) and may be part of an RNAP interaction patch for *ompU* activation (Fig. 3-8).

It should be noted that some mutations in the α-loop affected both *ompU* and *toxT* activation (ToxR-R65A, ToxR-G68A and ToxR-S74A) and two mutants, ToxR-R65Q and ToxR-D73A, affected *toxT* activation preferentially over *ompU* (Fig. 3-1 & Table 3-2). Thus the α-loop is not exclusively important for *ompU* activation. However, the ToxR-V71A mutant indicates this residue is particularly important for *ompU* activation as no other α-loop mutant

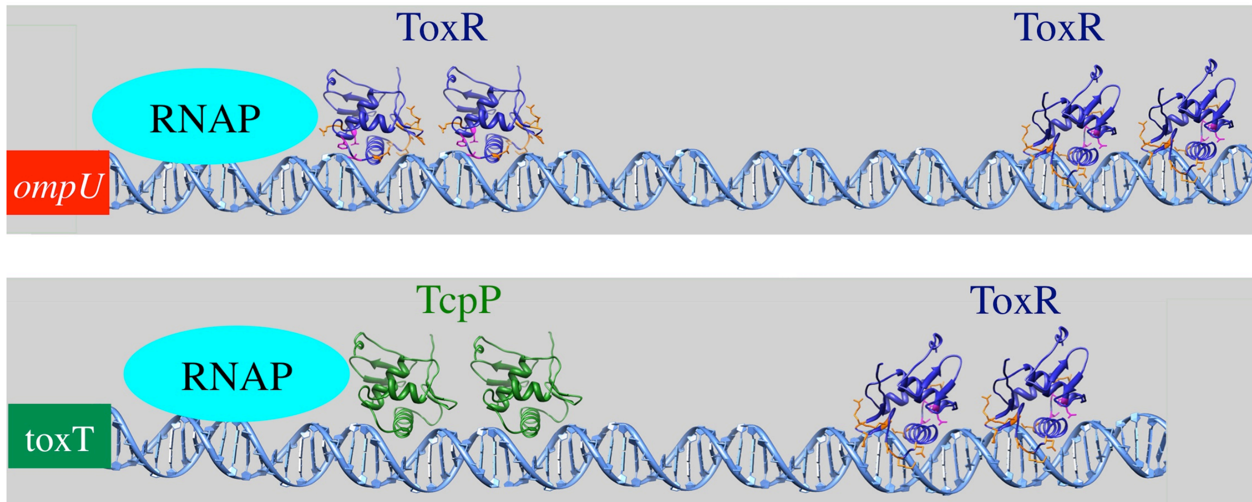


Figure 3-8. Model for differential activation of the *ompU* and *toxT* promoters of *V. cholerae* by ToxR. A) ToxR directly activates the *ompU* promoter in the absence of TcpP using residues F69 and V71 of the α-loop, and possibly E39 of α3, to stimulate RNA polymerase transcription. B) ToxR activates the *toxT* promoter in conjunction with TcpP by facilitating the ability of TcpP to interact with RNA polymerase. At the *toxT* promoter, ToxR and TcpP may interact via wing-wing contacts between the proteins. Thus, in this model, ToxR is inverted in its binding orientation on the DNA relative to the *ompU* promoter to present the wing face of ToxR to TcpP. Both promoters are shown from a backside view to allow easier visualization of the side chains that contribute preferentially to *ompU* (magenta) or *toxT* (orange) activation. The DNA template used is from the structure of PhoB bound to DNA (8).

tested had a comparable *toxT* activation defect (97% defective). Other members of the OmpR/PhoB family of winged-HTH activators also have activation-specific mutations identified in their α-loops (43, 44). In some cases, suppressor mutations in the gene encoding a component of RNA polymerase can rescue the activation-defective OmpR/PhoB family member (45).

Eight ToxR substitution mutants with intermediate defects in *ompU-lacZ* expression (ToxR-R65Q, ToxR-D73A, ToxR-D89E, ToxR-K92E, ToxR-S93P, ToxR-K98E, ToxR-P101L and ToxR-R103G) showed preferential defects in *toxT-lacZ* expression relative to *ompU-lacZ* expression. Six of eight substitutions affect residues adjacent to or within the putative wing domain of the winged-helix-turn-helix (winged-HTH) domain of ToxR. The wing domain in this family of proteins has been shown in some instances to be involved in DNA-binding, while in other cases it plays a role in protein-protein interactions (5, 8, 33, 35). When involved in DNA-binding, the wing binds the minor groove of the DNA helix while the DNA-recognition α-helix (α3, Fig. 3-2A) recognizes the major groove (8, 33). Our findings suggest the wing domain of ToxR is involved in DNA binding (Fig. 3-3) and may also play a role in TcpP interaction (Fig. 3-6). We hypothesize that ToxR interaction with TcpP allows ToxR to facilitate activation of the *toxT* promoter. One ToxR wing mutant that affected *toxT* activation preferentially, ToxR-P101L, was defective for this ToxR/TcpP interaction as assessed by a crosslinking assay, with ToxR-P101L expressed from its chromosomal locus (Fig. 3-6). On the other hand, a plasmid-based ToxR/TcpP bacterial two-hybrid assay revealed no significant defect for ToxR-P101L/TcpP interaction (Fig. 3-7). It was notable that wild-type ToxR consistently generated high levels of cAMP in the bacterial two-hybrid assay, resulting in *lacZ* expression levels ranging from 898-2922 Miller Units over 42 measurements, while ToxR-P101L gave more variable *lacZ* expression levels, ranging from 195-2975 Miller Units over 27 measurements (Fig. 3-7A).

Together we take these data to indicate that the ToxR-P101L mutant likely presents a slightly altered conformation of the ToxR wing domain that affects the ToxR/TcpP crosslinking assay more dramatically than the bacterial two-hybrid assay. We hypothesize that in the former assay, this altered wing conformation affects the presentation of a neighboring lysine residue, which is involved in ToxR/TcpP crosslinking.

Because no other wing mutants tested had a significant defect in TcpP interaction by either assay, the *toxT*-preferential defects of the other ToxR wing substitutions may reflect their slightly greater defects in *toxT* promoter binding as compared to *ompU* binding (Fig. 3-3 and 3-4) and/or some other aspect of promoter interaction required for full *toxT* activation. ToxR may have a lower affinity for the *toxT* promoter than the *ompU* promoter, thus the reduced DNA-binding activity of these ToxR wing mutants may largely explain their preferential defects in *toxT* activation. Modest reductions in DNA-binding affinity may also preferentially affect *toxT-lacZ* expression in *V. cholerae* relative to *ompU-lacZ* since ToxR must displace the global regulator H-NS bound to the *toxT* promoter (46). Alternatively, by affecting the wing domain, these mutants may be altered in their interaction with the minor groove of the DNA in a way that slightly changes the orientation of ToxR on the DNA rather than leading to a major defect in DNA-binding affinity. This could adversely affect the ability of ToxR to efficiently function with TcpP to facilitate *toxT* expression. It remains less clear why the ToxR α-loop mutant derivatives, ToxR-R65Q and ToxR-D73A, showed *toxT*-specific activation defects (Fig. 3-1 & Table 3-2), but their *toxT* activation defects were <2-fold of their *ompU* activation defects, making them the least dramatic of the *toxT*-specific defects. The ToxR-D73 side chain is predicted to be oriented towards the wing domain when modeled on other winged-HTH transcription factors (Fig. 3-2C), although assignment of side-chain orientation in loop regions is tentative. If oriented towards the

wing domain, D73 may function along with wing residues of ToxR to enhance TcpP-mediated *toxT* activation. R65 maintains some *toxT*-specific defects even when changed to alanine or leucine (Tables 3-1 & 3-2), although the preference diminishes with these other side-chain substitutions. When bound to the *toxT* promoter with α3 in the major groove it is possible that R65 can contribute to functions specific for TcpP-dependent activation as this side chain is predicted to extend to the edge of the α-loop (Fig. 3-2C) and potentially participate in interactions primarily involving the face of ToxR containing the wing domain. Our modeling of ToxR bound to DNA and results with other PhoB/OmpR family members (8, 33) indicate that the α-loop sits "side saddle" on the DNA (Fig. 3-8) and interacts with RNAP from that position. Thus, residues that extend in one direction from the α-loop like F69 and V71 (Fig. 3-2B) may interact with RNAP while those that extend in the other direction, like R65 and D73 may interact with other components of the activation complex.

Over the past several years a number of ToxR mutant derivatives have been generated with various affects on transcription and DNA binding. These studies were performed in various bacterial backgrounds including *E. coli*, *Salmonella typhimurium* and *V. cholerae* (1, 7, 9, 18, 23, 36-39, 47, 48). The promoter used in many of these studies was the cholera toxin (*ctx*) promoter, a promoter now believed to be directly activated by the AraC-like transcription factor, ToxT (10, 25, 26, 28). We reconstructed a number of these mutants from various studies using our pSK Bluescript expression vector with a C-terminal HA epitope tag to assess their activation using our *ompU-lacZ* and *toxT-lacZ V. cholerae* reporter strains. These two promoters represent those known to be directly activated by ToxR in *V. cholerae*.

In previous work, Ottemann *et al* reported a number of conserved residues in the DNA-binding and transactivation domain of ToxR were required for *ctx-lacZ* activation in *E. coli*,

OmpU and CT production in *V. cholerae* and *ctxA* promoter binding (7). Our findings using *toxT-lacZ* fusions (a promoter activated prior to CT production) and *ompU-lacZ* fusions in *V. cholerae* were largely in agreement with the previous findings. One difference we found was that ToxR-E39K had an intermediate defect on *ompU-lacZ* expression, but had little effect on *toxT-lacZ* expression (Table 3-1). Ottemann *et al* reported this mutant to be strongly defective for *ctx-lacZ* activation in *E. coli* and CT production in *V. cholerae* (7). It is unclear why we saw little to no effect on *toxT-lacZ* activation. It is possible that even in *V. cholerae*, there is a role for ToxR in direct *ctxAB* activation, and this mutation specifically affects *ctxAB* activation, while not affecting *toxT* activation. One other difference we found in our studies was that the ToxR-R84L mutation had no DNA-binding activity for either the *ompU* or *toxT* promoters (Fig. 3-4). Ottemann *et al* reported this mutant (ToxR-R96L by their numbering system) to maintain DNA-binding activity, yet be unable to activate transcription (7). Their gel shift assays were performed on *ctxA* promoter fragments, whereas ours used *ompU* and *toxT* promoter fragments. It is appears this mutation affects binding to the three promoters tested differently. Our data are in agreement that ToxR-R84L is unable to activate transcription.

Regarding the previous findings of DiRita *et al* where they identified a number of ToxS-blind alleles of *toxR* that failed to allow ToxR-ToxS interactions in the periplasm, the three mutants we constructed for testing, ToxR-K85E, ToxR-D89N and ToxR-T99M, behaved much like the mutants previously described (10). All three mutants were unable to activate both *ompU-lacZ* and *toxT-lacZ* fusions in *V. cholerae* (Table 3-1). We also showed that all three mutants were defective for *ompU* (Fig. 3-4A) and *toxT* promoter binding (Fig. 3-4B). In the case of ToxR-T99M, this is similar to the defect seen with two mutants identified in our original randomly-mutagenized pools affecting the same amino acid residue, ToxR-T99K and ToxR-

T99R. The defect of ToxR-D89N is much more severe than the ToxR-D89E mutant identified in our random screen. Thus, the nature of the side-chain substitution has a great effect on activity of ToxR substitutions at this position. Our randomly isolated ToxR-D89E derivative is a very conservative change, thus it is not surprising that it retains more activity than ToxR-D89N. Given that asparagine (N) is also closely related to aspartate (D), this further suggests the negative charge of residue D89 (which would also be present in the D89E substitution) plays an important role in transcription activation. Finally, ToxR-K85E is a substitution within the DNA-binding helix (α3) of ToxR. By changing a positively charged lysine to a negatively charged glutamate, DNA-binding is completely disrupted and the protein no longer activates transcription. A conservative ToxR-K85R mutation at this position has no effect on transcription activation (data not shown). Thus, all three tested "ToxS-blind" mutants disrupted DNA-binding. Why these substitutions affected the ability of ToxR to interact with ToxS in the periplasm remains unclear. It is possible that DNA-binding facilitates ToxR dimerization and dimerization enhances ToxS interaction. Alternatively, ToxS may act as a chaperone for ToxR until it finds a ToxR dimerization partner (39, 49). If ToxR is unable to bind DNA, then once it is released from ToxS it may not stably interact with its ToxR binding partner, rendering the periplasmic domain of ToxR susceptible to protease cleavage.

One last ToxR mutant to discuss is ToxR-K98E. This residue is predicted to lie in the first β-strand of the wing domain (Fig. 2C) and has an intermediate defect in DNA binding to the *ompU* and *toxT* promoters (Fig. 3-4). This mutation was isolated from a screen for ToxR derivatives with reduced *toxT-lacZ* activation capacity. ToxR-K98E mediates 26% of wild-type activation for the *toxT* promoter and 66% activation for *ompU* (Table 3-1). One possibility for the preferential defect of ToxR-K98E on *toxT* activation could be an impaired ability to interact

with TcpP. However, capture assay analysis and bacterial two-hybrid analysis demonstrated that ToxR-K98E had no defect in interaction with TcpP (Figs. 3-6 and 3-7). Thus, while this mutation negatively affects DNA binding, it maintains TcpP interaction. Since the crosslinker used in these studies, DSP, reacts with primary amines, this indicates crosslinking relies upon other lysines of ToxR. This could indicate that K98 and the wing domain of ToxR are not directly involved in ToxR/TcpP interactions, or other neighboring lysines (such as K92 or K102) can also facilitate ToxR/TcpP crosslinking.

In all, these studies define residue V71 of the α-loop of ToxR as being critical for activation of the *ompU* promoter and F69 playing a supporting role in *ompU* activation. Residues adjacent to and within the wing domain of ToxR also affect *ompU* activation, but in a number of cases more strongly affect *toxT* activation. Why some of these ToxR mutations affect *toxT* more dramatically than *ompU* remains to be determined. In at least one case, ToxR-P101L, presentation of the wing domain for ToxR/TcpP interaction was affected (Fig. 3-6). For the remainder of the ToxR derivatives preferentially defective for *toxT* activation, there was no statistically significant defect in TcpP interaction. Thus, the wing domain of ToxR appears to play a role on both *ompU* and *toxT* activation, but perhaps a dual role in *toxT* activation, DNA binding and TcpP interaction. It is notable that the mutation affecting ToxR/TcpP crosslinking was ToxR-P101L, which is predicted to perturb the structure of the wing domain by eliminating the proline turn residue at the tip of the wing. Mutations in the wing domain of TcpP (also an OmpR/PhoB family member) have been shown previously to affect ToxR interaction (5). Thus, we present a model where the wing domains of ToxR and TcpP are oriented towards each other on the *toxT* promoter (Fig. 3-8). ToxR binds the *toxT* promoter at a direct repeat three helical-turns upstream of the TcpP binding site (50). Thus, we model ToxR on the *toxT* promoter as a

head-to-tail dimer with its wing domain oriented toward TcpP (Fig. 3-8B). At the *ompU* promoter, we orient the promoter proximal ToxR molecule with residues F69 and V71 of the α-loop oriented towards the promoter for interaction with RNA polymerase (Fig. 3-8A). Orientation of the remaining ToxR molecules on the *ompU* promoter is speculative and future experiments will test the model we propose for ToxR binding throughout the *ompU* promoter (30, 31).

Materials and Methods

Bacterial strains and plasmids. A list of bacterial strains and plasmids is provided as Table 3-4. *V. cholerae* were grown in modified LB (with 5g/L NaCl rather than 10g/L) with 100 μg/ml streptomycin and 100 μg/ml ampicillin or 25 μg/ml chloramphenicol as appropriate.

Construction of an HA-epitope tagged toxR allele. *toxR* from *V. cholerae* strain O395 was PCR amplified from chromosomal DNA using a primer with a *Bam*HI restriction site and including the *toxR* ribosomal binding site (primer 5' ToxR-HA) and a 3' *Xho*I restriction site (primer 3' ToxR-HA) to allow in-frame ligation into the HA-tagging vector pcDNA3-HA (51)). The resulting C-terminally HA-tagged *toxR* allele was confirmed to encode wild-type *toxR* by sequencing. The *toxR-HA* allele was then liberated from pcDNA3-HA by *Bam*HI and *Apa*I digestion and ligated into the expression vector pSK Bluescript (Invitrogen) for expression in *V. cholerae*. Primers for these studies are listed in Table 3-5.

Isolation of sucrose-resistant toxR alleles using an ompU-sacB selection. The *toxR-HA* allele was subjected to random Taq polymerase-mediated mutagenesis by standard PCR amplification for 20 rounds of amplification using primers 5' ToxR-HA and 3' HA-tag ApaI. (Table 3-5). The *toxR-HA* mutant pool was cut with *Bam*HI and *Apa*I and ligated into fresh pSK

Table 3-4. Strains and plasmids.

V. cholerae strain		Source
EK307	O395 Δ*toxR*	(1)
EK406	EK307 *ompU-sacB*	this study
EK410	EK307 *ompU-lacZ*	(9)
EK459	O395 Δ*toxR*Δ*tcpP*	(1)
EK816	O395 Δ*toxRS toxT-lacZ*	this study
EK1072	EK307 *toxT-lacZ*	this study
E coli strain		
DH5a		lab strain
DHM1		(12)
BTH101		(21)
plasmids		
random mutants from *ompU-sacB*		
pSK-*toxR-HA* wild-type		this study
pSK-*toxR-HA*-W64R		this study
pSK-*toxR-HA*-R65Q		this study
pSK-*toxR-HA*-V71A		this study
pSK-*toxR-HA*-Q78R		this study
pSK-*toxR-HA*-L83P		this study
pSK-*toxR-HA*-D89E		this study
pSK-*toxR-HA*-K92E		this study
pSK-*toxR-HA*-S93P		this study
pSK-*toxR-HA*-T99K		this study
pSK-*toxR-HA*-T99R		this study
pSK-*toxR-HA*-P101L		this study
pSK-*toxR-HA*-R103G		this study
pSK-*toxR-HA*-G104S		this study
pSK-*toxR-HA*-L107S		this study
α-loop mutants		
pSK-*toxR-HA*-R65A		this study
pSK-*toxR-HA*-E66A		this study
pSK-*toxR-HA*-Q67A		this study
pSK-*toxR-HA*-G68A		this study
pSK-*toxR-HA*-F69A		this study
pSK-*toxR-HA*-E70A		this study
pSK-*toxR-HA*-D72A		this study
pSK-*toxR-HA*-D73A		this study
pSK-*toxR-HA*-S74A		this study
pSK-*toxR-HA*-S75A		this study

<u>Ottemann reconstructed mutants</u>	(7)
pSK-*toxR-HA*-E39K (formerly E51K)	this study
pSK-*toxR-HA*-R56K	this study
pSK-*toxR-HA*-R56L	this study
pSK-*toxR-HA*-R65L	this study
pSK-*toxR-HA*-R84K	this study
pSK-*toxR-HA*-R84L	this study
<u>DiRita ToxS-blind mutants</u>	(10)
pSK-*toxR-HA*-K85E	this study
pSK-*toxR-HA*-D89N	this study
pSK-*toxR-HA*-T99M	this study
<u>random mutants (*toxT-lacZ*)</u>	
pMMB66EH-*toxR*	this study
pSK-*toxR-HA*-R84C	this study
pSK-*toxR-HA*-D89Y	this study
pSK-*toxR-HA*-K98E	this study
<u>bacterial two-hybrid plasmids</u>	
pKT25	(21)
pUT18c	(21)
pUT18c-EpsM	this study
pUT18c-ToxRS	this study
pUT18c-ToxRS-D73A	this study
pUT18c-ToxRS-D89E	this study
pUT18c-ToxRS-K92E	this study
pUT18c-ToxRS-S93P	this study
pUT18c-ToxRS-K98E	this study
pUT18c-ToxRS-P101L	this study
pUT18c-ToxRS-R103G	this study
pKT25-TcpPH	this study
pKT25-TcpPH-L96S	this study
pKT25-TcpPH-I97T	this study
pKT25-TcpPH-K101E	this study

Table 3-5. Primers used in this study.

primer name	sequence 5' to 3'	purpose
5' ToxR-HA	gggggatcctcaaaagagatatcgatgag	cloning *toxR* into pcDNA-HA and pSK Bluescript
3' ToxR-HA	ggggggctcgagctcacacactttgatggc	cloning *toxR* into pcDNA-HA
3' HA-tag ApaI	cctgacgggcccactagcgaattcatctagaagcg	cloning *toxR* into pSK Bluescript
toxR K85R TOP	catttcgactctgcgcagaatgctcaaagattcg	separation of double mutants
toxR K85R BOTTOM	cgaatctttgagcattctgcgcagagtcgaaatg	separation of double mutants
toxR D89E TOP	caaaatgctcaaagagtcgacaaagtccccac	separation of double mutants
toxR D89E BOTTOM	gtggggactttgtcgactctttgagcattttg	separation of double mutants
toxR R65A	catgactttgtttgggctgagcaaggttttgaagtc	α-loop mutant construction
toxR E66A	gactttgtttggcgagcccaaggttttgaagtcgat	α-loop mutant construction
toxR Q67A	ctttgtttggcgagaggctggttttgaagtcgatgat	α-loop mutant construction
toxR D72A	caaggttttgaagtcgctgattccagcttaacc	α-loop mutant construction
toxR D73A	ggttttgaagtcgatgcttccagcttaacccaag	α-loop mutant construction
toxR S74A	gttttgaagtcgatgatgccagcttaacccaagc	α-loop mutant construction
toxR S75A	gaagtcgatgattccgccttaacccaagccatttc	α-loop mutant construction
toxR D72A BOTTOM	ggttaagctggaatcagcgacttcaaaaccttg	construction of chromosomal mutants
toxR D73A BOTTOM	cttgggttaagctggaagcatcgacttcaaaacc	construction of chromosomal mutants
toxR K92E TOP	cgcaaaatgctcaaagattcgacagagtccccacaatacgtca aaacggt	construction of chromosomal mutants
toxR K92E BOTTOM	accgttttgacgtattgtggggactctgtcgaatctttgagcatttt gcg	construction of chromosomal mutants
toxR S93P TOP	caaagattcgacaaagcccccacaatacgtc	construction of chromosomal mutants
toxR S93P BOTTOM	gacgtattgtgggggctttgtcgaatctttg	construction of chromosomal mutants
toxR K98E TOP	cccacaatacgtcgaaacggttccgaagcgc	construction of chromosomal mutants
toxR K98E BOTTOM	gcgcttcggaaccgtttcgacgtattgtggg	construction of chromosomal mutants
toxR P101L TOP	cgtcaaaacggttctgaagcgcggttacc	construction of chromosomal mutants
toxR P101L BOTTOM	ggtaaccgcgcttcagaaccgttttgacg	construction of chromosomal mutants
toxR R103G TOP	caaaacggttccgaagggcggttaccaattgatc	construction of chromosomal mutants
toxR R103G BOTTOM	gatcaattggtaaccgcccttcggaaccgttttg	construction of chromosomal mutants
ToxR XbaI Fwd	ggggtctagagatgagtcatattggtactaaattcattc	construction of BACTH system
ToxS BamH1 Rev	ggggggatccttaagaattactgaacagtacggtag	construction of BACTH system
TcpP XbaI Fwd	ggggtctagagatggggtatgtccgcgtga	construction of BACTH system
TcpH BamH1 Rev	ggggggatccctaaaaatcgctttgacaggaa	construction of BACTH system
EpsM XbaI Fwd	ggggtctagagatgatgaaagaattattggctcc	construction of BACTH system
EpsM EcoRI Rev	gggggaattctcagcctccacgcttcag	construction of BACTH system
tcpP L96S top	gatgaacataagacgtcgatcgaaaatgtaaag	construction of BACTH system
tcpP L96S bottom	ctttacattttcgatcgacgtcttatgttcatc	construction of BACTH system
tcpP I97T top	gaacataagacgttgaccgaaaatgtaaagttac	construction of BACTH system
tcpP I97T bottom	gtaactttacattttcggtcaacgtcttatgttc	construction of BACTH system
tcpP K101E top	gttgatcgaaaatgtagagttacaaggttatc	construction of BACTH system
tcpP K101E bottom	gataaccttgtaactctacattttcgatcaac	construction of BACTH system

Bluescript digested with *Bam*HI and *Apa*I. Three pools of ~300-400 clones were generated and used to transform the *V. cholerae* Δ*toxR ompU-sacB* strain, EK406. Transformants were plated onto LB plates containing 5% sucrose, 100 µg/ml streptomycin and 100 µg/ml ampicillin (to select for pSK Bluescript) and 100mM IPTG to induce *toxR-HA* expression. Within the three pools, the percentage of mutant pSK-*toxR-HA* alleles that resulted in sucrose resistance in strain EK406 was, 2.5%, 4.5% and 6.25%, respectively. About 160 colonies from the three pools were picked and screened individually for ToxR-HA expression by Western blot analysis after growth of the strain for 3hrs in 3mls LB + 100mM IPTG (data not shown). Those that expressed significant amount of full-length ToxR-HA as assessed by anti-HA antibody, were studied further. This amounted to about 25-35% of the total sucrose-resistant clones analyzed. Those mutants defective for *ompU-sacB* activation, that expressed full length ToxR-HA, were sent for sequencing at the University of Michigan Sequencing Core.

Isolation of ToxR mutants defective for activation of a toxT-lacZ fusion. A small pool of *toxR* mutants (about 130) expressed from the IPTG-inducible plasmid pMMB66EH (52) were screened for their ability to activate a *toxT-lacZ* reporter in *V. cholerae*, strain EK816 (Table 3-4). After transforming EK816 with the mutant pool, cells were grown for 1 hr in LB prior to plating on LB Agar supplemented with 10 µM IPTG, 100 µg/ml ampicillin (to select for the plasmid pMMB66EH-*toxR*) and 40 µg/ml X-gal at 30°C overnight. 24 white colonies were picked and analyzed for expression of full length ToxR protein by Western blot analysis using an anti-ToxR antibody. Plasmids expressing full-length protein were subjected to sequencing analysis at the University of Michigan Sequencing Core. Mutants of interest were removed from plasmid pMMB66EH-*toxR* and cloned into pcDNA3-HA and then pSK Bluescript as described for mutants from the sucrose selection strategy.

Measuring transcriptional activation activity of ToxR-HA derivatives. pSK-*toxR-HA* derivatives encoding various *toxR-HA* alleles were transformed into the *V. cholerae* Δ*toxR* *ompU-lacZ* reporter strain EK410 and the Δ*toxR* *toxT-lacZ* reporter strain EK1072. The latter is a Δ*toxR* derivative of a previously constructed O395 *toxT-lacZ* strain, a kind gift from Dr. Claudia Häse (19). Reporter strains harboring the various ToxR-HA derivatives were grown in triplicate overnight at 30°C and then diluted 1:50 and grown for 3-4 hrs at 30°C in the presence of 100 µg/ml streptomycin, 100 µg/ml ampicillin and 100 µM IPTG. Cells were harvested and 20 µl or 100 µl were used in a standard β-galactosidase assay (53). Measurements of *ompU* and *toxT* activation for each ToxR-HA mutant derivative were assessed in at least two separate experiments.

DNA gel mobility shift assays. Promoter-binding assays were performed essentially as described previously (1). The membranes used were from strain EK459 (Δ*toxR*Δ*tcpP*) carrying each pSK-*toxR-HA* allele and induced for 4-5 hours at 30°C in 500ml LB pH=6.5 with 100 µM IPTG. Increasing concentrations of membrane were mixed with either an *ompU* promoter probe extending from -211 to +22 relative to the transcriptional start site, a *toxT* promoter probe extending from -172 to +45 or a negative control *toxT* promoter probe extending from -46 to +45 (lacking the ToxR-binding site, (29)). Probes were labeled by Klenow fill-in of *Bam*HI or *Sal*I digested plasmids with α-^{32}P-dCTP and 3000 cpm of labeled probe were used in each reaction. Relative levels of each ToxR mutant protein were assessed using anti-HA monoclonal antibody (Covance), anti-HA polyclonal antibody (Covance), or anti-ToxR polyclonal antibody. Samples normalized to the same relative concentrations used in the gel shift assay were boiled in SDS-sample buffer and run in a 12% SDS-PAGE gel.

ToxR/TcpP-HSV Capture Assay. *V. cholerae* RY1 with various *toxR* alleles recombined at the normal *toxR* locus were transformed with plasmids expressing wild-type TcpP-HSV or the ToxR-interacting mutant TcpP-K101E. Strains were diluted 1:50 from an overnight culture at 30°C and grown for 4-6 hrs at 30°C in 500ml LB containing 100 μg/ml streptomycin, 25 μg/ml chloramphenicol and 100 μM IPTG. Membranes harboring ToxR and TcpP-HSV proteins of interest were prepared (18) and dialyzed into HEPES-buffered saline (HBS, 20mM HEPES pH=7.0, 150 mM NaCl). Three to five mg/ml membrane proteins were crosslinked using a 15-fold molar excess of DSP (Pierce) for 30' at room temp, blocked with 50mM Tris pH=7.4 and then solubilized in 1% Triton X-100 (Bio-Rad). The molarity of dialyzed membrane preparations was estimated by measuring the protein concentration and assuming a 50kD average protein size in the total membrane extract. After sonication on ice (3 x 5 seconds), 50 μl membrane extracts were added to microtiter plates coated with mouse anti-HSV antibody (Novagen, coated at 1:500 dilution in PBS) and binding proceeded overnight at 4°C. After washing five times with PBS, wells were incubated with 50 μl a 1:100 dilution of rabbit anti-TcpP antibody or a 1:1000 dilution rabbit anti-ToxR antibody. Primary antibody incubation proceeded from 3 hrs-overnight at 4°C. After five washes with PBS, wells were then incubated with a 1:3000 dilution of goat anti-rabbit-AP conjugated secondary antibody (Zymed) and binding was revealed by addition of 100 μl of the colorimetric substrate PNPP (Sigma) at 4 mg/ml following sequential washing with PBS (four times) Tris-buffered saline (100mM Tris pH=8.0, 150mM NaCl, one wash). Plates were read at ABS_{405}. Relative levels of each ToxR mutant protein were assessed using an anti-ToxR polyclonal antibody at a 1:1000 dilution. Relative levels of TcpP-HSV in each strain were assessed using an anti-HSV monoclonal antibody (Novagen) at a 1:5000 dilution. The amount of TcpP-HSV or ToxR captured is presented as % of wild-type after subtracting out the background

signal obtained for each protein in the absence of TcpP-HSV (using the pMMB207 empty vector). Samples diluted on the same day to the same relative concentrations used in the capture assay were boiled in SDS-sample buffer and run in a 12% SDS-PAGE gel for Western blot analysis of protein levels in the extracts. Statistical analysis was performed using the students' t-test comparing six or nine measurements relative to the strain expressing wild-type ToxR and wild-type TcpP-HSV.

ToxR/TcpP bacterial two-hybrid system. ToxRS and EpsM were PCR amplified from chromosomal DNA and cloned into the pUT18c vector (21) 3' of the cya18 fragment in DHM1. TcpPH was PCR amplified from chromosomal DNA and cloned into the pKT25 vector (21) 3' of the cya25 fragment in DHM1. Point mutations were created in ToxR and TcpP using site-directed mutagenesis (primers are listed in Table 3-5). Plasmids were transformed into the reporter strain BTH101 and grown at 37°C in the presence of 100 μg/ml ampicillin and 30 μg /ml kanamycin. Cultures were induced 16 hours at 30°C in LB broth in the presence of 100 μg/ml ampiclillin, 30 μg /ml kanamycin 0.5 mM and IPTG. 20 μl of culture was used in a standard β-galactosidase assay (53) in a minimum of 3 separate experiments with three replicates each. Stability of ToxR and TcpP mutants was determined by transforming the cya18-ToxRS mutant plasmid with pKT25 or cya25-TcpP mutant plasmid with pUT18c into BTH101. Cultures were induced 16 hours at 30°C in LB broth in the presence of 100 μg/ml ampicillin, 30 μg /ml kanamycin, 0.5 mM IPTG, and 1 mM cAMP. Samples normalized to the same relative concentrations by OD_{600} were boiled in SDS-sample buffer and run in a 10% SDS-PAGE gel. Samples were probed with anti-CyaA rabbit polyclonal antibody (Santa Cruz Biotechnology).

ToxR mutant modeling. Using a newly developed program I-TASSER (2-4), we threaded the ToxR sequence onto winged-HTH family members with solved structures. I-TASSER made a

secondary structure sequence prediction for ToxR and threaded that onto to several best matches in the PDB, compiling the best matching fragments from each structure. In creating the ToxR model, I-TASSER used parts of YycF (from *Bacillus subtilis*), OmpR (*E. coli*), MtrA (*Mycobacterium tuberculosis*), RegX3 (*M. tuberculosis*), ArsR (*Helicobacter pylori*) and VicRc (*Enterococcus faecalis*) as the top ten threading templates (results at http://zhanglab.ccmb.med.umich.edu/I-TASSER/output/S57294/). It then compiled these best fit pieces and performed energy/hydrogen bonding optimization. The best fit of the ToxR model is to YycF, with 25% sequence identity (with PhoB of *E. coli* as the third best fit). Using the protein structure analysis program Chimera (54), several key residues of ToxR were highlighted to show the predicted orientation of their side-chains to gain insights into their potential effects on ToxR function.

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Chapter 4

The wing of the ToxR winged helix-turn-helix domain is required for DNA binding

Note: All of the strains created and all of the assays performed in this chapter were done either by myself or by Emily French under my supervision.

Summary

ToxR and TcpP, two winged helix-turn-helix (w-HTH) family transcription factors, activate expression of the *toxT* promoter in *Vibrio cholerae*. ToxT then directly regulates a number of genes required for virulence. In addition to co-activation of *toxT*, ToxR can directly activate the *ompU* promoter and repress the *ompT* promoter. Based on a previous study suggesting that the wing of ToxR is preferentially involved in *toxT* co-activation compared to *ompU* direct activation, we employed alanine-scanning mutagenesis to determine which residues in the wing of ToxR are required for activation of each promoter. Although several residues in the wing were preferentially required for *toxT* activation when expressed from a plasmid, this phenotype was dependent upon overexpression and not observed when the same ToxR mutants were expressed from the chromosome. All of the ToxR wing residues tested that were critical for transcriptional activation of *toxT* and/or *ompU* were also critical for DNA binding. Furthermore, none of the ToxR wing residues tested were critical for ToxR-TcpP interaction indicating that the

wing of ToxR is likely not required for ToxR-TcpP interaction. Based on these findings we can conclude that the primary role of the wing of ToxR is to bind DNA, along with the DNA recogition helix of ToxR, and this function is required both for co-activation of *toxT* and direct activation of *ompU*.

Introduction

ToxR and TcpP are transmembrane transcription factors that coordinately activate transcription of *toxT*, the gene encoding the master virulence regulator in *V. cholerae*. TcpP is posited to be the direct activator of *toxT*, binding to the *toxT* promoter from -53 to -38, just upstream of the -35 element (8). Although, TcpP is able to activate intermediate levels of *toxT* expression when overexpressed (7, 9), ToxR is required for TcpP-mediated expression of *toxT* under native conditions. ToxR binds to the *toxT* promoter from -96 to -83 (11), approximately three helical turns upstream of the TcpP binding site, enhancing TcpP-mediated activation of the *toxT* promoter. In addition to acting as a co-activator of *toxT*, ToxR is also able to directly activate the *ompU* promoter (13) and repress the *ompT* promoter (15).

ToxR and TcpP both have cytoplasmic domains that are homologous with the w-HTH (winged helix-turn-helix) family of transcription factors. Most w-HTH proteins have a N-terminal regulatory domain and a C-terminal w-HTH domain. However, both ToxR and TcpP have an N-terminal w-HTH domain, which is linked to a periplasmic C-terminal domain through a single-pass transmembrane domain. The periplasmic domain of ToxR is involved in, but not required for, dimerization (16-21). The periplasmic domain of TcpP regulates stability and is proteolytically degraded under non-inducing conditions (22, 23). The functions of ToxR and TcpP periplasmic domains are enhanced by the periplasmic proteins ToxS and TcpP,

respectively (16, 22, 23). The w-HTH domains of ToxR and TcpP bind to the *toxT* promoter and activate transcription. The w-HTH domain consists of an N-terminal β-sheet, three α-helixes including the DNA-binding helix (α3) that is inserted into the major groove of the DNA, and a C-terminal wing (Fig. 4-1). The N-terminal β-sheet can be involved in protein-protein interaction as well as stabilizing the hydrophobic core (24-27). The first two α-helixes form part of the hydrophobic core as well as interact with the DNA backbone helping to stabilize protein-DNA interactions (3, 24, 28). Between the second α-helix (α2) and the DNA-binding helix (α3) is the α-loop. The α-loop of w-HTH proteins is hypothesized to interact with RNA polymerase (25). The α-loop of ToxR is critical for direct activation of the *ompU* promoter while less essential for co-activation of the *toxT* promoter (2). The wing of w-HTH proteins consists of a β-strand hairpin, which inserts into the minor grove of the DNA, thereby enhancing binding to the promoter (3, 24). The wing of w-HTH proteins is often also involved in dimerization (24, 29, 30).

Dimerization is critical for activation of w-HTH transcription factors and can be mediated by interactions between w-HTH domains, the N-terminal regulatory domain (of w-HTH proteins containing this domain) and the promoter architecture (24, 27, 29, 31, 32). w-HTH proteins have been found to dimerize in three different orientations. Both PhoB and OmpR have been shown to dimerize in a head-to-tail orientation on the promoter. In this orientation, the wing of the upstream w-HTH protein interacts with the β-sheet of the downstream w-HTH (24, 29). Alternatively, *Mycobacterium tuberculosis* PhoP has been shown by crosslinking to dimerize in a head-to-head orientation with the two β-sheets interacting (31). OmpR can also be found in head-to head orientations (27), although formation of OmpR dimers in head-to-head or head-to-tail orientation may be primarily dependant on the orientation of the OmpR binding site (32).

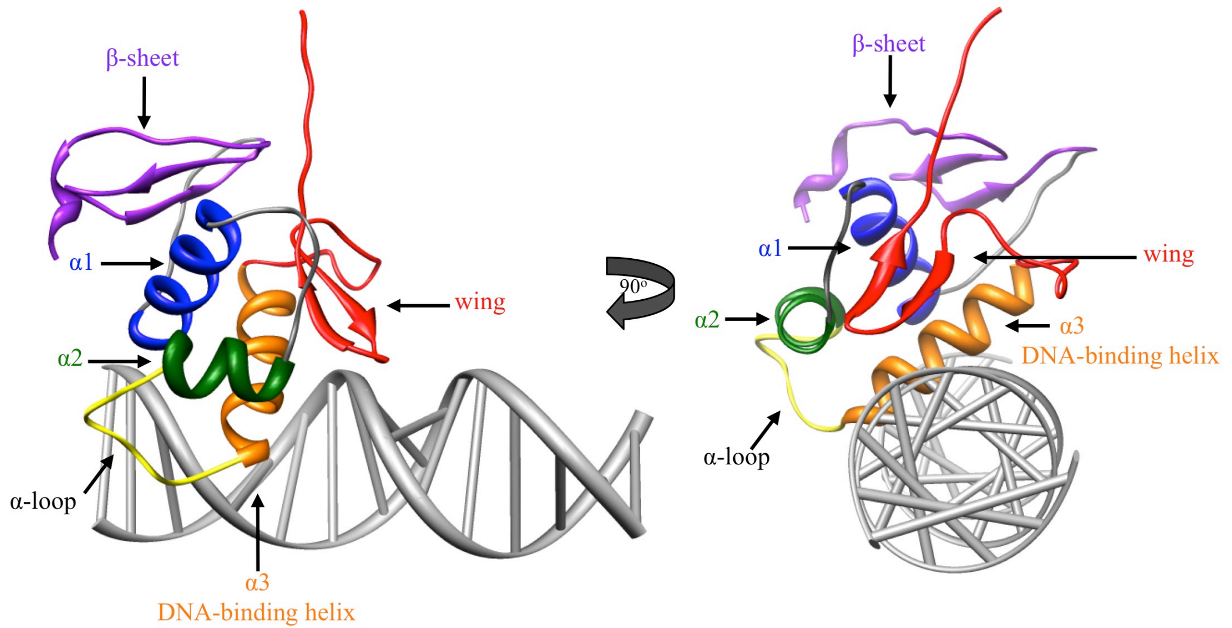


Figure 4-1. Model of ToxR bound to the promoter. ToxR bound to DNA was modeled using the I-TASSER modeling program (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) to determine ToxR structure based on the crystal structure of other w-HTH family members (2). Binding of ToxR to DNA was modeled using the NMR structure of PhoB bound to DNA (3)

Finally, HSF in *Kluyveromyces lactis* interacts in a tail-to-tail orientation in which the two wings interact (30). In this system the wings are only involved in protein-protein interaction and do not appear to interact with the DNA (30). Both ToxR and TcpP form homodimers (16-21, 33) as well as ToxR-TcpP heterodimers (2, 7). However, the orientation and interface(s) of these dimers has not been elucidated.

When ToxR is bound to the *toxT* promoter, a hypersensitivity site is created at the TcpP binding site (6) indicating an alteration of the structure of the *toxT* promoter upon ToxR binding. This hypersensitivity site indicates that upon ToxR binding to the *toxT* promoter, the TcpP-binding site may become more accessible, allowing for enhanced TcpP binding to the promoter. Additionally, the *toxT* promoter is repressed by H-NS, and deletion of H-NS results in a six-fold increase in *toxT* expression over wild-type in the presence of ToxR and TcpP (1). ToxR is particularly critical for *toxT* activation in the presence of H-NS, as it can increase expression of *toxT* over 1000-fold, whereas, in the absence of H-NS, ToxR only increases expression of *toxT* three-fold (1). Together, this indicates that one of the primary roles of ToxR may be to relieve H-NS repression, but that ToxR also plays an additional roll(s) in activation beyond relieving H-NS repression. ToxR also likely influences the localization of the *toxT* promoter by recruiting it to the membrane thereby enhancing TcpP binding. Although the cytoplasmic domain of ToxR is able to activate transcription of *ompU*, membrane localization is required for ToxR co-activation of *toxT* (5).

Another possible role for ToxR in co-activation of TcpP is recruitment of TcpP to the *toxT* promoter through ToxR-TcpP interaction. ToxR-TcpP interaction can be observed by DSP crosslinking in *V. cholerae* membranes (2, 7) and an membrane-localized *E. coli* bacterial two-hybrid (BACTH) system (2). ToxR-TcpP interaction likely occurs through the wing of TcpP,

since mutations in the wing of TcpP can disrupt ToxR-TcpP interaction resulting in a defect in transcriptional activation (7). The wing of TcpP plays a dual role, since residues in the wing of TcpP also are required for binding to the *toxT* promoter (6, 7). The wing of ToxR may also be involved in ToxR-TcpP interaction, as ToxR-P101L was preferentially defective for *toxT* activation, relative to *ompU* activation, and was defective in interaction with TcpP by DSP crosslinking (2). ToxR-TcpP interaction could be maintained on the *toxT* promoter, allowing ToxR to stabilize TcpP binding to the promoter and thereby enhancing transcriptional activation. Alternatively, ToxR-TcpP interaction could recruit TcpP to the *toxT* promoter. In this model, ToxR-TcpP interaction would be disrupted upon ToxR binding to DNA, allowing each protein to bind to the promoter independently.

The role of the ToxR-wing in co-activation of *toxT* could be due to DNA binding and/or ToxR-TcpP interaction. DNA binding by the wing of ToxR is critical for activation of both *toxT* and *ompU* since mutations in the wing of ToxR (T99K or G104S) that inhibit DNA binding inhibit transcriptional activation of both promoters (2). However, several mutants in the wing (P101L, and R103G) as well as mutants in the loop leading from the DNA-binding helix to the wing (D89E, K92E, S93P) are preferentially defective for transcription of *toxT*, compared to *ompU* (2). This could be due to differences in binding to the two promoters, ToxR-TcpP interaction, or other as yet unknown factors. The goal of this study was to determine the role of each residue in the wing of ToxR in transcriptional activation of both *toxT* and *ompU*. The requirement of ToxR wing residues for ToxR-TcpP interaction and DNA binding will also be used to determine what role the wing of ToxR plays in co-activation of *toxT*.

Results

The wing of ToxR is required for transcriptional activation of both *toxT* and *ompU*. To determine the role of each wing residue in ToxR co-activation of *toxT* and activation of *ompU,* we mutated each wing residue to alanine, including residues in the loop between the DNA-binding helix (α3) and the wing. Alanine scanning was used because residues in this region of ToxR can have different phenotypes depending on the amino acid to which they are mutated (2). Each ToxR-wing mutant was expressed from a plasmid and tested for transcriptional activation of chromosomal *ompU-lacZ* (Strain EK410) and *toxT-lacZ* (Strain EK1072) (Fig. 4-2) (2, 5).

Of the 25 residues tested, six were preferentially required for *toxT* co-activation as compared to *ompU* activation (K92, P94, Y96, P101, L107, and I108). Residues preferentially required for *toxT* co-activation were defined by mutation of that residue resulting in a 2-fold decrease in *toxT* co-activation relative to *ompU* activation and designated Class I mutants (Fig. 4-2 green). Three of the six residues (Y96, L107, and I108) were required for both *toxT* co-activation and *ompU* activation as mutation of these residues resulted in less than 50% activation of *ompU.* These residues are particularly critical for co-activation of *toxT*, since mutation of any of these residues decreased *toxT* transcriptional activation to less than 10% of wild-type. These residues are clustered at the hinge of the wing adjacent to the β-strands (Fig. 4-3). The two prolines present in the wing, P94 and P101, are partially required for *ompU* activation (50-70% activity when mutated), but critical for *toxT* co-activation (<30% activity when mutated). K92 is not required for *ompU* activation (maintained ~100% activity when mutated), but is involved in *toxT* co-activation (50% activity), similar to what has been previously described for this mutant (2). None of the residues were preferentially required for *ompU* activation relative to *toxT* activation, again highlighting the critical role for the wing of ToxR in co-activation of *toxT*.

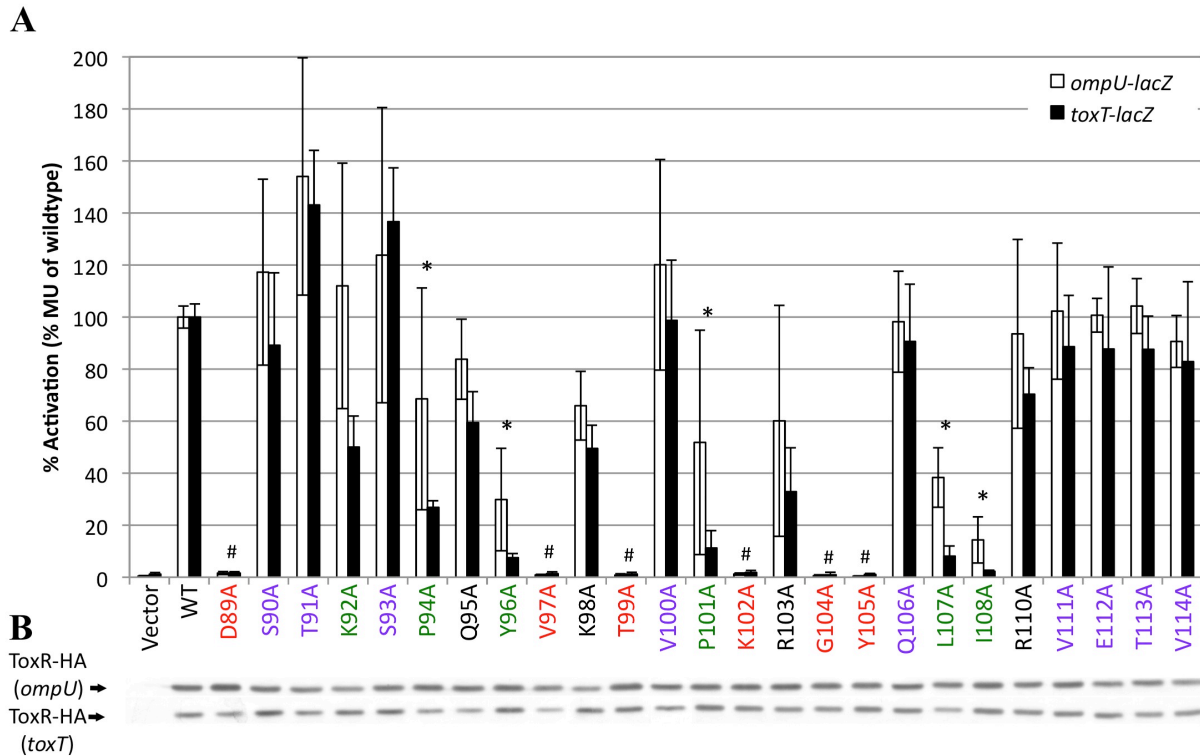


Figure 4-2. Requirement of each residue in the ToxR wing on *toxT* and *ompU* activation. A) ToxR-HA wing mutants were expressed from pBluescriptSK- in the presence of pREP4 to regulate expression. The level of activation both the *toxT-lacZ* and *ompU-lacZ* chromosomal reporters for each mutant was measured by β-galactosidase assay and normalized to wild-type ToxR. Mutants were divided into classes based on phenotype. Class I mutants (green) are greater than 2-fold defective for *toxT* activation relative to *ompU* activation. Class II mutants (purple) are not significantly defective for activation of either promoter ($P<0.05$). Class III mutants (red) are required for activation of both *toxT* and *ompU* promoters. * indicates ToxR mutants that are at least 2-fold more defective for *toxT* activation than *ompU* activation. # indicates ToxR mutants that were defective for activation of both promoters (< 10% activation). B) Stability of ToxR-HA was monitored by Western blot using monoclonal anti-HA. All strains were tested at least six times on at least two different days.

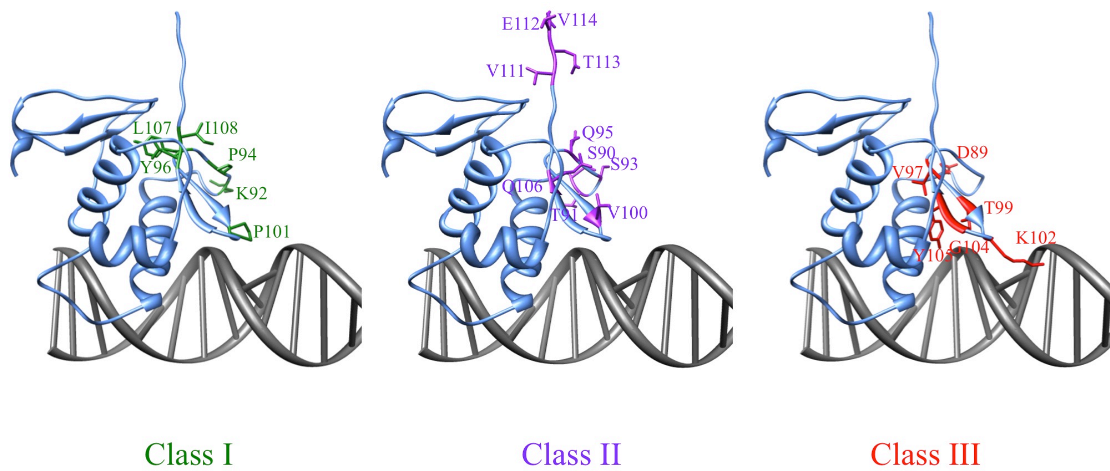


Figure 4-3. Location of ToxR wing residues. ToxR w-HTH domain was modeled using I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER/)(2). A) Wing residues belonging to Class I (green) are preferentially required for activation of *toxT* when expressed from a plasmid. However, this preferential defect was lost when ToxR was expressed from the *toxR* chromosomal locus. B) Class II residues (purple) are not required for transcriptional activation of either *toxT* or *ompU*. C) Residues belonging to Class III (red) are required for transcriptional activation of both *toxT* and *ompU* and DNA binding.

Several residue side chains in the wing region of ToxR are not required for transcriptional activation of either promoter (S90, T91, S93, V100, Q106, V111, E112, T113, and V114) and were designated Class II. When these residues were mutated to alanine >80% of transcriptional activation of both promoters was maintained (Fig. 4-2). The majority of these residues are either in the loop between the DNA-binding helix (α3) and the wing or after the β-hairpin of the wing (Fig. 4-3). The only two amino acid side-chains in the β-turn of the wing that are not required for transcriptional activation of at least one promoter are V100 and Q106, indicating that the β-hairpin is critical for transcriptional activation. This is expected since many w-HTH proteins insert the β-hairpin of the wing into the minor groove of promoter DNA (3, 24). In order to compound the effects of mutating these residues a quadruple mutant was constructed (ToxR-S90A/T91A/Q95A/V100A, designated ToxR-quad), however this mutant was still not defective for transcriptional activation of either promoter.

As was expected, since the wing of ToxR is involved in DNA binding (2), several residues wing of ToxR are required for transcriptional activation of both *ompU* and *toxT* (D89, V97, T99, K102, G104, Y105). Mutation of these residues, designated Class III, resulted in less than 5% activation of either *ompU* or *toxT* loci (Fig. 4-2). This results in *toxT* activation as low as the empty vector control ($p>0.05$). The majority of these residues are predicted to be involved in DNA binding, either directly or indirectly, and mutations in T99 and G104 have been previously shown to disrupt DNA binding to both promoters (2). All of the residues required for transcriptional activation of both promoters are found in the β-hairpin of the wing except D89, which is positioned at the end of the DNA-binding helix (Fig. 4-3).

Since overexpression can result in altered phenotypes, we analyzed the requirement of ToxR wing residues for transcriptional activation using chromosomally expressed ToxR wing

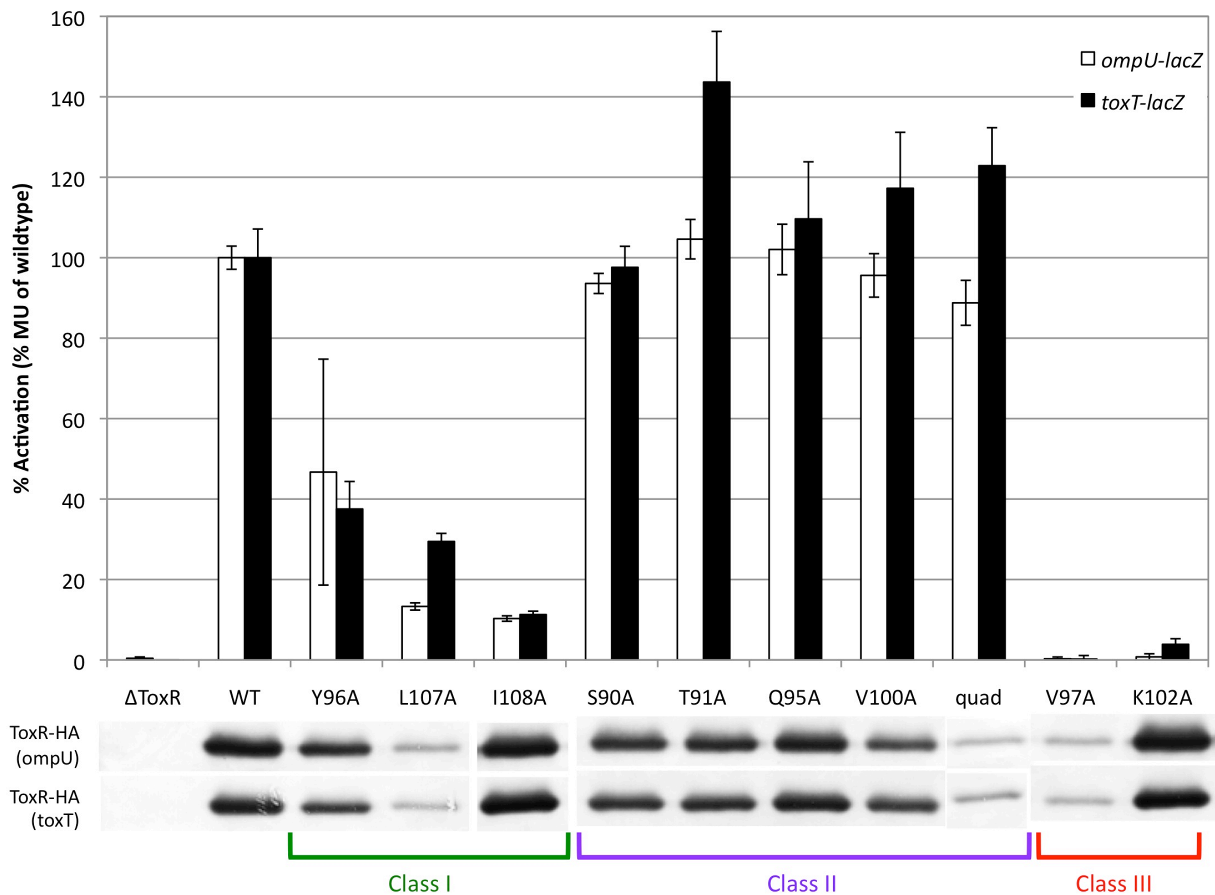
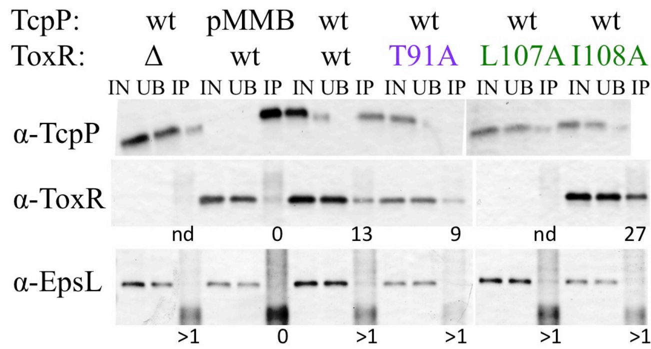
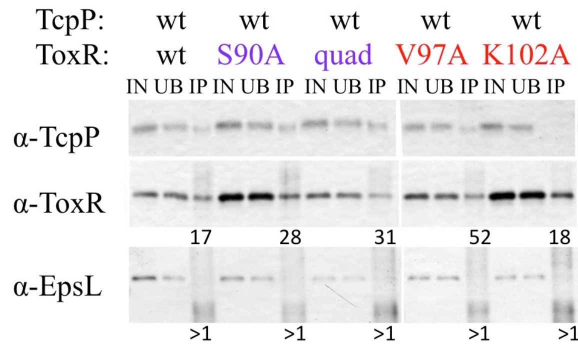


Figure 4-4. ToxR wing mutants expressed from the *toxR* chromosomal locus are not preferentially defective for *toxT* activation. A) Selected ToxR wing mutants representing each class of mutant were placed on the chromosome and assayed for transcriptional activation of *toxT-lacZ* and *ompU-lacZ* by β-galactosidase assay. Class I mutants were at least 2-fold more defective for *toxT* activation tan *ompU* activation when expressed from a plasmid, but equally defective for activation of both promoters when endogenously expressed. Class II mutants are not defective for activation of either promoter, and maintained 80% activity even when mutated. Class III mutants are defective for activation of both promoters. B) The stability of each ToxR wing mutant was monitored by Western blot using anti-ToxR antibodies.

mutants. Thus, we measured activation of chromosomal *toxT-lacZ* and *ompU-lacZ* reporter from each class of mutants expressed from the native *toxR* locus (Fig. 4-4). Three residues preferentially required for *toxT* co-activation under overexpression conditions, Y96, L107, and I108, were no longer preferentially required for *toxT* co-activation when expressed from the chromosome. L107 was even slightly more critical for *ompU* activation than *toxT* co-activation when endogenously expressed ($p<0.005$), although ToxR-L107A was unstable when expressed from the chromosome in the absence of the C-terminal HA tag. Q95 contributed to, but was not required for, *toxT* activation when expressed from a plasmid (59% of wild-type activation), was able to activate both *toxT* and *ompU* promoters at wild-type levels when chromosomally expressed (Fig. 4-2 and 4-4), and as such it was classified as a class II residue. All class II residues tested tolerated mutation to alanine and maintained transcriptional activation of both *toxT* and *ompU* promoters, whether expressed from the native *toxR* locus or a plasmid. Similarly, both of the class III residues tested were still required for transcriptional activation of *toxT* and *ompU* when expressed from the native locus. These results highlight the importance of monitoring transcriptional activation using ToxR expressed from the native locus, particularly when looking at subtle phenotypes or comparing activation of different promoters. It is particularly critical to note that mutations in ToxR can result in a preferential defect in *toxT* due to overexpression.

The wing of ToxR is not required for ToxR-TcpP interaction. Since one of the hypothesized roles for ToxR in facilitating TcpP-mediated *toxT* transcriptional activation is interaction with TcpP and recruitment of TcpP to the *toxT* promoter, we tested whether the wing of ToxR is involved in ToxR-TcpP interaction. Using DSP crosslinking, membranes containing ToxR and





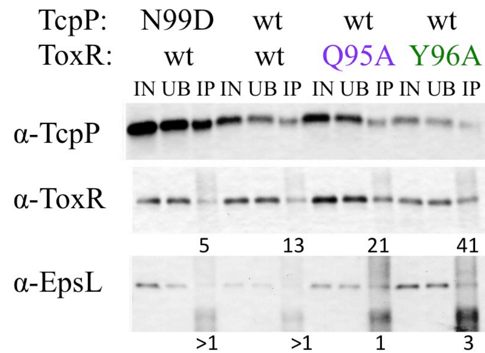


Figure 4-5. The wing of ToxR is not required for ToxR-TcpP interaction. Membranes from *V. cholerae* containing ToxR wing mutants and TcpP-HSV were crosslinked by DSP. TcpP-HSV was immunoprecipitated using anti-HSV. The amount of TcpP immunoprecipitated was monitored by Western blot using anti-TcpP. The amount of ToxR co-immunoprecipitated was monitored by Western blot using anti-ToxR. EpsL, a type 2 secretion system protein, which is not predicted to interact with ToxR or TcpP was monitored by Western blot as a negative control. As an additional control, TcpP-N99D was used which is defective for ToxR-TcpP interaction by the capture assay (7). The band intensities were quantified by image J and the % of ToxR co-immunoprecipitated by TcpP is denoted under the Western blot after subtracting the signal with the empty pMMB207 vector. Representative Western blots and quantification are shown from one of at least two independent experiments with similar results.

TcpP from *V. cholerae* expressing TcpP-HSV from a plasmid and chromosomally expressed ToxR wing mutants were immunoprecipitated using the HSV tag on TcpP, and the amount of co-precipitated ToxR was measured by Western blot (Fig. 4-5). EpsL, a transmembrane protein in the Type 2 secretion system of *V. cholerae*, which is not predicted to interact with ToxR or TcpP was also assayed as a negative control. 13-17% of ToxR crosslinked to TcpP (Fig. 4-5), which is not unexpected as ToxR regulates multiple loci in *V. cholerae,* independent of TcpP.

None of the eight stable ToxR wing residues tested (L107A was too unstable for quantification) were required for ToxR-TcpP interaction. This indicates that wing residues of ToxR that affect *toxT* transcriptional activation are not required for ToxR-TcpP interaction. As a secondary assay for ToxR-TcpP interaction, we employed a membrane localized bacterial two-hybrid system (BACTH) (2). The requirement of each of the 25 residues in the wing of ToxR for ToxR-TcpP interaction was tested using alanine mutants in this system (Fig. 4-6). Mutation of several of the residues in the wing of ToxR resulted in reduced ToxR-TcpP interaction by BACTH. However, the residue that was the most critical for interaction by BACTH (V97) is not required for interaction by co-immunoprecipitation (Fig. 4-5 and 4-6). Furthermore, the quad mutant (ToxR-S90A/T91A/Q95A/V100A) was also defective for interaction by BACTH (Fig. 4-6) but was co-immunoprecipitated by TcpP (Fig. 4-5). This made it difficult to interpret the results of the BACTH since it is either not sensitive enough to detect weakened but intact interactions between ToxR and TcpP, there are factors in *V. cholerae* that influence ToxR-TcpP interaction and are missing in the BACTH system, or mutation of the wing of ToxR affects the ability of the N-terminal and C-terminal fragments of Cya to productively reassemble rather than actual ToxR-TcpP interaction.

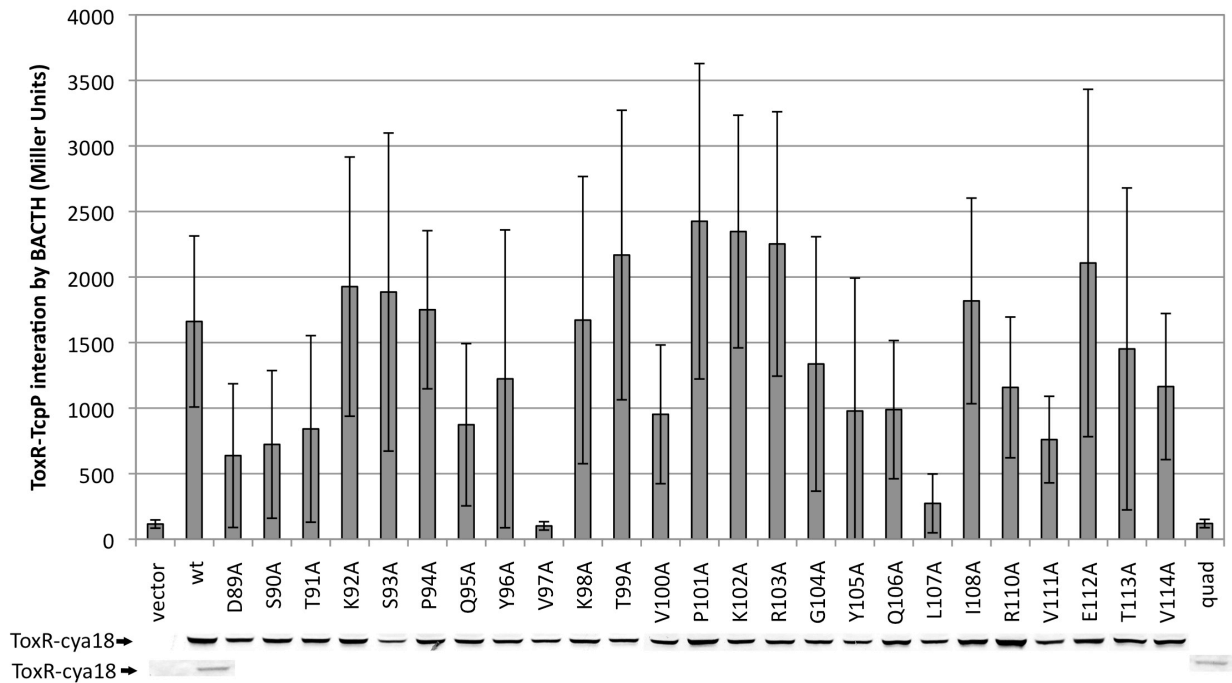
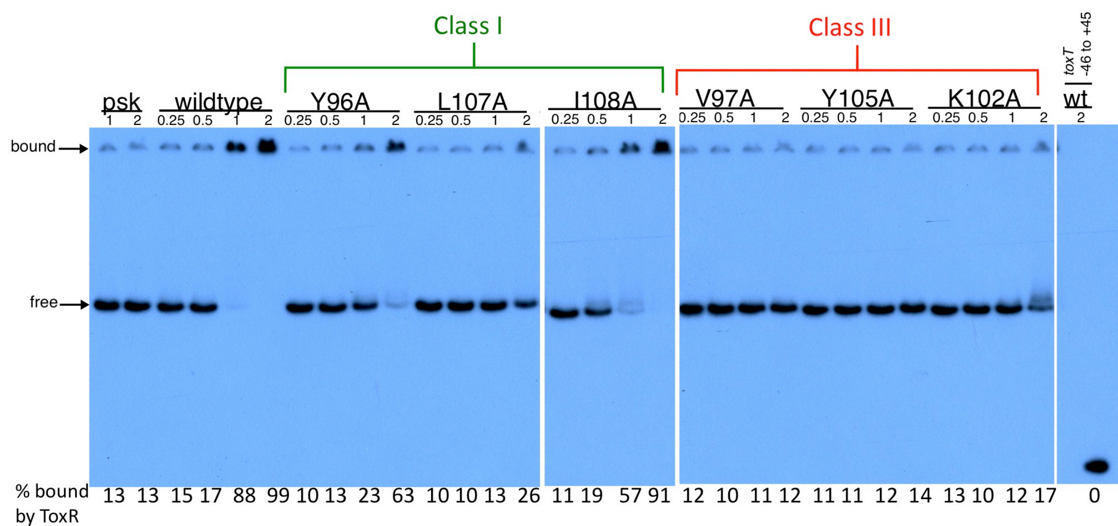


Figure 4-6. ToxR-TcpP interaction as monitored by BACTH varies. pKT25-TcpPH and pUT18c-ToxRS were expressed in the BACTH strain BTH101. Reassembly of adenylate cyclase due to ToxR-TcpP interaction bringing Cya18 and Cya25 together was monitored by β-galactosidase assay. Stability of these constructs was monitored by anti-CyaA Western blot on cultures which had been grown in the presence of adenylate cyclase to induce expression independent of interaction. The ToxR-quad mutant Western blot shown was performed (with wild-type ToxR and empty vector controls) on a separate day from the other BACTH western blots. All strains were replicated at least nine times on at least 3 different days.

Several ToxR wing residues are required for DNA binding. Because the wing of w-HTH proteins is often involved in binding to the minor groove of the DNA, we determined whether ToxR wing residues required for *toxT* activation are involved in DNA binding. We selected ToxR wing mutants from each class of ToxR wing residues and measured their DNA binding by EMSA (Electrophoretic Mobility Shift Assay). Shifting of the *toxT* and *ompU* promoters by wild-type ToxR was detectable at 0.5 mg/ml and 0.19 mg/ml respectively (Fig. 4-7). However, mutation of any of the class III residues (ToxR wing residues required for both *toxT* and *ompU* transcriptional activation) tested resulted in an inability of the ToxR mutant to shift 50% of either probe even at the highest concentrations tested (2 mg/ml for *toxT* and 1.5 mg/ml for *ompU*) (Fig. 4-7). The class I residues, which are involved in activation of both promoters, but preferentially affect *toxT* activation under some conditions, were also required for DNA binding (Fig. 4-7). High levels of membrane containing ToxR-Y96A or ToxR-I108A were required to shift the *toxT* and *ompU* promoters, indicating a dependence upon these residues for maximal DNA binding. Furthermore, these ToxR derivatives were enriched in their respective membrane preparations relative to wild-type ToxR, yet still required more membrane than wild-type ToxR to elicit similar gel shift activity. Mutation of ToxR-L107 resulted in an inability of ToxR-L107A to shift 50% of either probe even at the highest concentrations tested, indicating that this residue is a key residue for DNA binding (Fig. 4-7). Thus, all of the ToxR wing residues tested for DNA binding that were involved in activation were also involved in promoter binding.

Of the class II residues tested that are not required for transcriptional activation of either promoter (ToxR-S90, T91, Q95, V100, Q106, V111), none were required for DNA binding of either the *toxT* or *ompU* promoter. None of the class II mutants tested had any decrease in binding to either promoter relative to wild-type ToxR. ToxR-T91A resulted in almost a 2-fold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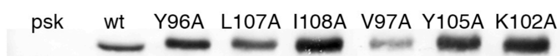
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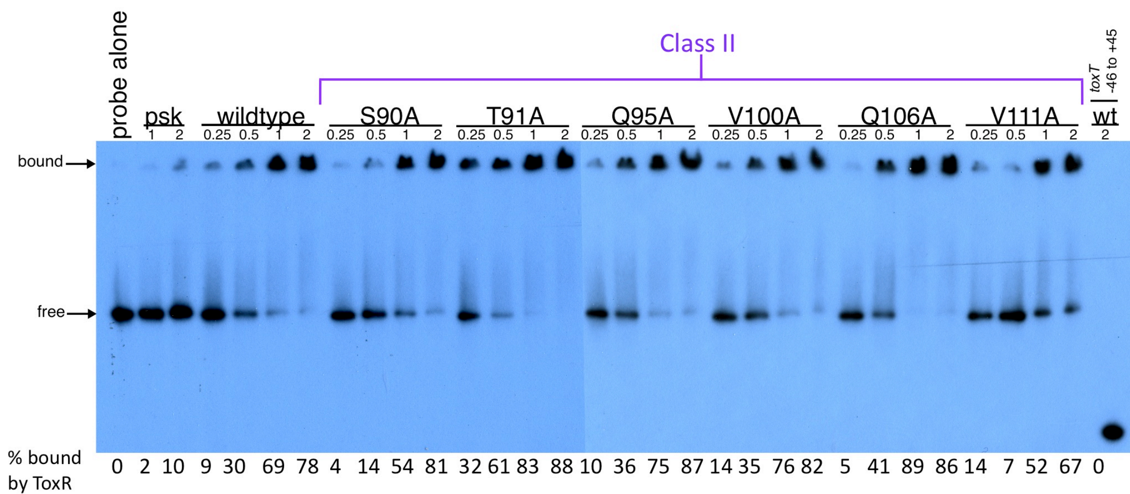
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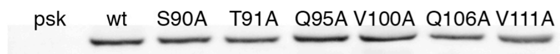
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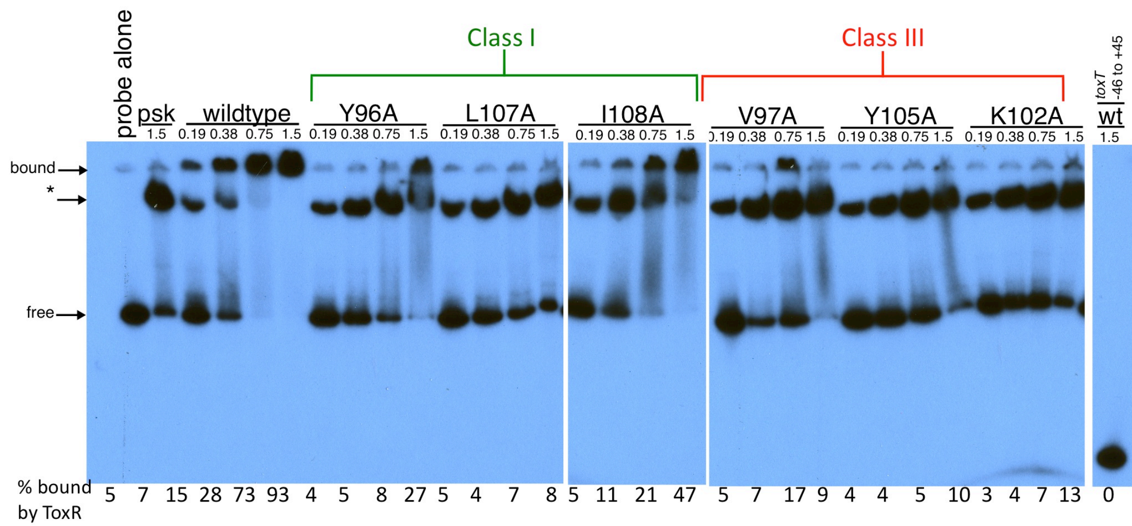
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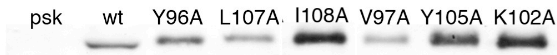
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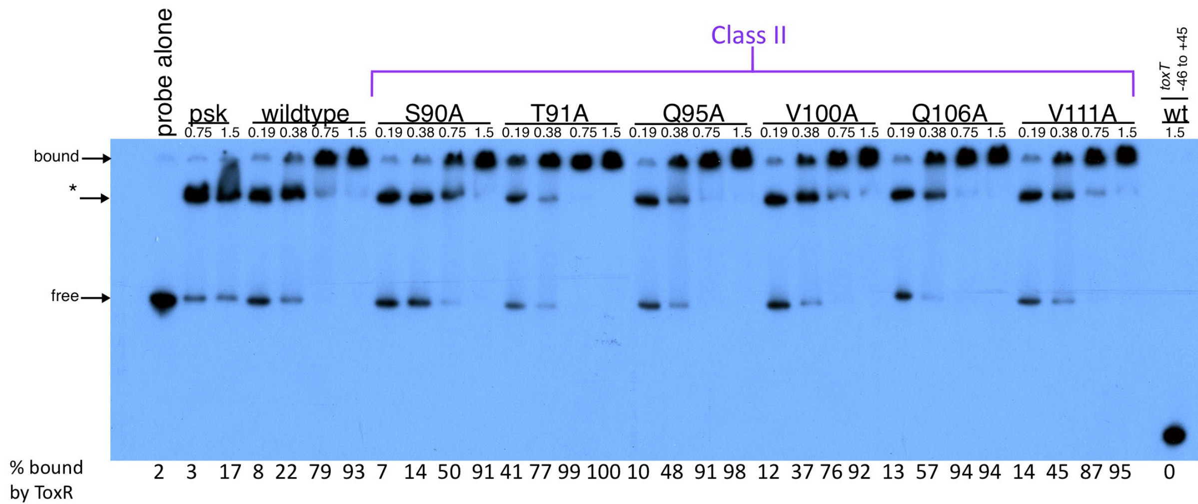
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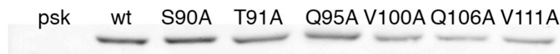
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Figure 4-7. Certain wing residues of ToxR are required for DNA binding. DNA binding by ToxR wing mutants was monitored using EMSA. *V. cholerae* membranes containing the ToxR wing mutants, but lacking TcpP, were bound to ^{32}P end-labeled probes containing either the *toxT* promoter (A) or the *ompU* promoter (B). ToxR stability for all membranes was monitored by anti-HA Western blot. Representative gels and quantification are shown from a minimum of two replicates. * indicates an intermediate shifted *ompU* promoter that is dependent on a factor in the membrane extract other than ToxR (2).

increase in DNA binding efficiency to both the *ompU* and *toxT* promoters. This corresponded to a slight, but significant ($p<0.05$) increase in transcriptional activation of both of these promoters (Fig. 4-2, 4-4, and 4-7). Based on all of the wing mutants tested, ToxR activity at both the *toxT* and *ompU* promoters corresponded to the ability of the ToxR wing to bind these promoters.

Discussion

The wing of ToxR is primarily required for DNA binding to both the *toxT* and *ompU* promoters. All of the residues in ToxR that were required for transcriptional activation of *toxT* and *ompU* were also required for DNA binding. Different regions of the wing are more or less critical for DNA binding and transcriptional activation by ToxR. The majority of the residues in the β-hairpin of the wing of ToxR are required for transcriptional activation and DNA binding (Fig. 4-2, 4-4, and 4-7). This is to be expected since this region of the wing is often inserted into the minor groove of the DNA (3, 24). Based on the modeled location of these residues, they may be involved in positioning of the wing relative to the rest of the w-HTH domain or actually interact with the DNA backbone. K102 is predicted to protrude from the tip of the wing and likely interacts with nucleotides in the minor groove, similar to R220 in PhoB (3, 24). ToxR-T99, G104, and Y105 correspond to PhoB-T217, G222, and Y223 which interact with the backbone of DNA (3, 24). ToxR-V97, Y105, and L107 may contribute to the hydrophobic core, since they correspond to residues that comprise the hydrophobic core in OmpR and PhoB (24, 28). Furthermore, mutation of two of these residues (ToxR-V97A and ToxR-L107A) renders the molecule unstable (Fig. 4-4), an anticipated phenotype of a mutant deficient in folding of the hydrophobic core. Mutation of a few residues near the hinge of the β-hairpin (Y96, L107 and I108), where it joins the rest of the w-HTH domain, were preferentially required for *toxT*

expression when expressed from a plasmid (Fig. 4-2). However, this is likely an effect of overexpression since these mutants lost the differential defect in *toxT* expression when expressed from the chromosomal *toxR* locus (Fig. 4-4). It is likely that these residues are involved in positioning of the wing relative to the DNA-binding helix. Although a crystal structure of the ToxR w-HTH domain is not currently available, I-TASSER modeling of ToxR, based on other members of the w-HTH family (2), places many of these residues aimed towards the core of the protein indicating that they are likely involved in positioning of the wing.

Some residues in the loop leading from the DNA-binding helix (α3) to the β-hairpin of the wing were critical for DNA binding and activation of *toxT* and *ompU*, while others were not (Fig. 4-2, 4-4, and 4-7). It is possible that these residues contribute to the hydrophobic core of ToxR, but this is not likely since no residues in this loop have been shown to be part of the hydrophobic core in either OmpR or PhoB (24, 28). The residues required for DNA binding and transcriptional activation may be involved in positioning the wing relative to the DNA-binding helix. D89 is positioned at the end of the DNA-binding helix and may affect helical structure or positioning of the DNA-binding helix. Since mutation of residues C-terminal to amino acid 109 had minimal effect on transcriptional activation of either promoter, it is likely that R110 marks the beginning of the variable linker region that tethers the w-HTH domain to the membrane (34).

Although some residues in the wing of ToxR were preferentially required for *toxT* activation, all of the residues tested lost this preferential requirement when expressed from the chromosome. Therefore, the preferential requirement of the wing for *toxT* activation is likely an effect of the overexpression system and not an actual increased dependence on the wing for *toxT* activation. Furthermore, the residues in the wing do not appear to preferentially be required for binding to one promoter or the other, since mutations leading to disruption of binding to the *toxT*

promoter result in similar disruption of binding to the *ompU* promoter. The consensus sequences for ToxR binding to *toxT* and *ompU* are similar (11). Additionally, a majority of the residues required for DNA binding are present in regions of the wing not likely to come into direct contact with the DNA, but instead are most likely involved in positioning of the wing such that it is able to insert into the minor groove. Together this shows that the role of the wing is to stabilize ToxR binding to the promoter.

None of the ToxR residues tested by ToxR-TcpP co-immunoprecipitation were required for TcpP interaction. Since ToxR-TcpP interaction should only be required for *toxT* activation, mutations disrupting ToxR-TcpP interaction would be expected to result in a preferential defect. However, five of the six ToxR residues that were preferentially required for *toxT* activation when expressed from a plasmid have been tested for interaction defects either in this or previous publications using DSP crosslinking (2). Additionally, any ToxR wing residues that were identified as being important for ToxR-TcpP interaction via the BACTH assay (Fig. 4-6) were not required for ToxR-TcpP interaction when tested by co-immunoprecipitation. Based on these findings it is likely that the wing of ToxR is either not required for ToxR-TcpP interaction or that ToxR-TcpP interaction is not required for *toxT* activation.

Since the wing of ToxR does not appear to contribute to ToxR-TcpP interaction, there are two likely ways in which ToxR and TcpP could interact based on the interaction of other w-HTH proteins (24, 29, 31, 27, 32): ToxR N-terminal β-sheet to TcpP N-terminal β-sheet or ToxR β-sheet to TcpP wing. Previous studies have shown that the wing of TcpP is required for ToxR-TcpP interaction, since mutation of several residues of the wing of TcpP results in decreased interaction (7). Furthermore, one such TcpP mutant, TcpP-N99D, showed reduced interaction with ToxR in ToxR-TcpP co-immunoprecipitation studies as well (Fig. 4-5). As we have found

the wing of ToxR does not appear to interact with TcpP, we predict ToxR and TcpP dimerize by interaction of the wing of TcpP with the β-sheet of ToxR. If ToxR and TcpP interact in this orientation on the promoter it would place the wing of TcpP oriented upstream, away from the promoter, in the opposite orientation to other w-HTH proteins bound to their promoters (3, 24, 29). Additionally, ToxR and TcpP bind to the *toxT* promoter three helical turns apart, making it difficult to picture how the w-HTH domains could interact when bound to the DNA (11). Finally, DNA binding by TcpP is required for *toxT* activation (8) and the wing of TcpP is required for DNA binding (6, 7). Thus, ToxR-TcpP interaction may enhance activation by recruiting TcpP to the *toxT* promoter along with ToxR, but this interaction may be disrupted upon DNA binding to allow TcpP to access the binding site three helical turns downstream of ToxR on the *toxT* promoter (Fig. 4-8).

It has been previously shown that ToxR co-activation of *toxT* is due in part to alteration of the *toxT* promoter. ToxR relieves H-NS repression, recruits the promoter to the membrane, and exposes a DNAse I hypersensitivity site at the TcpP-binding site (1, 5, 6). All of these functions are dependent on ToxR binding to the promoter, making the wing critical for ToxR co-activation of the *toxT* promoter.

Materials and Methods

Culture conditions. *V. cholerae* strains were routinely grown overnight in Vc LB (LB containing 5 g/L NaCl) at 37°C. Unless otherwise stated cultures were induced by backdilution into Vc LB pH 6.5 and grown at 30°C. Cultures were grown in the presence of 100 μg/ml ampicillin, 25 μg/ml chloramphenicol, or 100 μg/ml streptomycin as needed.

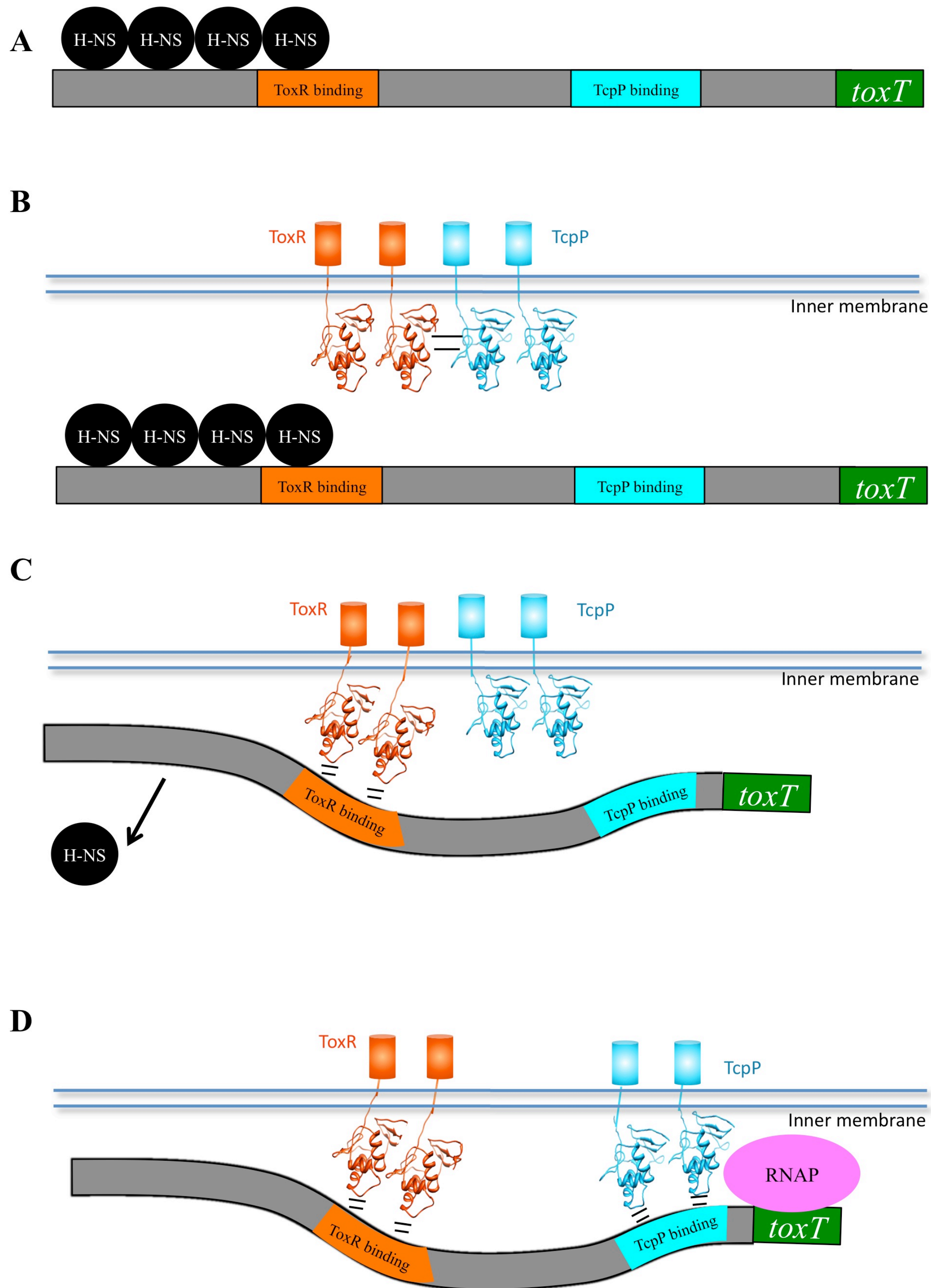
A
H-NS
H-NS
H-NS
H-NS
ToxR binding
TcpP binding
toxT
B
ToxR
TcpP
Inner membrane
H-NS
H-NS
H-NS
H-NS
ToxR binding
TcpP binding
toxT
C
ToxR
TcpP
Inner membrane
ToxR binding
TcpP binding
toxT
H-NS
D
ToxR
TcpP
Inner membrane
RNAP
ToxR binding
TcpP binding
toxT

Figure 4-8. Model of ToxR mediated co-activation of the *toxT* promoter. A) Prior to activation, the *toxT* promoter is silenced by H-NS (1). B) ToxR-TcpP interaction between the N-terminal β-sheet of ToxR and the wing of TcpP recruits TcpP to the *toxT* promoter. C) Upon ToxR binding to the *toxT* promoter, ToxR and TcpP no longer interact. ToxR binding to the promoter results in relieving H-NS repression (1), bending the DNA to make the TcpP binding site more accessible (6), and recruiting the *toxT* promoter to the membrane (5). D) This results in enhanced TcpP binding to the *toxT* promoter and transcriptional activation.

Construction of strains and plasmids. ToxR wing mutants were generated by PCR using complimentary muta genic primers as described previously (11). Chromosomal ToxR wing mutants were created using chromosomal recombination of the suicide plasmid pKAS32 and selected for by loss of streptomycin resistance as described previously (35).

β-galactosidase assay for transcriptional activation by ToxR. *V. cholerae* strains containing chromosomal *ompU-lacZ* or *toxT-lacZ* reporters (REF) were backdiluted 1:30 and induced as described above. ToxR expression from psk-ToxR-HA constructs was induced by addition of 100μM IPTG. For all transcriptional activation assays, activation was assayed after 4 hours by β-galactosidase assay as described previously (36). The OD_{600} was determined by spectrophotometry and used to normalize cultures for subsequent Western blot analysis using antibodies against HA (Covance) or ToxR (generated against the N-terminal 170 amino acids of ToxR by Covance).

Co-immunoprecipitation of ToxR and TcpP. pMMB-TcpP-HSV (7) was mated into Δ*tcpP V. cholerae* strains containing chromosomal ToxR wing mutations. Cultures were backdiluted 1:100 and grown under inducing conditions in the presence of 1mM IPTG for 6 hours. Membranes were isolated as described previously (37) and dialyzed into HEPES-buffered saline (HBS, 20 mM HEPES pH 7.0, 150 mM NaCl). Two mg/ml of membrane proteins was crosslinked using 5mM DSP (Pierce) for 30 minutes at room temperature. Subsequent crosslinking was blocked with 50mM Tris pH7.4 for 15 minutes at room temperature. Membrane proteins were denatured and solubilized by boiling in 1% SDS for 5 minutes prior to addition of IP buffer (1% Triton, 10mM MgCl, 5mM EDTA, 50mM Tris pH7.4). TcpP was immunoprecipitated by incubation for 2 hours at 4°C with 45μL of a 50:50 suspension protein G-agarose beads (Calbiochem), which had been pre-incubated with 3μg anti-HSV (Calbiochem) for

Table 1: Strains and Plasmids

***Vibrio cholerae* strains**	
0395 Δ*toxR toxT-lacZ* (EK1072)	(2)
0395 Δ*toxR ompU-lacZ* (EK410)	(5)
0395 *ompU-lacZ* (EK383)	
0395 *ompU-lacZ toxR*-Y96A	This study
0395 *ompU-lacZ toxR*-L107A	This study
0395 *ompU-lacZ toxR*-I108A	This study
0395 *ompU-lacZ toxR*-S90A	This study
0395 *ompU-lacZ toxR*-T91A	This study
0395 *ompU-lacZ toxR*-Q95A	This study
0395 *ompU-lacZ toxR*-V100A	This study
0395 *ompU-lacZ toxR*-S90A/T91A/Q95A/V100A (quad)	This study
0395 *ompU-lacZ toxR*-V97A	This study
0395 *ompU-lacZ toxR*-K102A	This study
0395 *toxT-lacZ* (EK733)	
0395 *toxT-lacZ toxR*-Y96A	This study
0395 *toxT-lacZ toxR*-L107A	This study
0395 *toxT-lacZ toxR*-I108A	This study
0395 *toxT-lacZ toxR*-S90A	This study
0395 *toxT-lacZ toxR*-T91A	This study
0395 *toxT-lacZ toxR*-Q95A	This study
0395 *toxT-lacZ toxR*-V100A	This study
0395 *toxT-lacZ toxR*-S90A/T91A/Q95A/V100A (quad)	This study
0395 *toxT-lacZ toxR*-V97A	This study
0395 *toxT-lacZ toxR*-K102A	This study
0395 Δ*toxT* Δ*toxR* (EK459)	(6)
0395 Δ*toxT* (RY1)	(10)
0395 Δ*toxT toxR*-T91A	This study
0395 Δ*toxT toxR*-L107A	This study
0395 Δ*toxT toxR*-I108A	This study
0395 Δ*toxT toxR*-S90A	This study
0395 Δ*toxT toxR*-S90A/T91A/Q95A/V100A (quad)	This study
0395 Δ*toxT toxR*-V97A	This study
0395 Δ*toxT toxR*-K102A	This study
0395 Δ*toxT toxR*-Q95A	This study
0395 Δ*toxT toxR*-Y96A	This study
***E. coli* strains**	
DH5α	Lab strain
DHM1	(14)
BTH101	(12)

Plasmids	
pBluescriptSK(-)	
pBluescriptSK(-)-*toxR-HA*	(2)
pMMB207	(4)
pMMB207-*tcpP-HSV*	(7)
pUT18c	(12)
pUT18c-ToxRS	(2)
pKT25	(12)
pKT25-TcpPH	(2)

Table 4-2. Primers used in this study

Name	Sequence
toxR D89A top	CGCAAAATGCTCAAAGCTTCGACAAAGTCCC
toxR D89A bottom	GGGACTTTGTCGAAGCTTTGAGCATTTTGCG
ToxR S90A top	CAAAATGCTCAAAGATGCGACAAAGTCCCCAC
ToxR S90A bottom	GTGGGGACTTTGTCGCATCTTTGAGCATTTTG
ToxR T91A top	ATGCTCAAAGATTCGGCAAAGTCCCCACAATAC
ToxR T91A bottom	ATTGTGGGGACTTTGCCGAATCTTTGAGCATTTTG
toxR K92A top	CTCAAAGATTCGACAGCGTCCCCACAATACGTC
toxR K92A bottom	GACGTATTGTGGGGACGCTGTCGAATCTTTGAG
toxR S93A top	CAAAGATTCGACAAAGGCCCCACAATACGTCAAAAC
toxR S93A bottom	GTTTTGACGTATTGTGGGGCCTTTGTCGAATCTTTG
toxR P94A top	GATTCGACAAAGTCCGCACAATACGTCAAAAC
toxR P94A bottom	GTTTTGACGTATTGTGCGGACTTTGTCGAATC
ToxR Q95A top	TCGACAAAGTCCCCAGCATACGTCAAAACGGTTC
ToxR Q95A bottom	GAACCGTTTTGACGTATGCTGGGGACTTTGTCGAATC
ToxR Y96A top	GACAAAGTCCCCACAAGCCGTCAAAACGGTTC
ToxR Y96A bottom	GAACCGTTTTGACGGCTTGTGGGGACTTTGTC
ToxR V97A top	CAAGTCCCCACAATACGCCAAAACGGTTCCGAAG
ToxR V97A bottom	CTTCGGAACCGTTTTGGCGTATTGTGGGGACTTG
toxR K98A top	GTCCCCACAATACGTCGCAACGGTTCCGAAGCG
toxR K98A bottom	CGCTTCGGAACCGTTGCGACGTATTGTGGGGAC
toxR T99A top	CACAATACGTCAAAGCGGTTCCGAAGCGCGG
toxR T99A bottom	CCGCGCTTCGGAACCCGTTTGACGTATTGTG
ToxR V100A top	CAATACGTCAAAACGGCTCCGAAGCGCGGTTAC
ToxR V100A bottom	GTAACCGCGCTTCGGAGCCGTTTTGACGTATTG
toxR P101A top	CAATACGTCAAAACGGTTGCGAAGCGCGGTTACC
toxR P101A bottom	GGTAACCGCGCTTCGCAACCGTTTTGACGTATTG
toxR K102A top	GTCAAAACGGTTCCGGCGCGCGGTTACCAATTG
toxR K102A bottom	CAATTGGTAACCGCGCGCCGGAACCGTTTTGAC
toxR R103A top	CAAAACGGTTCCGAAGGCCGGTTACCAATTGATC
toxR R103A bottom	GATCAATTGGTAACCGGCCTTCGGAACCGTTTTG
toxR G104A top	CGGTTCCGAAGCGCGCTTACCAATTGATCGC
toxR G104A bottom	GCGATCAATTGGTAAGCGCGCTTCGGAACCG
ToxR Y105A top	GTTCCGAAGCGCGGTGCCCAATTGATCGCCCGAG
ToxR Y105A bottom	CTCGGGCGATCAATTGGGCACCGCGCTTCGGAAC
ToxR Q106A top	CCGAAGCGCGGTTACGCATTGATCGCCCGAGTG
ToxR Q106A bottom	CACTCGGGCGATCAATGCGTAACCGCGCTTCGG
ToxR L107A top	GAAGCGCGGTTACCAAGCGATCGCCCGAGTGGAAAC
ToxR L107A bottom	GTTTCCACTCGGGCGATCGCTTGGTAACCGCGCTTC
ToxR I108A top	CGCGGTTACCAATTGGCCGCCCGAGTGGAAAC
ToxR I108A bottom	GTTTCCACTCGGGCGGCCAATTGGTAACCGCG
ToxR R110A top	GTTACCAATTGATCGCCGCAGTGGAAACGGTTGAAG
ToxR R110A bottom	CTTCAACCGTTTCCACTGCGGCGATCAATTGGTAAC

ToxR V111A top	CAATTGATCGCCCGAGCGGAAACGGTTGAAGAAG
ToxR V111A bottom	CTTCTTCAACCGTTTCCGCTCGGGCGATCAATTG
ToxR E112A top	GATCGCCCGAGTGGCAACGGTTGAAGAAGAG
ToxR E112A bottom	CTCTTCTTCAACCGTTGCCACTCGGGCGATC
ToxR T113A top	GATCGCCCGAGTGGAAGCGGTTGAAGAAGAGATG
ToxR T113A bottom	CATCTCTTCTTCAACCGCTTCCACTCGGGCGATC
ToxR V114A top	GCCCGAGTGGAAACGGCTGAAGAAGAGATGGCTC
ToxR V114A bottom	GAGCCATCTCTTCTTCAGCCGTTTCCACTCGGGC
ToxR V111A top	CAATTGATCGCCCGAGCGGAAACGGTTGAAGAAG
ToxR V111A bottom	CTTCTTCAACCGTTTCCGCTCGGGCGATCAATTG
ToxR E112A top	GATCGCCCGAGTGGCAACGGTTGAAGAAGAG
ToxR E112A bottom	CTCTTCTTCAACCGTTGCCACTCGGGCGATC
ToxR T113A top	GATCGCCCGAGTGGAAGCGGTTGAAGAAGAGATG
ToxR T113A bottom	CATCTCTTCTTCAACCGCTTCCACTCGGGCGATC
ToxR V114A top	GCCCGAGTGGAAACGGCTGAAGAAGAGATGGCTC
ToxR V114A bottom	GAGCCATCTCTTCTTCAGCCGTTTCCACTCGGGC

2 hours at 4°C. Samples were taken prior to addition of protein G-agarose beads and anti-HSV, after incubation with protein G-agarose beads and anti-HSV, and after three washes with PBS and designated “INput, “UnBound,” and “ImmunoPrecipitated,” respectively. Samples were loaded onto SDS-PAGE gels in either a 1:1:1 ratio (IN:UB:IP) for anti-TcpP (generous gift from the DiRita Laboratory) Western blots or 1:1:5 (IN:UB:IP) for anti-ToxR (generated against the N-terminal 170 amino acids of ToxR by Covance) and anti-EpsL (generous gift from the Sandkvist Laboratory) Western blots. Western blots were developed using Alkaline Phospatase and band intensity was quantified using ImageJ. TcpP-dependant co-immunoprecipitation was calculated by subtracting the band intensity in the IP lane Δ*tcpP* + pMMB207 negative control from the band intensity in the IP lane of each strain tested.

DNA mobility shift assay. DNA binding assays were performed essentially as described previously (6). Membranes were isolated from Δ*toxR*Δ*tcpP V. cholerae* strains (EK459) with pSK-*toxR-HA* which had been induced for 6 hours in 1mM IPTG as described previously (37). Membrane concentrations ranging from 0.19 to 2 mg/ml were incubated with either the *ompU* promoter probe (extending from -211 to +22 relative to the transcriptional start site), the *toxT* promoter probe (extending from -172 to +45 relative to the transcriptional start site), or the negative control probe (*toxT* promoter extending from-46 to +45 relative to the transcriptional start site). 3000 cpm of probe labeled with ^{32}P-dATP was used in each reaction. Western blotting with anti-HA (Covance) was used to monitor ToxR protein levels.

Adenylate cyclase bacterial two-hybrid (BACTH). BACTH assay was performed as described previously (2). ToxR wing mutants expressed from the pUT18c-ToxRS and TcpP expressed from pKT25-TcpPH were transformed into the reporter strain BTH101. Cultures were induced by addition of 0.5 mM IPTG to LB and grown 16 hours at 30°C. 20μL of culture was used for β-

galactosidase assay as described previously (36). Stability was determined by induction of expression of pUT18c-ToxR in BTH101 in the presence of pKT25 with 0.5mM IPTG and 1mM cAMP added to induce expression. Samples were normalized by OD_{600} and analyzed by Western blot using anti-cyaA (Santa Cruz Biotechnology).

Modeling of ToxR wing residues. ToxR wing residues were modeled using the program Chimera based on the threaded structure of ToxR as determined by I-TASSER (2).

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Chapter 5

An intramolecular periplasmic disulfide bond in TcpP prevents premature degradation and enables virulence gene expression in *Vibrio cholerae*

Note: All of the strains created and all of the assays performed in this chapter were done either by myself or by Emily French under my supervision.

Summary

TcpP and ToxR coordinately regulate transcription of *toxT*, the master regulator of many virulence factors in *Vibrio cholerae*. TcpP and ToxR are both membrane-localized transcription factors each with a periplasmic domain containing two cysteines. In ToxR, it has been previously demonstrated that these cysteines form an intramolecular disulfide bond, and substitution of one of these cysteine results in an intermolecular disulfide bond affecting activity. We determined that the two periplasmic cysteines of TcpP also form an intramolecular disulfide bond. Disruption of this intramolecular disulfide bond by mutation of either cysteine resulted in formation of intermolecular disulfide bonds. Furthermore, disruption of the intramolecular disulfide bond in TcpP resulted in decreased stability by increasing TcpP sensitivity to proteolytic degradation by YaeL. The decreased stability of TcpP mutants lacking one or both periplasmic cysteines resulted in levels of *toxT* activation similar to that of a Δ*tcpP* mutant,

culminating in an ~10,000-fold decrease in cholera toxin production. Thus, the periplasmic intramolecular disulfide bond in TcpP is critical for TcpP stability and virulence gene expression.

Introduction

Cholera is caused by ingestion of the aquatic gram-negative bacterium *Vibrio cholerae*. The profuse watery diarrhea characteristic of cholera is induced by cholera toxin (CT), an ADP-ribosolating toxin that induces cyclic AMP production in intestinal epithelial cells resulting in massive secretion of water and electrolytes. Microcolony formation and colonization of the intestine requires expression of the toxin co-regulated pilus (TCP) (7). Transcription of the genes encoding CT and TCP, as well as several other virulence factors, is regulated by the transcription activator ToxT (9). *toxT* expression is regulated by two transmembrane winged-helix-turn-helix (w-HTH) transcription factors, ToxR and TcpP, and their co-activators ToxS and TcpH, respectively (1, 8, 11-16). Based on current models, upon entry into the intestine, environmental signals activate expression of *tcpPH* (18, 19). TcpPH, along with constitutively expressed ToxRS, activates transcription of *toxT* resulting in colonization and CT secretion in a temporally coordinated fashion (21). Under non-inducing conditions, TcpP is specifically targeted for degradation by an undefined periplasmic protease followed by the membrane-localized metalloprotease, YaeL. (8, 20).

TcpP and ToxR have three distinct domains: an N-terminal cytoplasmic DNA-binding domain, a single pass transmembrane domain and a C-terminal periplasmic domain of unknown structure and function. The cytoplasmic domain of TcpP and ToxR is homologous to the OmpR/PhoB family of w-HTH transcription factors (22). Mutations in several key residues of the cytoplasmic domains of both TcpP and ToxR have been identified that inhibit DNA binding

and transcriptional activation (1, 4, 5, 23). TcpP binds to a direct repeat in the *toxT* promoter just upstream of the predicted RNA polymerase (RNAP) binding site (1, 24), whereas ToxR binds to a direct repeat, three turns upstream of the TcpP-binding site (1, 25). Based on the location of these binding sites, TcpP likely interacts with RNA polymerase and is the direct activator of *toxT*. This model is further supported by the fact that overexpressed TcpP can activate *toxT* in the absence of ToxR, but ToxR cannot activate *toxT* expression in the absence of TcpP (1, 5, 11, 12). Membrane localization of ToxR is required for *toxT* activation in conjunction with TcpP, but not activation of the TcpP-independent *ompU* promoter (26-28). Thus, ToxR is believed to serve as a co-activator, enhancing transcriptional activation of *toxT* by promoting TcpP recruitment and/or binding to the *toxT* promoter (1, 4, 5).

Two periplasmic co-activators TcpH and ToxS coordinate with the periplasmic domains of TcpP and ToxR, respectively, for full activation of *toxT* (8, 12, 13, 16)}(11, 15). ToxS has been shown to increase ToxR dimerization and transcriptional activation of *toxT* by ToxR (16), while TcpH is required for TcpP stability and enhances transcriptional activation of *toxT* by protecting TcpP from degradation (8, 20). The periplasmic domain of TcpP is particularly vulnerable to proteolytic degradation resulting in instability of the entire protein (8, 20). Evidence for the role of the periplasmic domain in TcpP instability was provided when the periplasmic domain of TcpP was fused to ToxR, making a ToxR-$TcpP_{peri}$ chimeric protein. This resulted in an unstable ToxR species unless TcpH was present (8). Conversely, replacement of the periplasmic domain of TcpP with the periplasmic domain of ToxR resulted in increased TcpP stability (8). Proteolysis of TcpP is regulated in a multi-step process in which initially an unknown periplasmic protease recognizes the C-terminus and cleaves TcpP. This partially degraded TcpP, denoted TcpP*, is then further cleaved by the membrane-localized

metalloprotease YaeL resulting in complete degradation of TcpP (20). In *E. coli*, YaeL cleaves RseA, a transmembrane protein that tethers σ^E to the membrane. Cleavage and subsequent degradation of RseA releases σ^E from the membrane, thereby allowing σ^E to activate its target promoter (29-31). In the case of TcpP, TcpP is active in the membrane, and cleavage of TcpP by YaeL results in inactivation and degradation (20).

Proper disulfide bond formation is important for the function of many periplasmic proteins (32). In *V. cholerae*, the disulfide isomerase DsbA is required for proper formation and activity of TCP, CT, ToxR and other virulence factors (33-36). The periplasmic domain of ToxR contains two cysteines that form an intramolecular disulfide bond, and disruption of this periplasmic intramolecular disulfide in ToxR by mutation of one of the cysteines to serine in the classical *V. cholerae* strain O395 resulted in formation of an intermolecular disulfide likely between two ToxR molecules (37). The resulting mutant, when expressed from a plasmid, was found to be 30-fold defective in induction of cholera toxin (CT) expression (37). A recent study expressing ToxR from the chromosome found that mutating both periplasmic cysteines of ToxR in the classical strain O395 led to a dramatic decrease in OmpU production, but less than a two-fold defect in CT production in LB medium at pH 6.5 30°C (toxin inducing conditions, (36)). Differences in CT production in these two studies may be due to differences in the effects of replacing one (37) or both (36) cysteines or due to differences between plasmid-expressed and chromosomally-expressed ToxR. TcpP also has cysteines at similar positions in its periplasmic domain. In this study we determined that the TcpP periplasmic cysteines also form an intramolecular disulfide bond. In addition, we examined the role of TcpP periplasmic cysteines in *toxT* transcriptional activation and found that the intramolecular periplasmic disulfide bond of

TcpP is critical for TcpP stability and therefore transcriptional activation of *toxT* and *V. cholerae* virulence gene expression.

Results

TcpP forms an intramolecular disulfide bond. To determine whether TcpP, like ToxR, forms primarily intramolecular disulfide bonds, *V. cholerae* lysates containing pMMB207-expressed TcpP-HSV (TcpP which contains a C-terminal HSV tag) were run on non-reducing SDS-PAGE and analyzed by Western blot. To preserve disulfide bond formation and prevent additional disulfide bonds from forming during sample preparation, *V. cholerae* lysates were treated with iodoacetamide as described previously (37). Non-reduced TcpP-HSV ran slightly faster than reduced TcpP-HSV due to the presence of an intramolecular disulfide bond (Fig. 5-1 lanes 1 and 4). Two fainter bands at approximately 70 kDa were also visible on the non-reduced gel; these bands are consistent with formation of intermolecular disulfide bonds between two TcpP molecules. These dimers could be the result of formation of a single intermolecular disulfide bond between two TcpP molecules or two disulfide bonds between two TcpP molecules. To determine whether the disulfide bonds observed were formed between the two periplasmic cysteines present in TcpP, each periplasmic cysteine was mutated to serine (C207S and C218S). Because these mutants were no longer able to form intramolecular disulfide bonds, TcpP-C207S and TcpP-C218S migrated either at the same position as reduced TcpP, or at a higher molecular weight consistent with a TcpP dimer formed by intermolecular disulfide bonds (Fig. 5-1 lanes 6-9). The TcpP dimer bands migrated slightly differently depending on whether C207 or C218 is present, likely due to differences in the location of the disulfide bond relative to the C-terminus of the protein. Since proteins in this family tend to dimerize (27, 37-42), and this band was the

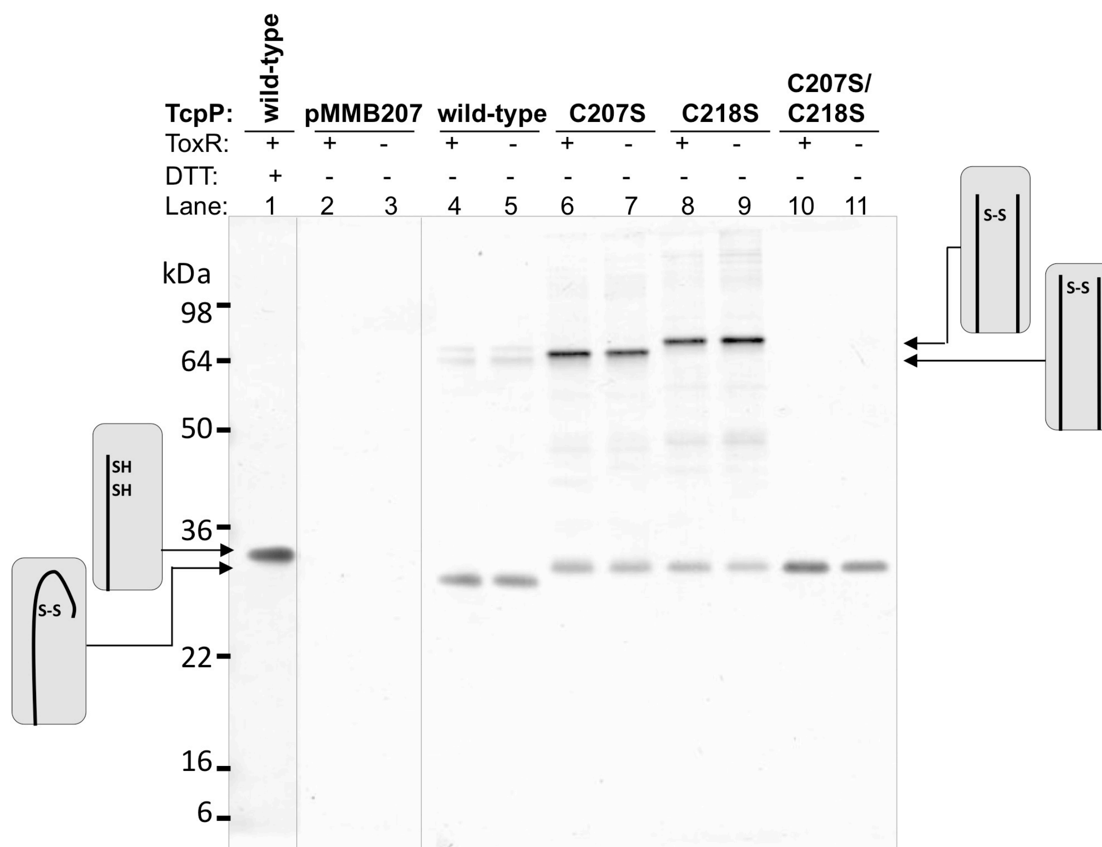

Figure 5-1. **TcpP forms intramolecular and intermolecular disulfide bonds.** Lysates of *V. cholerae* (EK459 Δ*toxR*Δ*tcpP*) expressing pMMB-TcpP-HSV and either pSK or pSK-ToxR-HA were incubated with iodoacetamide prior to resuspension in sample buffer with or without DTT. Blots were probed with anti-HSV primary antibody and alkaline phosphatase conjugated anti-mouse secondary antibody. Mutations in either or both, periplasmic cysteines were used to perturb disulfide bond formation. The arrows and diagrams along the side of the blot indicate the state of the periplasmic cysteines in each form of TcpP detected.

expected size of a TcpP dimer, we predicted that this band was likely the result of an intermolecular disulfide bond formed between two TcpP molecules. To confirm this, the complex was isolated by immunoprecipitation and analyzed by mass spectrometry (data not shown). The only peptides detected by mass spectrometry were from TcpP, indicating that this band is likely the result of a TcpP homodimer. When both periplasmic cysteines were mutated to serine only a single band at the expected size for reduced TcpP was observed (Fig. 5-1 lanes 10-11), confirming that the disulfide bonds observed were formed by the periplasmic cysteines and not any of the four cytoplasmic cysteines in TcpP. The presence of ToxR or TcpH had no effect on disulfide bond formation (Fig. 5-1 and data not shown). In conclusion, the periplasmic cysteines in TcpP form intramolecular disulfide bonds. However, when only one periplasmic cysteine is available, TcpP forms crosslinked homodimers.

TcpP containing intermolecular disulfide bonds maintains *toxT* activation. The TcpP periplasmic cysteine mutants were tested for transcriptional activation of a chromosomal *toxT-lacZ* reporter to determine whether intramolecular periplasmic disulfide bonds are required for activity. TcpP-C218S was able to activate transcription at >80% of wild-type levels (Fig. 5-2A). Since TcpP-C218S is not able to form intramolecular disulfide bonds, this demonstrates that the intramolecular disulfide bond is not required for transcriptional activation of *toxT.* Additionally, the presence of an intermolecular disulfide bond only partially interfered with *toxT* activation by TcpP-C207S, which maintained 48% activity. TcpP-C207S/C218S, on the other hand, was nearly completely defective for *toxT* activation (13% of wild-type), perhaps in part due to the modest instability of this mutant (Fig. 5-2B). However, TcpP-C207S has similar levels of protein present and maintained 48% *toxT* activation (Fig. 5-2). Thus, either intramolecular or

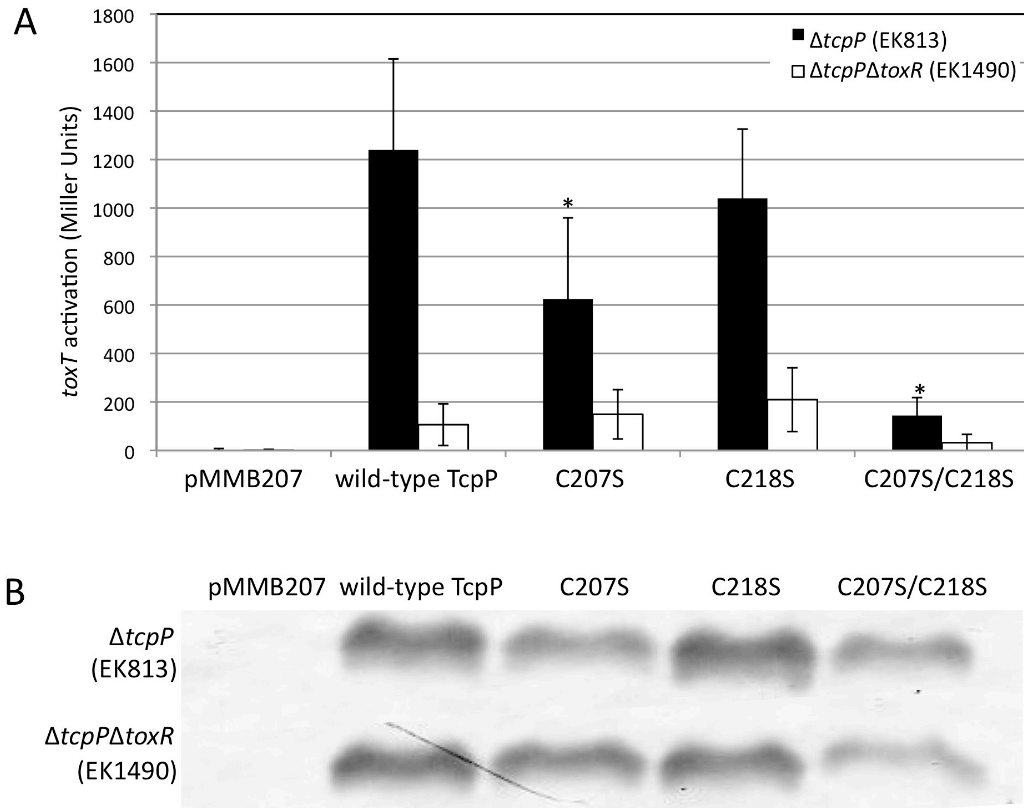

Figure 5-2. TcpP-HSV is able to activate transcription of *toxT* in the presence of intramolecular or intermolecular periplasmic disulfide bonds. A) *toxT* activation by the TcpP-HSV periplasmic cysteine mutants expressed from the pMMB207 plasmid was measured by β-galactosidase assay using chromosomal *toxT-lacZ* reporters (EK813 and EK1490, Table S1, (5)). Activation was measured both in the presence (black bars) and the absence (white bars) of chromosomally-expressed ToxR. B) TcpP-HSV expression was monitored by Western blot using anti-HSV antibody. * $p < 0.005$ relative to wild-type TcpP.

intermolecular disulfide bonds in the periplasmic domain of TcpP are required to maintain *toxT* activation capacity.

TcpP-mediated *toxT* activation in the absence of ToxR decreased 10-fold, as ToxR facilitates TcpP-mediated *toxT* activation (1). ToxR-independent activation of *toxT* by overexpressed TcpP-C207S and TcpP-C218S was at or above wild-type levels (Fig. 5-2A white bars), again demonstrating that maintenance of either intramolecular or intermolecular periplasmic disulfide bonds in TcpP are sufficient for *toxT* activation activity. Mutation of both cysteines, TcpP-C207S/C218S, resulted in loss of *toxT* activation by TcpP overexpressed in the absence of ToxR (Fig. 5-2A), although in this strain background TcpP-C207S/C218S was particularly unstable, maintaining just 34% of the level of TcpP protein as wild-type TcpP (as determined by ImageJ software).

Stability of TcpP periplasmic cysteine mutants is greatly reduced when expressed from the chromosome without a C-terminal epitope tag. Because our initial experiments were performed with C-terminally HSV-tagged TcpP, and the C-terminus of TcpP is known to be susceptible to periplasmic proteases (8, 20), we determined the consequences of disrupting TcpP periplasmic disulfide bond formation with untagged chromosomally expressed TcpP. *tcpP*-C207S, *tcpP*-C218S or *tcpP*-C207S/C218S alleles were introduced on the chromosome, and *toxT* activation was measured using a plasmid-based reporter containing the *toxT* promoter. Expression of TcpP-C207S and the TcpP-C207S/C218S mutant resulted in a dramatic decrease in *toxT* expression, approaching the levels seen in the Δ*tcpP* strain RY1 (Fig. 5-3). This decrease in activity corresponded to profound TcpP instability, since little to no TcpP-C207S or TcpP-C207S/C218S was detectable by Western blot (Fig. 5-3). TcpP-C218S was the most stable of all

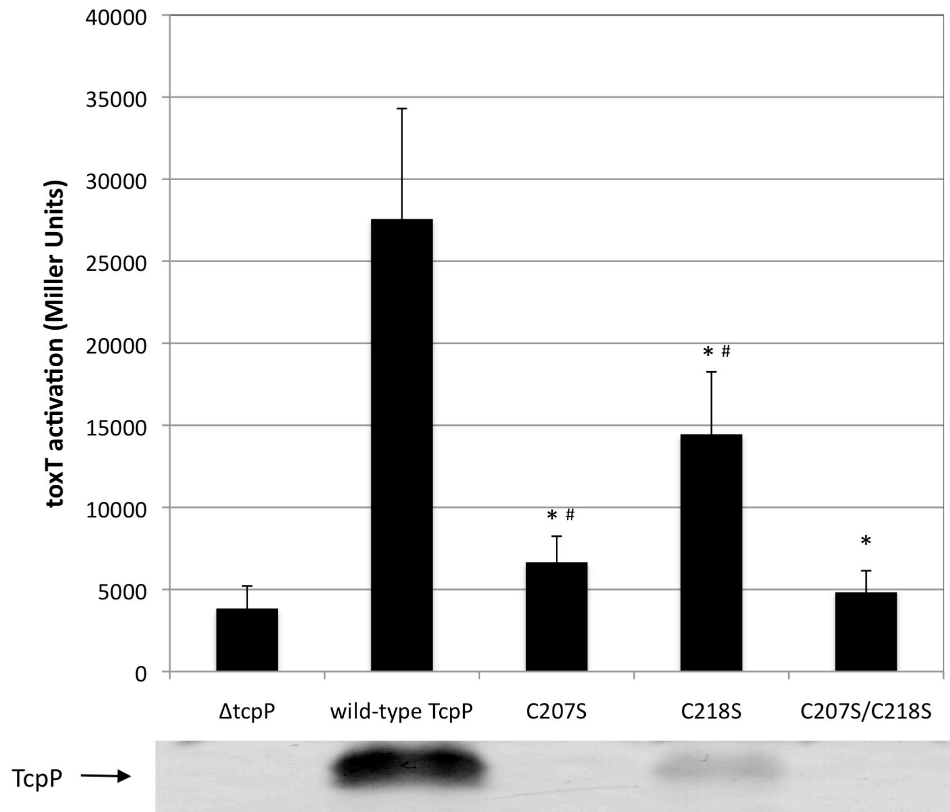

Figure 5-3. The instability of chromosomally-expressed TcpP cysteine mutants lacking a C-terminal epitope tag results in a *toxT* activation defect. A) Transcription activation of *toxT* was monitored by β-galactosidase assay in *V. cholerae* strains in which the wild-type allele of *tcpP* was replaced with alleles encoding TcpP cysteine mutants. *toxT* activation was monitored using the plasmid-based *toxT-lacZ* reporter, pTLI2 (3). A Western blot of lysates from the β-galactosidase assay were probed with anti-TcpP antibodies demonstrating the instability of these constructs. While TcpP-C207S and TcpP-C207S/C218S were undetectable, TcpP-C218S was present at 13% the level of wild-type TcpP as determined by ImageJ software. * $p < 0.005$ relative to wild-type TcpP. # $p < 0.005$ relative to Δ*tcpP*.

the chromosomally expressed mutants (although only present at 13% of wild-type levels) and maintained ~50% of *toxT* activation relative to wild-type TcpP. While TcpP-C207S was not detectable by Western blot, it was able to activate *toxT* expression 2-fold above background. Addition of extra TcpH from a plasmid did not increase stability of any of the mutants (data not shown). These findings suggest TcpP can be artificially protected from proteolysis by addition of a C-terminal HSV tag, and this likely accounts for the differences observed in stability between the mutants expressed from the chromosome and the pMMB207-*tcpP*-HSV plasmid (Figs. 5-2B and 5-3).

To determine whether the defect in TcpP stability, and therefore *toxT* transcription, resulted in a defect in virulence gene production, CT secretion directed by each chromosomally-expressed TcpP mutant was measured. As expected from the *toxT* activation data (Fig. 5-3), TcpP-C207S and TcpP-C207S/C218S directed background levels of CT production, a nearly 10,000-fold decrease relative to the parental strain 0395 (Table 5-1). TcpP-C207S was able to produce CT levels just above the limit of detection for this assay indicating that the low levels of *toxT* induced in this strain (Fig. 5-3) may be sufficient to induce a small amount of CT production (Table 5-1). The dramatically reduced levels of CT produced by TcpP-C207S and TcpP-C207S/C218S corresponded to undetectable TcpP protein levels in their respective strains (Fig. 5-4), as was observed in the *toxT* activation assay (Fig. 5-3). TcpP-C218S was the most stable chromosomally expressed cysteine mutant in both the *toxT* activation and CT assays, although expression levels were still well below wild-type (Fig. 5-3 and 5-4). This resulted in a slight (2-fold) defect in *toxT-lacZ* induction (Fig. 5-3). This level of *toxT* expression is sufficient for levels of CT production approaching the wild-type, as CT levels were only reduced 6-fold in this strain (Table 5-1). Thus, the decrease in *toxT* expression and CT production by the TcpP

Table 5-1. Some chromosomally-encoded TcpP periplasmic cysteine mutants are severely defective for CT production. Levels of cholera toxin secretion by each chromosomally-encoded TcpP periplasmic cysteine mutant were detected by a CT-ELISA on overnight cultures of *V. cholerae*. CT levels in TcpP-C207S and TcpP-C207S/C218S were not significantly above those in the Δ*tcpP*Δ*toxR* negative control strain (p=0.06 for TcpP-C207S). CT was measured in duplicate in two separate experiments (n=4).

Strain	CT (ng/mL/OD_{600})
0395	1467 ± 583
ΔtcpPΔtoxR	<0.2 ± 0.2
TcpP-C207S	0.39 ± 0.04
TcpP-C218S	238 ± 103
TcpP-C207S/C218S	<0.2 ± 0.2

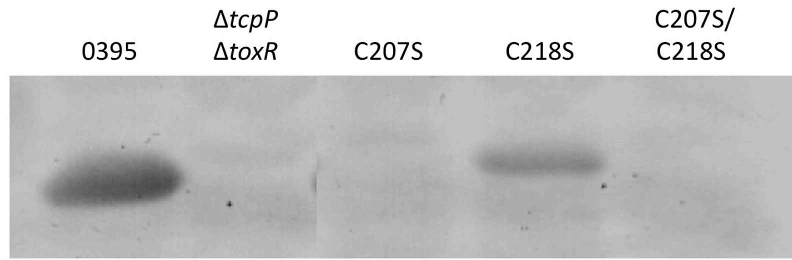

Figure 5-4. TcpP expression in strains assessed for CT production. Western blot analysis of TcpP expression from 0395, EK459 (Δ*tcpP*Δ*toxR*) and chromosomally-encoded TcpP mutants.

periplasmic cysteine mutants corresponds to their relative instability, indicating that defects in *toxT* transcription and virulence factor production by these mutants is directly related to their decreased stability.

Degradation of the TcpP periplasmic mutants is mediated by YaeL. Since YaeL is a known protease of TcpP (20) we determined whether the periplasmic disulfide bond of TcpP protects it from degradation by YaeL. Thus, we constructed an 0395 Δ*tcpP* strain into which we added back pBAD18-TcpPH plasmids encoding the TcpP periplasmic cysteine mutants. Upon plasmid-based expression, the untagged TcpP-C207S, TcpP-C218S, and TcpP-C207S/C218S were still unstable (Fig. 5-5A). A residual degradation band at approximately 17 kDa was observed in the periplasmic cysteine mutant strains, as proteolysis was likely incomplete due to plasmid-based overexpression. When these constructs were expressed in a Δ*tcpPH*Δ*yaeL* background, the TcpP periplasmic cysteine mutants were processed to a protein of ~20 kDa, previously designated TcpP* (Fig. 5-5B) (20). This degradation product was previously described upon shifting wild-type O395 cultures to non-inducing conditions in a Δ*yaeL* strain (20). The same pattern was observed when TcpP periplasmic mutants were expressed from the chromosome of a Δ*yaeL* strain (data not shown). The increase in levels of TcpP* in the Δ*yaeL* background of TcpP-C207S, TcpP-C218S, and TcpP-C207S/C218S mutants indicates they are more vulnerable to cleavage by YaeL than wild-type TcpP. Their susceptibility under TcpP-inducing conditions is similar to YaeL-mediated degradation of wild-type TcpP under non-inducing conditions.

Cleavage by YaeL is a two-step process, with a site-1 protease initiating cleavage followed by cleavage by the site-2 protease, YaeL. One likely candidate site-1 protease is DegS. DegS is the *V. cholerae* homolog of the site-1 protease responsible for initial cleavage of proteins

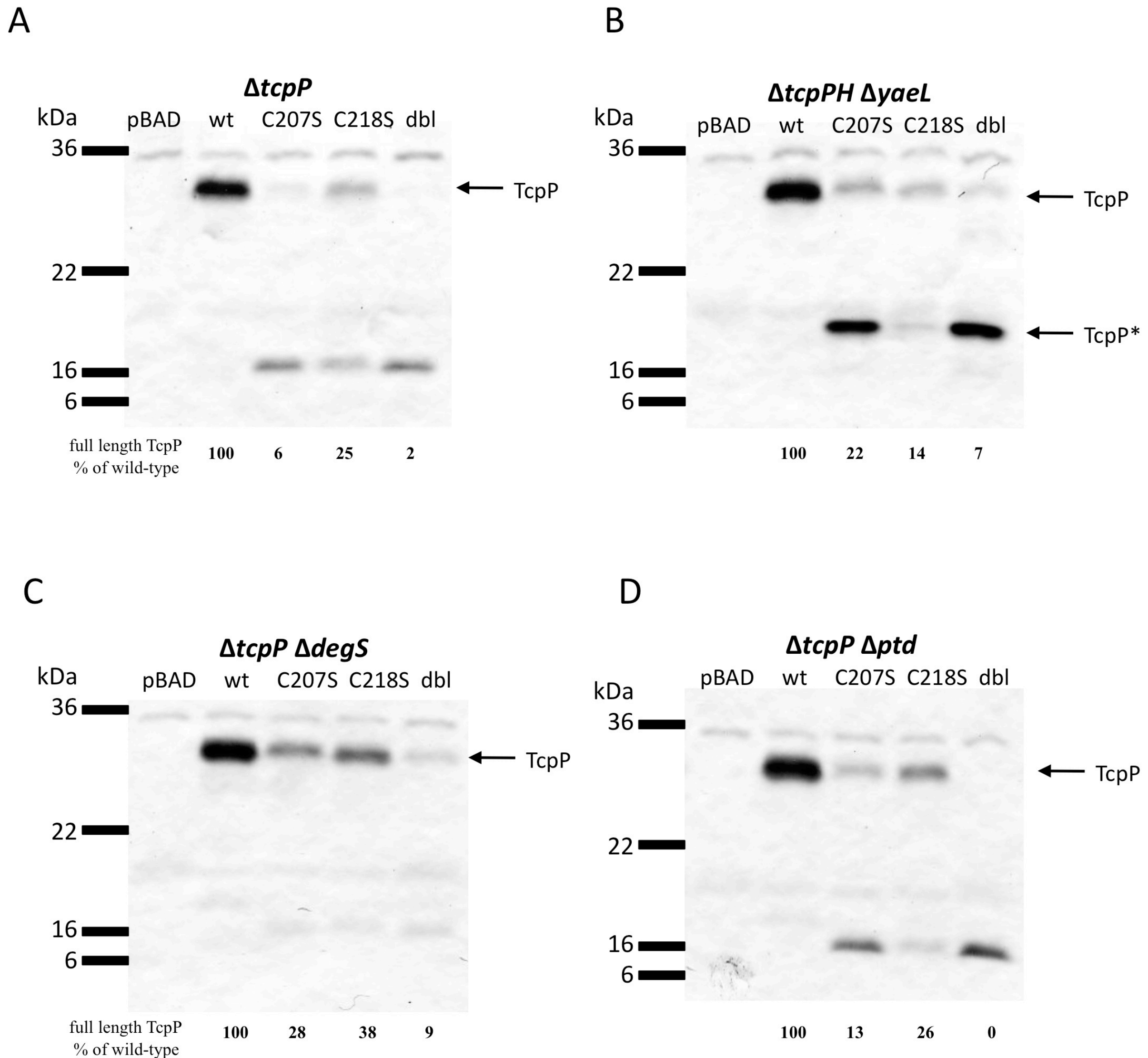

Figure 5-5. TcpP mutants with intermolecular or no periplasmic disulfide bonds are degraded through the YaeL pathway. A) Stability of untagged TcpP periplasmic cysteine mutants expressed from the pBAD18 plasmid in a Δ*tcpP* strain was monitored by Western blot. B) Deletion of *yaeL* from *V. cholerae* strains expressing TcpP with periplasmic cysteine mutations resulted in accumulation of partially degraded TcpP (designated TcpP*). The stability of the plasmid-expressed TcpP cysteine mutants was also monitored by Western blot in Δ*tcpP*Δ*degS* (C) or Δ*tcpP*Δ*ptd* (D) strains. TcpP was detected by Western blot probed with anti-TcpP. A cross-reactive, non-TcpP band running at ~36 kDa was present in all lanes, even the Δ*tcpP* strain harboring the empty vector pBAD18. The % of full length TcpP remaining in each lane relative to wild-type TcpP for each panel is provided below each lane and was determined by ImageJ software (http://rsbweb.nih.gov/ij/).

prior to YaeL cleavage in *E. coli* (20, 30). Although deletion of DegS in *V. cholerae* did not result in increased stability of wild-type TcpP under non-inducing conditions (20), DegS could be involved in degradation of the more proteolytically-sensitive TcpP periplasmic cysteine mutants. Using the pBAD18 expression system we observed modest stabilization of TcpP-C207S and TcpP-C218S in the Δ*tcpP*Δ*degS* strain (present at 28% and 38% of wild-type TcpP levels, respectively), but minimal stabilization of TcpP-C207S/C218S (Fig. 5-5C). This indicates that although deletion of *degS* may have partially stabilized the TcpP periplasmic cysteine mutants, there is another periplasmic protease that is primarily responsible for the enhanced proteolytic degradation of these mutants. Another candidate for the site-1 protease mediating degradation of the TcpP periplasmic cysteine mutants is protease-DO (Ptd). Ptd is a periplasmic chaperone responsible for degradation of unstable or misfolded proteins in the periplasm (8, 43, 44). Deletion of *ptd* resulted in modest if any increased stability of TcpP-C207S and TcpP-C218S (Fig. 5-5D) indicating that Ptd is not the site-1 protease for misfolded TcpP. Thus, another yet to be identified periplasmic protease or combination of proteases is likely responsible for degradation of the TcpP periplasmic cysteine mutants.

ToxR periplasmic cysteines also form intramolecular and intermolecular disulfide bonds. Previous work has shown that wild-type ToxR forms an intramolecular disulfide bond and that disruption of that bond by mutation of one of the cysteines results in intermolecular disulfides and a corresponding decrease in cholera toxin production (37). We wanted to re-examine the role of ToxR periplasmic disulfides using chromosomally expressed ToxR and looking at multiple loci activated by ToxR. As was seen by Ottemann and Mekalanos, under non-reducing conditions, wild-type ToxR migrates slightly faster than the fully reduced form indicating the

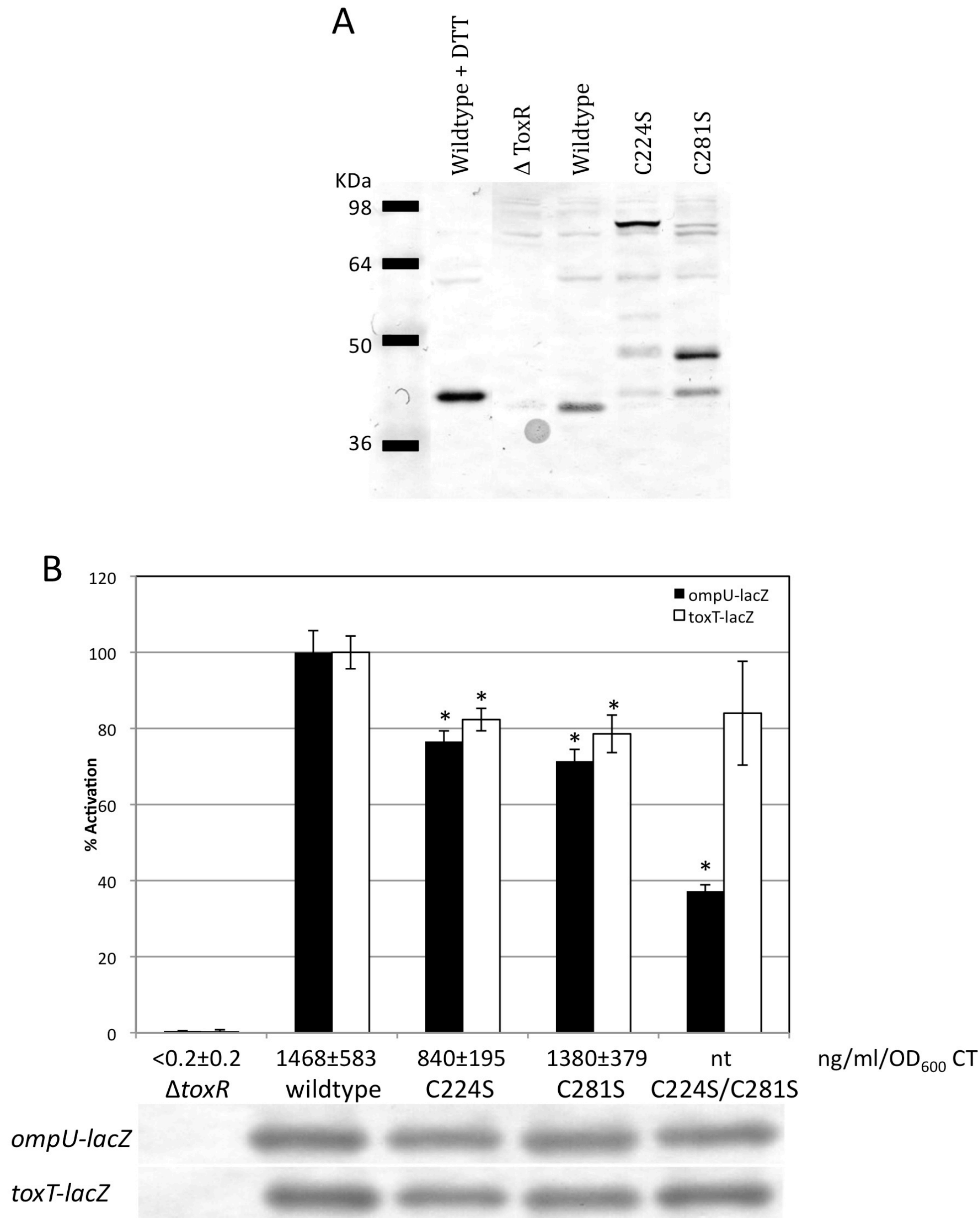

Figure 5-6: Chromosomally-encoded ToxR periplasmic mutants have modest effects on *ompU* expression and minimal effects on *toxT* expression and CT production. A) Chromosomally expressed ToxR periplasmic cysteine mutants were assayed for formation of intermolecular and intramolecular disulfide bonds using non-reducing western blots. B) Chromosomally expressed ToxR periplasmic cysteine mutants were assessed for transcription activation of *toxT-lacZ* and *ompU-lacZ* using chromosomal reporters *p < 0.005 relative to wild-type ToxR. The ng/ml/OD_{600} of cholera toxin produced by each strain is designated under the graph as measured by a CT-ELISA assay. No significant difference between CT production of wild-type ToxR and ToxR-C224S or ToxR-C281S was observed. Western blots using anti-ToxR antibody are shown below the graph to indicate stability of these mutants. nt=not tested. Transcription activation was tested at least twice in triplicate (n=6) for each strain. CT assays were performed twice in duplicate (n=4).

formation of an intramolecular disulfide bond between C224 and C281 (37) (Fig. 5-6A). ToxR-C224S resulted in formation of a large molecular weight band at approximately 80 KD consistent with a ToxR homodimer formation of an intermolecular disulfide bond. A small portion of ToxR-C224S is found as a ToxR monomer. Despite its ability to form intermolecular disulfide bonds, ToxR-C281S is not found in disulfide linked homodimers and instead migrates at the same position as reduced ToxR (Figure 5-6A). Both ToxR-C224S and ToxR-C281S were slightly defective for activation of *toxT-lacZ* and *ompU-lacZ*. ToxR-C224S/C218S was preferentially defective for *ompU-lacZ* (approximately 60% activity) compared to *toxT-lacZ* (80% activity) (Fig. 5-6B). This may be due to the difference in roles of ToxR in activating these two promoters. The transcriptional activation defect is not likely connected to the formation of inappropriate disulfide bonds since both ToxR-C224S and ToxR-C218S were equally defective for activation despite only ToxR-C224S forming detectable intermolecular disulfide bonds. The observed transcriptional activation defect may be a result of a lack of proper intramolecular disulfide bond formation resulting in incorrect periplasmic structure or a slight instability.

Discussion

The purpose of this study was to determine whether TcpP forms periplasmic disulfide bonds, and if so, to determine whether the disulfide bonds are required for *toxT* promoter activation. We found that TcpP, like ToxR, has two periplasmic cysteines that form an intramolecular disulfide bond (Fig. 5-1). Disruption of this disulfide bond by mutation of either of the two TcpP periplasmic cysteines to serine results in an intermolecular disulfide, forming a TcpP homodimer (Fig. 5-1). An additional, faint band can be detected at approximately 50 kDa in both the TcpP-C207S and TcpP-C218S mutants (Fig. 5-1), which is the predicted size of a

TcpP-TcpH heterodimer. TcpH is somewhat unusual in that it is a periplasmic protein with a single cysteine and may serve a chaperone-like function for TcpP (45). TcpP mutants lacking the ability to form an intramolecular disulfide bond are unstable when chromosomally expressed (Fig. 5-3 and 5-4). This instability results in decreased *toxT* activation and ToxT-dependent CT production (Fig. 5-3 and Table 5-1). Disulfide bonds formed with C207 are particularly crucial for stability as can be seen by the increase in stability of the TcpP-C218S mutant relative to the TcpP-C207S mutant. The *tcpP-C218S* mutation overlaps the start site of *tcpH* (changing ATG to ATC) and may decrease translation of *tcpH*. However, expression of additional TcpH did not further stabilize the TcpP-C218S mutant (or any other periplasmic cysteine mutant, data not shown). The hypersensitivity of TcpP-C207S to proteolytic degradation may indicate that formation of a disulfide bond by this residue partially masks the unknown proteolytic recognition site on TcpP, thereby enhancing TcpP stability.

Under inducing conditions TcpP is protected from degradation by TcpH, allowing it to induce expression of *toxT* (8, 20). When switched to non-inducing conditions, TcpP is cleaved by an unknown site-1 periplasmic protease. This results in further cleavage by YaeL and degradation of TcpP, thus decreasing induction of *toxT* (20). When the intramolecular disulfide in TcpP is disrupted, TcpP is also readily degraded by the YaeL pathway, even under inducing conditions (Fig. 5-5 and 5-7). This could be due to decreased interaction with TcpH and/or improper folding of the periplasmic domain. The periplasmic domain of the TcpP cysteine mutants is degraded by an unknown site-1 periplasmic protease resulting in production of TcpP*. In the presence of YaeL this intermediate form is cleaved and degraded, preventing detection of the increased levels of TcpP* except in a *ΔyaeL* background (Fig. 5-5B). YaeL is a site-2 protease, and therefore is only active on previously cleaved substrates. DegS, a periplasmic

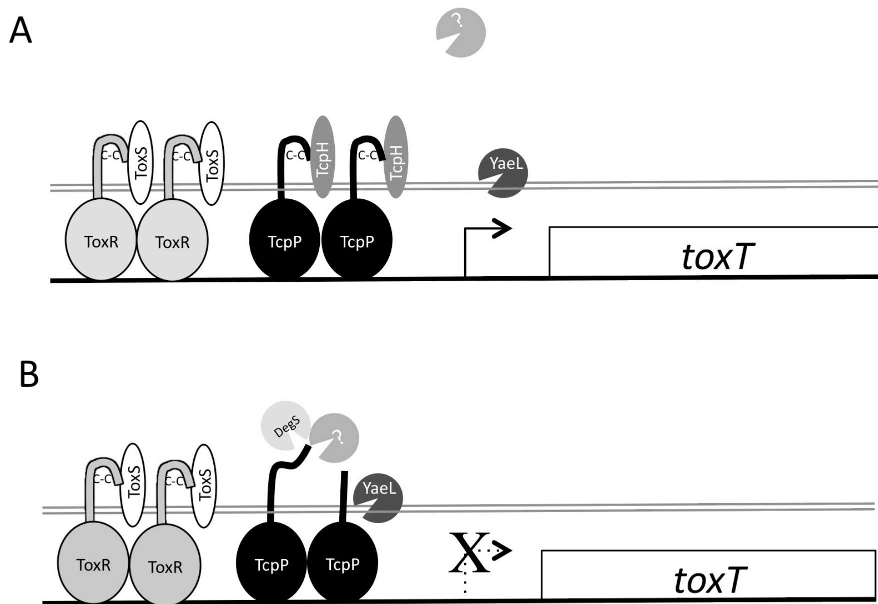

Figure 5-7. A model for disulfide bond and TcpH-mediated protection of TcpP from degradation. The intramolecular periplasmic disulfide bond, along with TcpH, protects TcpP from degradation under TcpP-inducing conditions A) A model showing protection of wild-type TcpP under inducing conditions. TcpH protects TcpP from proteolysis by an unknown site-1 protease and YaeL. B) When the intramolecular periplasmic disulfide bond in TcpP is disrupted, TcpH is no longer able to protect TcpP leaving it venerable to degradation by DegS and another yet to be identified site-1 protease. This allows for degradation by the membrane-localized protease YaeL.

protease which is the site-1 protease for YaeL in other systems (30), while not required for proteolytic degradation of wild-type TcpP (20), plays a partial role in degradation of TcpP lacking proper periplasmic disulfide bonds (Fig. 5-5C). This appeared to be particularly true for TcpP-C207S and TcpP-C218S, which are able to form intermolecular disulfide bonds. TcpP-C207S/C218S, which is unable to form any periplasmic disulfide bonds, was poorly stabilized by deletion of *degS*. Although proteolysis of the TcpP periplasmic cysteine mutants points to instability in the periplasmic domain, deletion of the gene encoding the general protease typically responsible for degradation of misfolded periplasmic proteins, Ptd (43, 44), did not increase stability of the TcpP periplasmic cysteine mutants (Fig. 5-5D).

Disruption of the periplasmic disulfide bond of ToxR, a protein of similar structure to TcpP in *V. cholerae*, is reported to not affect stability (37). In a ToxR overexpression system, Ottemann and Mekalanos found a 30-fold decrease in CT production when they disrupted the periplasmic disulfide bond by mutating a periplasmic cysteine to serine (37). However, using a chromosomally-expressed allele, Fengler *et al.* recently found that mutation of both ToxR periplasmic cysteines prevented *ompU* expression and allowed enhanced *ompT* expression (*ompU* is activated by ToxR, while *ompT* is repressed by ToxR) (36), under similar growth conditions to the Ottemann and Mekalanos studies (LB medium). Furthermore, chromosomally-expressed ToxR lacking both periplasmic cysteines still directed nearly wild-type levels of CT (36). Using chromosomally-expressed *toxR* alleles in the *V. cholerae* classical strain O395, we found that mutation of either periplasmic cysteine in ToxR resulted in a 20% decrease in *ompU* transcription activation, and mutation of both cysteines in ToxR resulted in a 40% decrease in *ompU* transcription activation in LB (Fig. 5-6), similar to the findings by Fengler *et al.* Disruption of the periplasmic disulfide bond in ToxR did not have much effect on *toxT* activation

when ToxR was expressed from its chromosomal locus, as we observed only a 20% defect in activation of *toxT* in all of our ToxR periplasmic mutants. This corresponded to no significant defect in CT production in any of the strains we tested, similar to the results found by Fengler *et al.* in both classical and El tor *V. cholerae* strains (36). Thus, although both ToxR and TcpP contain similar periplasmic intramolecular disulfide bonds, these disulfide bonds appear to play different roles in these proteins. In TcpP, the periplasmic disulfide bond is particularly critical for stability of TcpP and therefore expression of *toxT* and production of CT. The TcpP periplasmic intramolecular disulfide, in combination with TcpH, enhances stability, allowing TcpP to be present long enough to induce expression of *toxT* (Fig. 5-7). Because the periplasmic disulfide bond is critical for TcpP stability and therefore virulence expression, formation of these disulfide bonds may be a good candidate as a future target for anti-cholera therapeutics.

Materials and Methods

Bacterial strains and plasmids: All strains and plasmids used in this study are listed in Table 5-2. Specific mutants were generated by site-directed mutagenesis using primers listed in Table 5-3 and *Pfu* Turbo (Stratagene) followed by *Dpn*I digestion as described previously (24). Plasmids containing the mutants were cloned into DH5α. The sequences of all constructs were verified at the University of Michigan sequencing core. Plasmids were then transferred to reporter strains. For chromosomal mutations, the sequence containing the mutation was cloned into the suicide plasmid pKAS32 (17). The plasmids were mated into *V. cholerae* and chromosomal recombination was selected as described previously (17). This same system was used to delete *tcpP* from the chromosome of various *V. cholerae* protease deletion strains. The locus encoding non-epitope-tagged TcpPH was digested from of pMMB207-*tcpPH* (pEK32, Table 5-2) using

Table 5-2. Strains and plasmids

Strains	Genotype/Characteristics	Source
E. coli		
DH5α	*supE44* D*lacU169(F80lacZDM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Laboratory collection
DH5α λpir	*supE44* D*lacU169(F80lacZDM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1* (l*pir*)	Laboratory collection
SM10 λpir	*thi-1 thr leu tonA lacY supE recA::RP4-2-Tc::Mu* Km^R (l*pir*)	Laboratory collection
EK3034	Rosetta (DE3) pLysS: F^- *ompT* $hsdS_B$(R_B^- m_B^-) *gal dcm* λ(DE3 [*lacI lacUV5*-T7 gene1 *ind1 sam7 nin5*]) + pLysSRARE + pET30b+*toxR*cyt2-TEV	this study
V. cholerae		
O395	0395, wild-type Classical biotype	Laboratory collection
RY1	0395 Δ*tcpP*	(6)
EK459	O395 Δ*toxR*Δ*tcpP*	(1)
EK813	0395 Δ*tcpP toxT-lacZ*	(5)
EK1490	0395 Δ*tcpP* Δ*toxR toxT-lacZ*	(5)
SM269	0395 *tcpP*-C207S	this study
SM492	0395 *tcpP*-C218S	this study
SM495	0395 *tcpP*-C207S/C218S	this study
JM84	0395 Δ*yaeL*	(20)
JM128	0395 Δ*degS*	(20)
NB43	0395 Δ*ptd*	(8)
JM160	0395 Δ*tcpPH* Δ*yaeL*	(20)
SM1651	0395 Δ*tcpP* Δ*degS*	this study
SM1664	0395 Δ*tcpP* Δ*ptd*	this study
EK410	0395 Δ*toxR ompU-lacZ*	(26)
EK383	0395 *ompU-lacZ*	(26)
SM1301	0395 *ompU-lacZ toxR*-C224S	this study
SM1264	0395 *ompU-lacZ toxR*-C281S	this study
SM1303	0395 *ompU-lacZ toxR*-C224S/C281S	this study
EK733	0395 *toxT-lacZ*	(12)
EK1072	0395 Δ*toxR toxT-lacZ*	(4)
SM1299	0395 *toxT-lacZ toxR-C224S*	this study
SM1260	0395 *toxT-lacZ toxR-C281S*	this study
SM1263	0395 *toxT-lacZ toxR-C224S/C281S*	this study
EK307	0395 Δ*toxR*	(1)
SM488	0395 *toxR-C224S*	this study
SM486	0395 *toxR-C281S*	this study

Plasmids	**Source**
pEK41 (pMMB207-*tcpP*-HSV)	(1)
pMMB207	(2)
pMMB207-*tcpP*-HSV-C207S	this study
pMMB207-*tcpP*-HSV-C218S	this study
pMMB207-*tcpP*-HSV-C207S/C218S	this study
pSK-*toxR*-HA	(4)
pACYC184-*tcpH*	(8)
pTLI2 (pTL61T-$toxT_{\mathrm{pro}}$)	(3)
pEK32 (pMMB207-*tcpPH*)	(1)
pBAD18 (Kan^{R})	(10)
pBAD18-*tcpPH* (wt)	this study
pBAD18-*tcpPH*-C207S	this study
pBAD18-*tcpPH*-C218S	this study
pBAD18-*tcpPH*-C207S/C218S	this study
pKAS32	(17)
pKAS32-*tcpP*-C207S	this study
pKAS32-*tcpP*-C218S	this study
pKAS32-*tcpP*-C207S/C218S	this study
pKAS32-Δ*yaeL*	(20)
pKAS32-Δ*tcpP*	(1)
pET30b+*toxR*cyt2-TEV	this study

Table 5-3. Primers used in this study.

toxR C224S top	CGTCAATCGAACTGTCCGTTAAAAAATACAATG
toxR C224S bottom	CATTGTATTTTTTAACGGACAGTTCGATTGACG
toxR C281S top	GATGCCATCAAAGTGTCTGAGCTCGAGTACCC
toxR C281S bottom	GGGTACTCGAGCTCAGACACTTTGATGGCATC
tcpP C207S top	CTATTGATCAACATCAGTCTTCCGTGAATTATG
tcpP C207S bottom	CATAATTCACGGAAGACTGATGTTGATCAATAG
tcpP C218S top	CAGAAGACATTAGAATCCACAAAAAATGCCCC
tcpP C218S bottom	GGGGCATTTTTTGTGGATTCTAATGTCTTCTG
toxR C281S chrom top	GATGCCATCAAAGTGTCTGAGTAGgatcttgc
toxR C281S chrom bottom	GCAAGATCCTACTCAGACACTTTGATGGCATC
tcpP C218S chrom top	CAGAAGACATTAGAATCCACAAAAAATTAAAAGC
tcpP C218S chrom bottom	GCTTTTAATTTTTTGTGGATTCTAATGTCTTCTG
pBAD NoBAMH1 top	GATTAGCGGATCGTACCTGACGCTTTTTATC
pBAD NoBamH1 bottom	GATAAAAAGCGTCAGGTACGATCCGCTAATC
pBAD NoNhe1 top	ACCCGTTTTTTTGGGCAAGCGAATTCGAGC
pBAD NoNhe1 bottom	GCTCGAATTCGCTTGCCCAAAAAAACGGGT
TEV site for pET30b+ BOTTOM	CCAGATCTGGGTACCGAGAACCTGTACTTCCAGGGCG CCATGGCGATATCGG
TEV site for pET30b+ BOTTOM	CCGATATCGCCATGGCGCCCTGGAAGTACAGGTTCTC GGTACCCAGATCTGG
NdeI-ToxR orf-1 Forw	GGAATTCCATATGAGTCATATTGGTACTAAATTC
KpnI-ToxR orf-170 Rev	GGGGTACCTCGATTCCCCAAGTTTGGAG

*Eco*RI and *Bam*HI and cloned into the *Eco*RI and *Bam*HI sites in a pBAD18 (Kan) plasmid in which the second *Bam*HI site and the *Nhe*I site at the upstream end of the multiple cloning site were removed by site-directed mutagenesis.

Culture conditions: *V. cholerae* strains were routinely grown overnight in Vc LB (LB containing 5 g/L NaCl rather than 10 g/L) at 37°C. To induce virulence gene expression and promote *tcpP* expression and stability, all samples were assayed after induction in Vc LB pH 6.5 at 30°C. Cultures were grown in the presence of 100 μg/ml ampicillin, 25 μg/ml chloramphenicol, 100 μg/ml streptomycin, and 30 μg/ml kanamycin as needed.

β-galactosidase assays: Cultures were diluted from overnight growth in Vc LB at 37°C and induced at 30°C for 4 hours in Vc LB pH 6.5 with 100 μM IPTG (as required). To monitor induction from plasmid-based TcpP, the previously described reporter strains EK813 (Δ*tcpP toxT-lacZ*), EK1490 (Δ*tcpP*Δ*toxR toxT-lacZ*), (5) were used. For quantification of *toxT* expression directed by chromosomally-encoded *tcpP*, a plasmid containing a *toxT-lacZ* reporter was utilized (pTLI2, Table 5-2, (3)). 20-100 μl of culture was used to measure β-galactosidase activity as described previously (46). For quantification of ToxR activation of *ompU-lacZ* and *toxT-lacZ*, the chromosomal reporter strains EK383 and EK733 were used (12, 26). The OD_{600} was determined by spectrophotometry and used to normalize cultures for subsequent Western blot analysis.

Western blot: Samples were resuspended in SDS-PAGE sample buffer containing 1mM DTT adjusted for OD_{600} and boiled for 5 minutes before being loaded onto a 10% polyacrylamide gel. TcpP-HSV was detected using mouse monoclonal anti-HSV at 1:10,000 dilution (Novagen) or rabbit polyclonal anti-TcpP at a 1:500 dilution. ToxR was detected using rabbit polyclonal anti-

ToxR antibodies at a 1:10,000 dilution. Alkaline phosphatase conjugated anti-mouse (Invitrogen) or anti-rabbit (Invitrogen) secondary antibody was used at a 1:3000 dilution.

Production of rabbit anti-ToxR antibodies: Two rabbits were immunized by Covance with a purified form of ToxR-His_6 containing the first 170 amino acids of the ToxR cytoplasmic domain (ToxRcyt2) followed by a TEV protease cleavage site and C-terminal 6xHis tag, according to standard procedures. ToxRcyt2-His_6 protein was expressed and purified from the E. *coli* overexpression strain Rosetta (DE3) pLysS (Table 5-2) following cloning of *toxR*cyt2 into the pET30b+ vector (Novagen) via *Nde*I and *Kpn*I restriction sites (Table 5-2 and 5-3).

Non-reducing Western blot: 1:50 dilutions of overnight cultures were grown in Vc LB broth with 100 μM IPTG for 3 hours at 30°C. 1 ml of culture was treated on ice for 15 min with 10 μM iodoacetamide (Sigma) to block free cysteines before being pelleted. The OD_{600} of the culture was used to determine the appropriate volume of SDS sample buffer with or without DTT for resuspension. Samples were analyzed by Western blot as described above.

Immunoprecipitation and Mass Spectrometry: Membranes containing TcpP-HSV were prepared as previously described (13) and boiled for 5 min in 1% SDS. Samples were diluted 1:100 in 50 mM Tris pH 7.4, 300 mM NaCl, 1% Triton X-100 and centrifuged to remove any precipitates. Mouse monoclonal anti-HSV (Novagen, 1:500 dilution) was used to bind TcpP-HSV to protein A/G agarose beads (CalBiochem). After washing, the beads were resuspended in sample buffer +/- DTT. Samples were analyzed by Western blot and Coomassie and select bands were removed and analyzed by mass spectrometry by the University of Michigan Proteomics Consortium.

TcpP stability in protease deletion strains: Cultures were induced for 16 hours in Vc LB pH 6.5 with 0.25% arabinose at 30°C. Samples were adjusted for OD_{600} and analyzed by Western blot using the antibody against TcpP as described above.

ELISA for cholera toxin production: ELISAs for CT were performed as described previously (1, 47). The supernatant from induced cultures was allowed to bind to GM1 ganglioside-coated wells in a 96 well plate. Bound CT was then detected using an anti-CT primary antibody and anti-rabbit-AP conjugate secondary antibody. Color development of the substrate para-Nitrophenylphosphate (pNPP) was measured after 20 min and the average of 2 replicate wells was used to determine CT content for each sample.

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Chapter 6

Discussion

Summary

The goal of this study was to understand how ToxR and TcpP co-activate transcription of *toxT*, and therefore virulence gene expression in *V. cholerae*. In order to do this, one of the critical questions that needed to be answered was how ToxR and TcpP bind to and interact on the *toxT* promoter. We identified a direct repeat on the *toxT, ompU, ompT,* and *ctxA* promoters that is bound by ToxR and required for ToxR-dependent regulation of these promoters. ToxR binding to a direct repeat is consistent with the tendency of ToxR to dimerize (3-6). Furthermore, we found that the ToxR-binding site is three helical turns upstream of the promoter proximal TcpP-binding site on the *toxT* promoter. This brings into question whether ToxR and TcpP are able to interact while on the promoter. To better understand the role of ToxR in transcriptional activation, we investigated the dual role of ToxR as a direct activator of transcription of *ompU*, and as a co-activator of transcription of *toxT*. Two residues in the α-loop of ToxR are preferentially required for transcriptional activation of *ompU*. This is consistent with the role of the α-loop of other w-HTH proteins interacting with the RNA polymerase (7, 8). Both the DNA-binding helix (α3) and the wing of ToxR are required for DNA binding and therefore transcriptional activation. Although the wing of many w-HTH family members is involved in protein-protein interaction, no residues in the wing of ToxR were found that were required for ToxR-TcpP interaction. Finally, we investigated the importance of the

intramolecular disulfide bond in the periplasmic domain of TcpP on stability and therefore activation of *toxT*. Disruption of the periplasmic disulfide, particularly by the TcpP-C207S mutation, resulted in an increase in the YaeL mediated stepwise proteolysis of TcpP. This results in TcpP instability, even under inducing conditions. Together this new information gives us insight into how ToxR and TcpP interact, bind to the *toxT* promoter, and activate transcription.

Promoter Architecture

ToxR binds to the *toxT* promoter three helical turns upstream of the TcpP-binding site. One of the keys to understanding how *toxT* transcription is activated is to understand the promoter architecture. Previous studies showed that TcpP binds to a promoter proximal direct repeat spanning from -53 to -38 (7, 9). The footprint of ToxR spanned from -104 to -68, partially encompassing an inverted repeat (from -94 to -78 and -73 to -58) (10, 11). However, mutational analysis indicates that ToxR actually binds to a direct repeat within the footprinted region from -93 to -83 (Fig. 2-1 and 2-3)(12). There is a non-consensus ToxR-binding site in the opposite orientation (from -69 to -56), however ToxR does not appear to bind this site (Fig. 2-5). The discovery of the ToxR-binding site from -93 to -83 places the ToxR-binding site three helical turns upstream of the TcpP-binding site. This makes it unlikely that the w-HTH domains of ToxR and TcpP can interact with each other while bound to the promoter. However, the ToxR and TcpP-binding sites are spaced on the *toxT* promoter such that they are both on the same face of the DNA. This would allow membrane-localized ToxR and TcpP to simultaneously bind the promoter. Because ToxR and TcpP require membrane localization to co-activate transcription of *toxT* (13, 14), it has been hypothesized that ToxR recruits the *toxT* promoter to the membrane, thereby bringing it to TcpP. Due to spatial constrictions, it would be difficult for this to occur if

ToxR and TcpP did not bind to the same face of the DNA. Additionally, we have shown that the ToxR-binding site can be moved two helical turns upstream or downstream without disrupting transcriptional activation of *toxT* (Fig. 2-6). Further work is required to determine if the ToxR-binding site can be moved further from the TcpP-binding site, and if ToxR and TcpP must be bound to the same face of the DNA to allow for transcriptional activation.

One of the roles of ToxR is to bind to the *toxT* promoter and relieve H-NS repression (15). Truncation of the *toxT* promoter shows that the majority of the H-NS repression occurs upstream of -172 (15). However, in *E. coli,* the *toxT* promoter fragment from -172 to +45 is repressed approximately 2-fold by H-NS (15), indicating that H-NS can bind to this portion of the promoter, although the majority of H-NS recognition sites are further upstream. Based on this information it can be hypothesized that ToxR binds to the *toxT* promoter at the promoter proximal end of the H-NS bound region. Binding by ToxR to the *toxT* promoter would displace H-NS from this region, likely resulting in a cascade of H-NS released from the promoter, and decreased repression of *toxT*. More work is required to understand where H-NS binds to the *toxT* promoter, and determine if this binding site does overlap with the ToxR and/or TcpP-binding sites.

Another hypothesized role for ToxR co-activation of the *toxT* promoter is to bend the DNA, resulting in increased accessibility of the TcpP-binding site. ToxR binding to the *toxT* promoter results in a DNAse I hypersensitivity overlapping the TcpP-binding site (10). Furthermore, other w-HTH proteins have been observed to bend the DNA due to insertion of the DNA-binding helix (α3) into the major groove and the wing into the minor groove (1). Moving the ToxR-binding site relative to the TcpP-binding site on *toxT* does result in approximately a 20% defect in transcriptional activation of this promoter. This may be due to the ToxR induced

bend in the *toxT* promoter no longer being in the appropriate location to expose the TcpP-binding site. This could be confirmed by DNAse I footprinting of *toxT* promoters with the ToxR-binding site moved relative to the TcpP-binding site. One important question that still needs to be addressed is to determine if ToxR binding to the *toxT* promoter enhances TcpP binding. Traditionally, this would be determined by looking for supershifting of the *toxT* promoter by EMSA assay, however supershifting by two transmembrane proteins cannot be observed in this assay due to retention of membrane-localized proteins in the well. Therefore, ToxR facilitation of TcpP binding of the *toxT* promoter must be tested with a cytoplasmic form of ToxR. Such reagents are currently being developed for these studies.

ToxR-binding sites are found in two different orientations on ToxR-regulated promoters. It is also important to examine how the orientation and binding site location differs between ToxR-regulated promoters to provide insights concerning the different roles of ToxR. ToxR is a co-activator that facilitates TcpP-mediated activation of *toxT*, a direct activator of *ompU* and *ctxA*, and a repressor of *ompT*. On the *toxT* promoter, where ToxR is a co-activator, ToxR binds to a single direct repeat in the orientation TNAAA-N_5-TNAAA (Fig. 2-1)(Fig. 6-1), three helical turns upstream of the TcpP-binding site. Similarly, ToxR binds to two sets of direct repeats on the *ompU* promoter in the same orientation as the *toxT* promoter (TNAAA-N_5-TNAAA) five and seven helical turns upstream of the promoter proximal ToxR-binding site on the *ompU* promoter (Fig. 2-7 and 6-1). However the promoter proximal ToxR-binding site of the *ompU* promoter, which is required for transcriptional activation (Fig. 2-7), is in the opposite orientation (TTTNA-N_5-TTTNA) (Fig. 6-1). The promoter proximal ToxR-binding site of *ctxA* is also in this

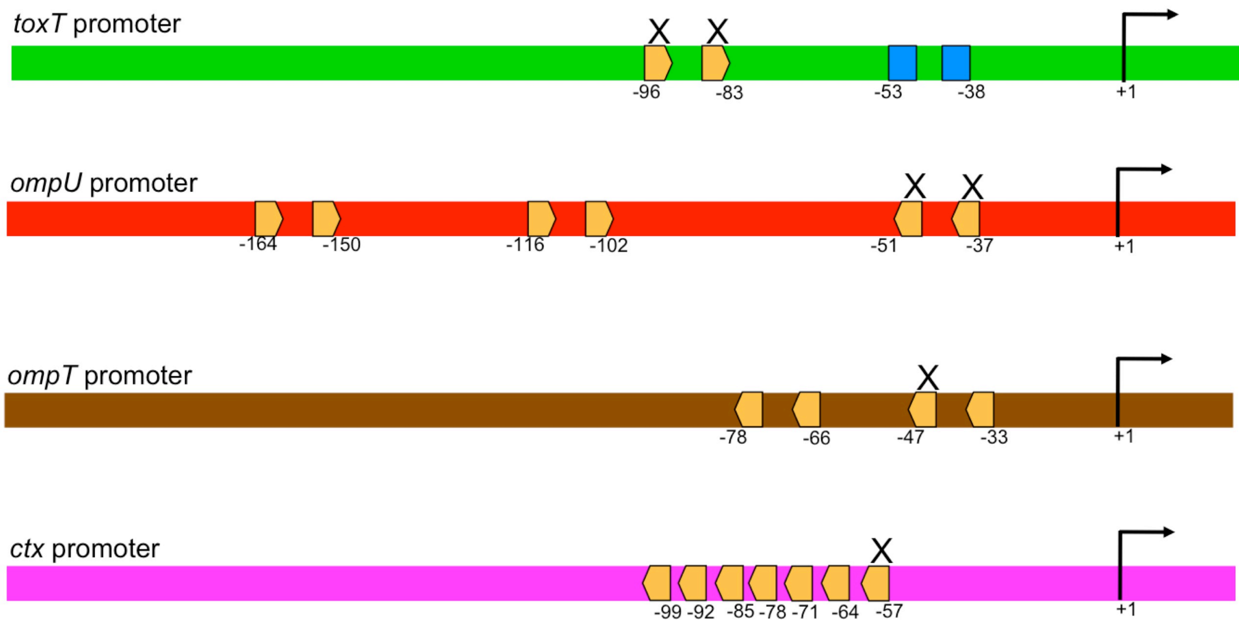

Figure 6-1. Orientation of ToxR binding-sites on ToxR regulated promoters. ToxR-binding sites are shown as orange pentagons indicating the orientation of the ToxR sequence (TNAAA sequences have the point towards the promoter and TTTNA sequences have the point facing away from the transcriptional start site. An X above a ToxR-binding site indicates a site we have shown to be required for ToxR binding and ToxR-dependent regulation (Chapter 2). Blue boxes indicate TcpP-binding sites. Location of ToxR and TcpP binding sites relative to the transcriptional start site (indicated with an arrow and +1) are designated below each promoter.

orientation (TTTNA), indicating that ToxR must bind to the promoter in a specific orientation in order to directly activate transcription. However, the *ctxA* promoter does not contain any ToxR-binding sites in the co-activation orientation (TNAAA), but instead contains multiple repeats of the ToxR-binding site in the direct activator orientation (TTTNA) with non-consensus spacing (N_2 between repeats) (Fig. 6-1). The combination of the non-consensus spacing and the lack of a co-activation oriented ToxR-binding site may explain why ToxR only activates this promoter under over-expression conditions (16). The *ompT* promoter is repressed by ToxR, and contains two ToxR-binding sites in the TTTNA-N_5-TTTNA orientation (Fig. 2-9 and 6-1). The promoter proximal ToxR-binding site spans from -47 to -33, placing ToxR on top of the -35 element, and likely resulting in disruption of RNAP binding when ToxR is bound. Since all of the promoters that are directly activated by ToxR contain a promoter proximal ToxR-binding site in the TTTNA-N_5-TTTNA orientation, it is likely that this orientation is required for ToxR activation, presumably because it places ToxR in the correct orientation to interact with RNAP. More research is needed to determine if the more promoter distal ToxR-binding sites are able to co-activate transcription in either orientation. Additionally, crystal structures of ToxR bound to the ToxR recognition sequence should be used to determine the orientation with which ToxR binds to these promoters.

ToxR and TcpP structure and function

The hydrophobic core of w-HTH proteins is conserved in ToxR. Much of the structure and function of the cytoplasmic w-HTH domain of ToxR and TcpP can be hypothesized based on the structure and function of other w-HTH proteins. The hydrophobic core of w-HTH proteins is primarily comprised of residues in the β-sheet, the three α-helixes, and the wing (Fig. 6-2). The

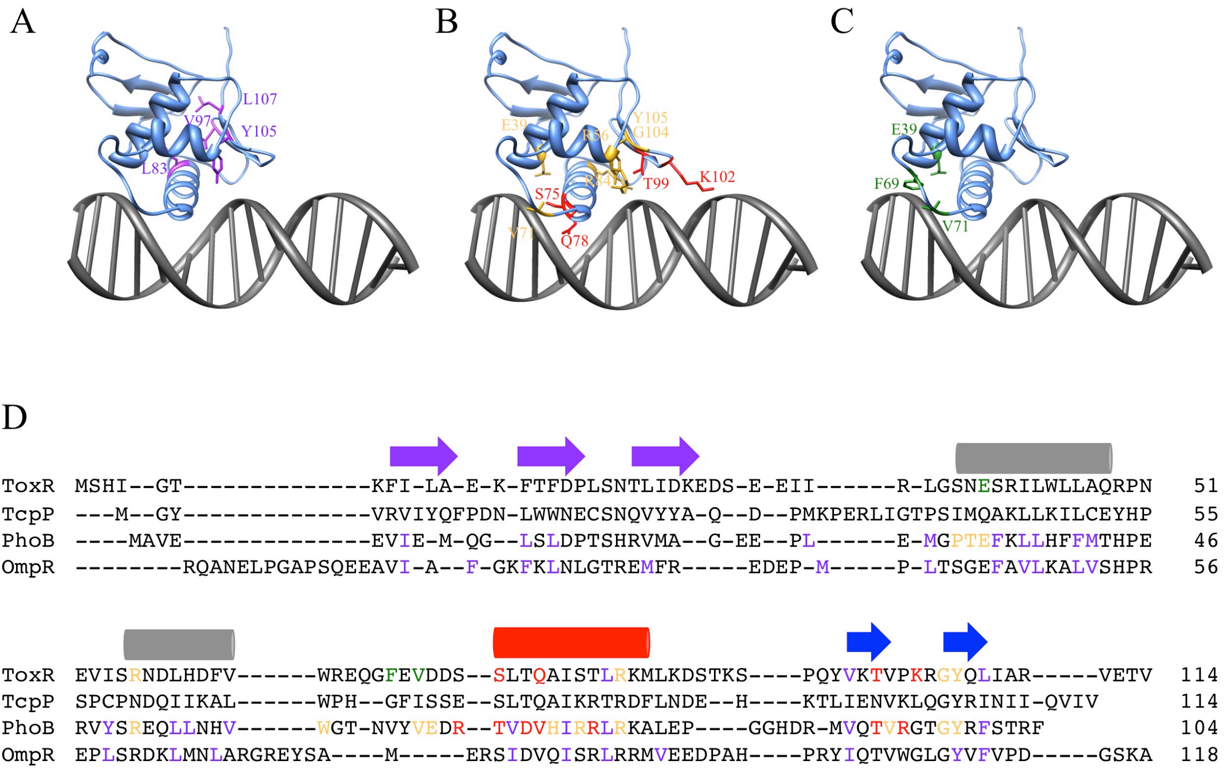

Figure 6-2. Model of ToxR w-HTH residues by function. Putative ToxR residues comprising the hydrophobic core are shown in purple. B) ToxR residues required for DNA binding and transcriptional activation are shown in yellow for putative DNA backbone contacts and red for putative contacts with DNA bases (based on PhoB contacts with DNA) (1, 2). C) ToxR residues preferentially required for *ompU* activation are shown in green. D) Alignment of the ToxR, TcpP, PhoB, and OmpR w-HTH domain. ToxR residues in A, B, and C are highlighted with the same colors as in the above figures. PhoB and OmpR hydrophobic (purple) and DNA contact (yellow for backbone contacts and red for base contacts) are highlighted (1, 2, 7).

hydrophobic core maintains w-HTH structure and likely positions the DNA-binding helix (α3) and the wing in the proper orientation to bind to DNA (17). Although our mutational screens were not designed to identify hydrophobic core residues, we have found four mutants (ToxR-L83P, V97A, Y105A, and L107A) that we believe disrupt the hydrophobic core since these residues correspond to the hydrophobic core of OmpR and PhoB. One of the hydrophobic core residues in the DNA-binding helix of OmpR and PhoB corresponds to ToxR-L83. The three residues in the wing of PhoB and OmpR that are part of the hydrophobic core correspond to three residues in the wing of ToxR (ToxR-V97A, ToxR-Y105A, and ToxR-L107A) that are required for DNA binding, transcriptional activation, and stability. As would be expected, the four putative hydrophobic core mutants are defective for DNA binding and transcriptional activation (Fig. 3-1, 3-3, 4-2, and 4-7). Further evidence that these residues are involved in the hydrophobic core of ToxR is that mutation of any of these residues results in ToxR instability. Additionally, all of these residues are pointed inwards in the ToxR model (Fig. 6-2A).

DNA binding by ToxR is primarily mediated by the DNA-binding helix (α3) and the wing. Our screens for ToxR transcriptional activation mutants have identified several residues that we believe to directly contact DNA. Mutation of residues required for DNA binding prevents transcriptional activation, of both *ompU* and *toxT* since these promoters contain identical or nearly identical ToxR-binding sites (Fig. 2-1 and 2-7). Unlike mutation of the hydrophobic core residues, mutation of DNA binding residues does not affect ToxR stability. The majority of residues that come into direct contact with DNA are in the DNA-binding helix (α3) and the wing of w-HTH proteins, as these features insert into the major and minor groove of the DNA, respectively (1, 2). However, additional contact sites occur where the α1 and α2 helixes and the

α-loop contact the backbone of the DNA. Three residues of ToxR have been found in the α1 helix, the α2 helix, or the α-loop that correspond to PhoB residues that contact the backbone of promoter DNA. Mutation of any of these residues (ToxR-E39K, R56K/L, V71A) resulted in decreased DNA binding to both the *ompU* and *toxT* promoters (Fig. 3-3 and 3-4) although, two of the mutants (ToxR-E39K and ToxR-V71A) were only modestly defective for promoter binding. ToxR-E39K was able to fully activate transcription of *toxT*, but was only able to activate transcription of *ompU* at 19% of wild-type (Table 3-1). ToxR-V71A was approximately 4 fold more defective for *ompU* activation than *toxT* activation, but was defective in activating both promoters (Fig. 3-1). Because these mutants were preferentially defective for activation of *ompU* relative to *toxT,* these backbone interactions may be more critical for *ompU* activation. Alternatively, this could be an artifact of the ToxR overexpression system used to analyze these mutants.

The DNA-binding helix (α3) and the wing of w-HTH proteins sit in the major and minor grooves, respectively, and both regions can interact with the DNA backbone and the nucleotide bases. We have found three residues, one in the DNA-binding helix (ToxR-R84) and two in the wing (ToxR-G104 and ToxR-Y105) that correspond to residues in PhoB that contact the DNA backbone (Fig. 6-2). These residues are required for DNA binding and transcriptional activation of both the *toxT* and *ompU* promoters Fig. 3-4, 4-2, 4-7 and Table 3-1). We found three residues, two in the DNA-binding helix (ToxR-S75 and ToxR-Q78) and one in the wing (ToxR-T99), which correspond to PhoB residues that interact directly with the nucleotide bases of the DNA. Mutation of any of these residues also prevents DNA binding and transcriptional activation of *ompU* and *toxT* (Fig. 3-1, 3-3, 3-5, 4-2, 4-7 and Table 3-2). Additionally, ToxR-K102A is positioned at the tip of the wing, similar to PhoB-R219, which protrudes from the tip of the wing

into the minor groove of the promoter to make base specific contacts. ToxR-K102 is modeled to protrude from the tip of the wing and is required for DNA binding and transcriptional activation of *ompU* and *toxT* (Fig. 4-2, 4-4, and 4-7). Although these two residues are in slightly different locations on the tip of the wing, it is likely that they both bind DNA in a similar manner. All of the ToxR residues hypothesized to make contact with the DNA are oriented towards the DNA in the ToxR model (Fig. 6-2B) further indicating that these residues likely interact with the DNA, however a crystal structure of ToxR bound to DNA would confirm this.

Direct activation of the *ompU* promoter. ToxR directly activates transcription at the *ompU* promoter, but does not directly activate transcription at the *toxT* promoter. We can use this to identify residues that are particularly required for direct activation, and therefore possibly involved in ToxR-RNAP interaction. Three ToxR mutants were identified that are preferentially defective for *ompU* activation compared to *toxT* activation (ToxR-E39K, ToxR-F69A, and ToxR-V71A). Surprisingly, two of these mutants (ToxR-E39K and ToxR-V71A) map to residues that correspond to PhoB residues that interact with the backbone of the promoter, and have modest defects on DNA binding. However, all three of these residues are modeled to point towards the same region of the w-HTH domain (Fig. 6-2C). Mutation of either of these residues does result in decreased DNA binding on both the *ompU* and *toxT* promoters, despite being preferentially defective for activation of the *ompU* promoter. ToxR-F69A maintains DNA binding activity on both the *ompU* and *toxT* promoters. It is tempting to speculate that ToxR-F69 interacts with RNAP, since it is not involved in DNA binding, but is required for direct activation of the *ompU* promoter, and it is in the α-loop of the w-HTH domain, which is

presumed to interact with RNAP (12). However, further study is required to confirm this hypothesis.

The wing of ToxR is not required for ToxR interaction with TcpP. Based on the interaction of other w-HTH proteins, ToxR and TcpP might interact in four different orientations: ToxR N-terminal β-sheet to TcpP N-terminal β-sheet, ToxR N-terminal β-sheet to TcpP wing, ToxR wing to TcpP N-terminal β-sheet, or ToxR wing to TcpP wing. A previous study identified mutations in the wing of TcpP that disrupt ToxR-TcpP interactions indicating that ToxR and TcpP interact either via ToxR β-sheet to TcpP wing or ToxR wing to TcpP wing (18). However, we found that no residue in the wing of ToxR is required for ToxR-TcpP interaction (Fig. 4-5 and 4-6), leading us to hypothesize that ToxR-TcpP interaction occurs between the N-terminal β-sheet of ToxR and the wing of TcpP. Based on the tendency of ToxR and TcpP to form homodimers (Morgan, unpublished)(Fig. 5-1)(3-6) it is likely that oligomers of ToxR and TcpP occur with a ToxR dimer interacting with a TcpP dimer. Despite multiple mutational screens of ToxR (Chapter 3 and 4), no ToxR mutant has been found that is defective for ToxR-TcpP interaction. However, no mutations in the ToxR N-terminal β-sheet, which we now hypothesize to be the region of ToxR involved in ToxR-TcpP interaction, have been characterized at this time.

Periplasmic cysteines in both TcpP and ToxR form intramolecular disulfide bonds. It is challenging to predict structure/function relationships of the periplasmic domains of ToxR and TcpP since these domains are not homologous to other known proteins. The periplasmic domain of TcpP, along with TcpH, is primarily involved in regulating stability of TcpP. Under inducing conditions TcpH protects the periplasmic domain of TcpP, preventing degradation (13).

However, under non-inducing conditions TcpP is proteolytically cleaved in a stepwise fashion, first by a site-1 protease and then by YaeL (19). We have shown that the periplasmic domain of TcpP contains two periplasmic cysteines, which form an intramolecular disulfide bond. Mutation of a single cysteine (TcpP-C207S or TcpP-C218S) results in formation of an intermolecular disulfide bond (Fig. 5-1), however this disulfide bond is not able to protect TcpP from proteolysis as effectively as the intramolecular disulfide bond, particularly when TcpP-C207 is mutated (Fig. 5-3). This instability appears to be the result of an increase in the stepwise proteolysis TcpP undergoes under non-inducing conditions because deletion of the protease YaeL results in accumulation of TcpP cysteine mutants in the TcpP* form, which has been cleaved by the site-1 protease, but not fully degraded (Fig. 5-5) (19). Yang et. al. found no defect in stability of TcpP when either or both periplasmic cysteines were mutated in *V. cholerae* El Tor strains, however this may have been due to their use of a C-terminal FLAG tag. Furthermore, they observed intermolecular disulfide bonds only in the presence of bile or upon mutation of TcpP-C218 (20). The absence of an intermolecular disulfide bond correlated to an decrease in transcriptional activity (20), however this may have been due to instability of TcpP-C207S in the absence of a C-terminal tag similar to our observations (Fig. 5-3).

The periplasmic domain of ToxR has long been hypothesized to sense environmental conditions, but this hypothesis has never been confirmed. The periplasmic domain of ToxR appears to be involved in ToxR dimerization (21), although the cytoplasmic domain of ToxR is able to dimerize independently of the periplasmic domain (3-5). As was found with TcpP, ToxR forms intramolecular disulfide bonds, and disruption of this disulfide by mutation of either periplasmic cysteine results in formation of an intermolecular disulfide bond (Fig. 5-6) (22). Unlike with TcpP, the periplasmic intramolecular disulfide bond of ToxR is not required for

stability. Furthermore, mutation of either ToxR periplasmic cysteine does not result in a dramatic decrease in transcriptional activation of *ompU* or *toxT* or a decrease in CT production (Fig. 5-6). Under inducing conditions in minimal media, disruption of the ToxR periplasmic disulfide results in a partial defect in activation of *ompU* and repression of *ompT*, however this defect was not detected when the same strains were grown in inducing (AKI) conditions in rich media (23). Since the ToxR periplasmic disulfide bond is required for ToxR activity only under specific conditions, it is possible that this disulfide bond is part of an environmental signaling system. However, understanding of ToxR responsiveness to environmental signals has been obscured historically by laboratory induction conditions, which do not reflect *in vivo* conditions.

Transcriptional activation of the virulence cascade

At the beginning of this study we proposed three possible models for how ToxR co-activates transcription of *toxT* with TcpP, two of which were dependent on ToxR-TcpP interaction (Fig. 1-3). In the "hand-holding model" ToxR-TcpP interaction is maintained on the promoter, helping to position TcpP on the *toxT* promoter. However, we have found that ToxR binds to the *toxT* promoter three helical turns upstream of the TcpP-binding site. It is therefore unlikely that the w-HTH domains of ToxR and TcpP are able to interact while both ToxR and TcpP are bound to the promoter. Furthermore, moving the ToxR-binding site two helical turns upstream or downstream does not dramatically reduce transcriptional activation of *toxT*, even though this would potentially disrupt any ToxR-TcpP interaction maintained on the promoter. We cannot rule out interaction between the periplasmic domains of ToxR and TcpP, however, mutational analysis of TcpP indicates that the wing of TcpP is required for ToxR-TcpP interaction (18). Additional evidence against the "hand-holding model" is that residues in the

wing of TcpP are required for both ToxR interaction and DNA binding (18) indicating that TcpP likely interacts with ToxR and the *toxT* promoter sequentially, not simultaneously.

The second model for ToxR co-activation of the *toxT* promoter is the "catch and release" model in which ToxR-TcpP interaction recruits TcpP to the *toxT* promoter, but ToxR-TcpP interaction is released upon DNA binding (Fig. 1-3). Based on the lack of identification of any ToxR wing residues that are required for ToxR-TcpP interaction, we propose that ToxR-TcpP interaction occurs between the N-terminal β-sheet of ToxR and the wing of TcpP. Several mutations in the wing of TcpP have been previously identified that disrupt ToxR-TcpP interaction (18). However, mutation of these residues also disrupts binding to the *toxT* promoter (18). Since TcpP binding to DNA is required for transcriptional activation (9), we are unable to determine if transcriptional activation defects by these TcpP mutants are due only to DNA binding defects or due to a combination of DNA binding defects and ToxR-TcpP interaction defects. Although it is possible that ToxR-TcpP interaction is only an artifact of similarities between the structures of ToxR and TcpP, it is likely that ToxR-TcpP interaction aids in transcriptional activation of *toxT*. Identification of ToxR mutants defective for TcpP interaction, but competent for DNA binding will be required to test this hypothesis.

The third model for ToxR co-activation of the *toxT* promoter is the "promoter modification model" in which ToxR binding to the *toxT* promoter modifies it, thereby enhancing TcpP binding (Fig. 1-3). One way that this occurs is by ToxR recruitment of the *toxT* promoter to the membrane. Evidence for this mechanism is that ToxR membrane localization is required for TcpP-mediated transcriptional activation of *toxT*, but not transcriptional activation of *ompU* or repression of *ompT*, both of which are TcpP independent (14). This is consistent with our finding that ToxR and TcpP-binding sites are on the same face of the *toxT* promoter and therefore likely

both face the membrane. Additionally, ToxR relives H-NS repression (15). H-NS repression of A/T rich DNA is a common method of repressing foreign DNA (24), so it is not unexpected that the *toxT* promoter, which was likely acquired from a mobile genetic element (25) is regulated in this manner. Finally, ToxR likely bends the DNA to expose the TcpP-binding site. ToxR binding to the *toxT* promoter results in a DNAse I hypersensitivity site corresponding to the TcpP-binding site (10). Since w-HTH protein binding can introduce curvature into the DNA (1), it is likely that ToxR binding to the *toxT* promoter introduces a curvature into the promoter thereby exposing the TcpP-binding site. All of these modifications are dependent on ToxR binding to the *toxT* promoter, but independent of ToxR-TcpP interaction. Since ToxR binding to the promoter is required for *toxT* activation, it is likely that all three of these mechanisms are critical for ToxR co-activation of *toxT*.

Based on these findings, it is likely that ToxR co-activation of *toxT* occurs as a combination of the "catch and release" and "promoter modification" models. When *V. cholerae* is in the aquatic environment, the virulence cascade is turned off and the *toxT* promoter is repressed by H-NS. Upon ingestion of *V. cholerae*, environmental signals induce expression of *tcpP* and possibly increase activity of ToxR (Fig. 1-1). The N-terminal β-sheet of ToxR interacts with the wing of TcpP, recruiting TcpP to the *toxT* promoter. ToxR binding to the *toxT* promoter releases ToxR-TcpP interaction, relieves H-NS repression, brings the *toxT* promoter to the inner membrane, and bends the DNA to expose the TcpP-binding site. This enables TcpP binding to the *toxT* promoter and transcriptional activation (Fig. 4-8).

Activation of *toxT* by ToxR and TcpP represents a decision point for *V. cholerae*. Environmental signals from a variety of sources coalesce to activate ToxR and expression of *tcpP*. Upon activation of these two transcription factors the gene encoding the master virulence

regulator (*toxT*) is induced allowing for intestinal colonization and secretion of cholera toxin. Because activation of both ToxR and TcpP is required to activate the virulence cascade virulence, gene expression only occurs under specific conditions, making this a carefully regulated system. The majority of virulence genes in *V. cholerae* are encoded in two pathogenicity islands, one of which encodes genes for TcpP, ToxT and TCP and the other encodes the genes for cholera toxin and the CTX phage. TcpP does not appear to regulate expression of any other genes besides *toxT*. ToxR is present in environmental non-toxigenic, *Vibrio spp*. and regulates expression of the outer membrane porins *ompU* and *ompT* in addition to *toxT*. It is likely that ToxR regulation of *toxT* is an adaption that allows for tighter regulation of the *toxT* promoter and therefore both pathogenicity islands. By cooperative activation of *toxT*, the virulence genes in *V. cholerae* can be carefully regulated and only activated under the appropriate environmental conditions.

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