N-terminal Residues of the Vibrio cholerae Virulence Regulatory Protein ToxT Involved in Dimerization and Modulation by Fatty Acids*

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The regulatory protein ToxT is an AraC family protein that is responsible for activating transcription of the genes encoding cholera toxin and toxin coregulated pilus, which are required for virulence by the human pathogen Vibrio cholerae. The N terminus of ToxT contains dimerization and regulatory elements, whereas the C terminus contains the DNA binding domain. Bile and long chain fatty acids negatively regulate ToxT activity. Utilizing a comprehensive alanine substitution mutant library of ToxT, 19 N-terminal residues were found to be critical for dimerization and transcriptional activation. One of these mutant proteins (F151A) was confirmed to be monomeric via centrifugation and exhibited a weakened ability to bind to the tcpA promoter in a gel mobility shift assay. Moreover, a V. cholerae toxTF151A mutant failed to colonize the infant mouse intestine, emphasizing the importance of ToxT N-terminal dimerization to cholera pathogenesis. Six N-terminal alanine substitutions allowed ToxT transcriptional activity in the presence of inhibitory concentrations of bile, palmitoleic acid, and the small molecule inhibitor virstatin. Two of these mutations (N106A and L114A) enhance N-terminal dimerization in a bacterial two-hybrid system reconstituted in V. cholerae, which is otherwise disrupted by bile, palmitoleic acid, and virstatin. We demonstrate that V. cholerae toxTN106A and toxTL114A strains colonize the infant mouse intestine at significantly higher levels than the wild type strain. Our results demonstrate that ToxT N-terminal dimerization is required for transcriptional activation and cholera pathogenesis and that fatty acids modulate ToxT activity via modulation of dimerization.

Vibrio cholerae causes the disease cholera, a life-threatening diarrheal illness that affects thousands of people annually (1). The bacterium is acquired through the consumption of contaminated food or water and colonizes the small intestine (2). In the intestinal environment, V. cholerae expresses two critical virulence factors that facilitate colonization and disease symptoms, cholera toxin (CT) and toxin-coregulated pilus (TCP). CT is an ADP-ribosylating toxin that is translocated into host cells and modifies Gαi, causing an ion imbalance that leads to the profuse diarrhea associated with the disease (2). TCP is a type IV bundle-forming pilus that is required for intestinal colonization (3).

Expression of CT and TCP is coordinately regulated by the transcriptional activator ToxT (4). ToxT, an AraC/XylS family activator, directly binds to the promoters of the ctx and tcp genes (which encode CT and TCP) and activates their transcription (5). V. cholerae strains lacking ToxT express no CT or TCP and are unable to colonize the intestine and cause disease, emphasizing the central role this regulatory protein plays in cholera pathogenesis (6). Transcription of toxT is regulated by a virulence cascade commonly referred to as the ToxR regulon, which responds to various environmental stimuli to ensure that ToxT is only expressed within the intestine (7).

ToxT is composed of two domains, an N-terminal domain (amino acids 1–164) that contains dimerization determinants and environmentally responsive elements and a C-terminal domain (amino acids 165–276) that contains the DNA-binding determinants (two HTH motifs) that share homology with other AraC family members (8, 9). The N terminus has been demonstrated to dimerize by dominant inhibition, LexA fusion, and two-hybrid analyses (8, 10), and inhibition or enhancement of N-terminal dimerization inhibits or enhances ToxT-dependent transcription (8–12). The C terminus is able to bind to the tcp promoter region in a gel mobility shift assay but only when it is fused to a heterologous dimerization domain (8). These observations suggest that ToxT must be dimerized to activate transcription. However, the isolated (monomeric) C terminus has been shown to bind to the mshA promoter region, which is repressed by ToxT, suggesting that monomeric ToxT, although not transcriptionally active, can still bind certain promoters and repress their expression (13).

Evidence has accumulated that ToxT transcriptional activity is modulated by environmental conditions found within the intestine. Initially, bile was shown to negatively modulate ToxT-dependent transcription activation (14). Subsequently, fractionation of bile components revealed that fatty acids present in bile, such as oleic acid, repressed ToxT-dependent ctx transcription.

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2 The abbreviations used are: CT, cholera toxin; MBP, maltose-binding protein; TEV, tobacco etch virus; CI, competitive index; POA, palmitoleic acid; TCP, toxin-coregulated pilus; IPTG, isopropyl 1-thio-β-D-galactopyranoside.
and tcp transcription (15). Capsaicin, the vanilloid compound isolated from chili peppers, is also capable of repressing ToxT-dependent cts and tcp transcription (16), as is a synthetic small molecule inhibitor, virstatin (17). The recent resolution of the ToxT crystal structure revealed a monomer tightly bound to palmitoleic acid (POA) (9), and this is presumed to be an inactive state, because POA disrupts ToxT DNA binding activity and transcription activation. POA is completely buried within the monomer, sandwiched between the N and C termini, leading to a model that predicts that certain fatty acids, such as POA, modulate ToxT dimerization, and hence activity, by binding ToxT and “locking” ToxT into a monomeric and inactive state (closed conformation) (9, 10).

We previously performed comprehensive scanning alanine mutagenesis on ToxT (11), and we have used this mutant library to identify residues important for dimerization and modulation by fatty acids. We demonstrate that bile, POA, virstatin, and capsaicin, and other fatty acids inhibit ToxT activity by a common mechanism, namely inhibition of N-terminal dimerization. Our results confirm a tight correlation between ToxT dimerization and virulence.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains—**Escherichia coli strains DH5α and DH5αpir were used for cloning (18); strain WM3046 (gift of William Metcalf, University of Illinois) was used for conjugation; strain BL21(DE3)pLysS was used for overexpression of His-MBP-ToxT (19), and strain KDZifΔz was used for the bacterial two-hybrid system (20). All *V. cholerae* strains used are isogenic with the classical O1 strain O395. The *V. cholerae* two-hybrid reporter strain KKV2297 was created by first introducing the ΔtoxT::Cm and rpoZ mutations from strains SY1002 (21) and EC14799 (22) into O395 via CPT1ts transduction (23) to create strain KKV2296, and then replacing the endogenous lacZ promoter in KKV2296 with the Zif-dependent promoter from KDZifΔz via conjugation with plasmid pKEK1448, to create reporter strain KKV2297. The *V. cholerae* toxT mutant strains were created by first introducing the ΔtoxT::Cm mutation from strain SY1002 (21) into the ΔlacZ O395 strain KKV598 via CPT1ts transduction (23) to create strain KKV2297, and then replacing the ΔtoxT::Cm with the various mutant forms of toxT via conjugation (24) with plasmids pKEK1445, pKEK1496, pKEK1497, pKEK1498, and pKEK1499 to create strains KKV2289 (toxTwt), KKV2291 (toxTNI06A), KKV2292 (toxTLL114A), KKV2293 (toxTFT151A), and KKV2294 (toxTS223K), respectively. Strains were grown overnight at 30 °C in LB to induce virulence factor expression.

**Plasmids—**Plasmid pPK160, which expresses MBP-ToxT from the pBAD promoter (25), was used as template for site-directed mutagenesis with the primers listed in supplemental Table S1 to introduce the various ToxT mutations. The plasmid used to create the chromosomal toxT mutant *V. cholerae* strains was constructed by PCR amplification of toxT and 1-kb flanking region on each side using primers ToxT Flank Up NotI and ToxT Flank Dn NotI (supplemental Table S1) and then cloning the resulting fragment into the NotI site of pKA546 (24) to create pKEK1445. This plasmid was used in site-directed mutagenesis with the appropriate primers listed in supplemental Table S1 to introduce the F151A, N106A, L114A, and S223K mutations, which were subsequently introduced into the *V. cholerae* chromosome.

The plasmid used to construct the *V. cholerae* two-hybrid reporter strain was created by first amplifying a splicing by overlap extension PCR product (26) consisting of 1 kb of DNA upstream of the *V. cholerae* lacZ translation start site, the chloramphenicol resistance gene flanked by Kasl and Ndell sites, and the DNA corresponding to 1 kb of the *V. cholerae* lacZ gene from the start codon. This product was created using the primers LacZ Up PstI, LacZ Dn PstI, Uni Up, Uni Dn, LacZp Uni Dn, and LacZp Uni Up (supplemental Table S1) (27) and cloning into the plasmid pGEM-T to form pKEK1446. The Zif-dependent promoter region from KDZif1Δz was then PCR-amplified with primers KanZifrrnBUp and KanZifrrnB Dn, digested with Kasl and Ndell, and ligated to pKEK1446 digested similarly, to create pKEK1447. The entire construct was then amplified using LacZp Up PstI Up and LacZp Dn PstI and cloned into pDS132 (28) digested similarly to create pKEK1448. pKEK1445, carrying the full *V. cholerae* lacZ gene, and 1-kb flanking homology on each side, was created by PCR amplification of this region from the wild-type chromosome using primers LacZ. ReplaceUp BamHI and LacZ Replace SacI and then cloning the resultant fragment into the BamHI and SacI sites of pKA546.

**Purification of ToxT from an *E. coli* Expression System—**The genes encoding the full-length wild type, N106A, and F151A ToxT proteins were cloned into a modified pMal-c2x plasmid (New England Biolabs) and expressed as fusions with maltose-binding protein (MBP). The N-terminal MBP and C-terminal ToxT components were separated by a tobacco etch virus (TEV) protease cleavage site and the MBP component also possessed a 6× histidine tag fused to its N terminus. The MBP-ToxT fusion proteins were expressed in *E. coli* strain BL21(DE3)pLysS at 37 °C. Cells were grown to an *A*$_{600}$ of 0.6 before induction using 1 mM IPTG. The cells were shaken overnight before being harvested by centrifugation. The pellet cells were resuspended in 20 mM Tris-HCl, 200 mM NaCl, 2 mM DTT, and 1 mM EDTA, pH 7.5 (column buffer), and lysed by sonication on ice. Cell debris was pelleted by centrifugation, and cleared lysate was loaded onto an amylose resin (New England Biolabs), washed with 5 volumes of column buffer, and eluted with column buffer made 10 mM in maltose. Fractions containing the MBP-ToxT fusion proteins were pooled, and His$_6$-tagged TEV protease (purified in-house) was added such that the fusion protein:TEV protease ratio was 10:1 and allowed to incubate overnight. The resulting solutions were loaded onto a Ni$^{2+}$-nitrilotriacetic acid affinity column (GE Healthcare) and the His$_6$-MBP and His$_6$-TEV protease components were retained, whereas the cleaved ToxT proteins were collected in the flow-through and dialyzed against 50 mM Tris-HCl, 150 mM...
NaCl, 2 mM tris(2-carboxyethyl)phosphine, pH 8.0. The free ToxT proteins were purified to homogeneity on a HiTrap Q anion exchange resin (GE Healthcare) for use in analytical ultracentrifugation and electrophoretic mobility shift assays.

**Analytical Ultracentrifugation**—Sedimentation velocity experiments were performed at 20 °C using a Beckman Optima XL-I centrifuge. All samples were scanned in intensity mode at 280 nm. ToxT wild type was measured at 16.5 μM, corresponding to an absorbance of 0.79 A280, whereas ToxT mutant F151A was measured at a loading concentration both above and below that of the wild type, at 7.5 and 21.6 μM, corresponding to an absorbance of 0.36 and 1.03 A280. Measurements were performed at 50,000 rpm for the wild type, whereas ToxT F151A was measured at 40,000 rpm. Hydrodynamic parameters were corrected for buffer density, viscosity, and partial specific volume according to methods outlined by Laue et al. (29) and as implemented in UltraScan. All sedimentation velocity data were analyzed with UltraScan (30, 31) by two-dimensional spectrum analysis with simultaneous removal of time and radially invariant noise (32), followed by analysis with the enhanced van Holde-Weischt method (33). The two-dimensional spectrum analysis results were further refined by genetic algorithm (34, 35) and Monte Carlo analyses (36). Analysis of sedimentation velocity data by the van Holde-Weischt method effectively removes the contribution of diffusion to boundary spreading to yield G(s), a model independent, integral distribution of s20,w of all species in the sample. Consequently, a G(s) plot of the boundary fraction versus s20,w is vertical when the sample is homogeneous and has a positive slope when the sample is heterogeneous (37). The S value is directly proportional to molecular mass and inversely proportional to the frictional ratio (f/f0). The frictional ratio is 1 for a sphere and increases for elongated molecules (with values usually in the range of 1.25–2.5 (38)). The two-dimensional spectrum analysis and genetic algorithm methods further characterize each species in the distribution by partial concentration, frictional ratio, and molecular weight, and the Monte Carlo analysis provides statistical confidence limits for each parameter.

For the bacterial two-hybrid assays, plasmids expressing ToxTN-Zif and ToxT N-ω were cotransformed into either KDZif1Δz or KKV2297. Overnight cultures of these strains were diluted 1:100 into LB containing 0.3 mM IPTG, with or without 0.4% bile, 0.1% POA, 0.01% visrtatin, 0.05% 9-decanoic acid, 0.05% decanoic acid, 0.01% 7-octenoic acid, 0.05% myristoleic acid, 0.2% oleic acid, 1% elaidic acid, and 0.01% capsasacin, grown at 30 °C to A600 0.2–0.4, and assayed for β-galactosidase activity (39). To measure CT and TCP expression, overnight cultures of *V. cholerae ΔtoxT* strain V740 (40) carrying plasmids expressing the various ToxT mutant proteins were diluted 1:100 in LB with 0.1% arabinose, with or without 0.4% bile, 0.1% POA, 0.01% visrtatin, 0.05% 9-decanoic acid, 0.05% decanoic acid, 0.01% 7-octenoic acid, 0.05% myristoleic acid, 0.2% oleic acid, 1% elaidic acid, and 0.01% capsasacin, grown at 37 °C overnight, and then assayed for CT expression by GM1-ELISA (41) and TCP expression by Western immunoblot with rabbit polyclonal antisera directed against TcpA, utilizing ECL detection reagent (Amersham Biosciences). Significance was calculated using Student’s two-tailed t test.

**RESULTS**

**Amino Acids Critical for ToxT N-terminal Dimerization**—We previously constructed a comprehensive scanning alanine mutant ToxT library, to identify residues critical for ToxT function (11). 20 Ala substitution mutations within the N terminus (amino acids 1–164) prevented transcriptional activity (<1% wild type activity) at two different ToxT-dependent promoters, indicating that these residues are essential for ToxT function. We and others have shown that the N terminus of ToxT dimerizes (8, 10), so we utilized a bacterial two-hybrid system to determine the effect these 20 Ala substitutions have upon N-terminal dimerization. This assay has been used previously to demonstrate ToxT N-terminal dimerization (10). The ToxT N terminus was fused to both the ω subunit of *E. coli* RNA polymerase (ToxTN-ω) and a zinc finger DNA-binding protein (ToxTN-Zif), and interaction between the two fusion proteins was measured in an *E. coli* reporter strain with a Zif-dependent promoter-lacZ transcriptional fusion (20). As demonstrated previously (10), the N termini of ToxT in these fusion proteins interact and induce lacZ transcription (Fig. 1).

We then introduced each of the 20 N-terminal Ala substitutions that prevent ToxT activity (11) into the ToxTN-Zif and ToxTN-ω fusion proteins, and we measured dimerization of each Ala substitution mutant paired with the cognate wild type DNA Binding Assay— tcpAp probe was generated by first annealing complementary oligonucleotides based on the following sequence corresponding to tcpAp (only the 5′–3′ strand is shown): 5′-TCAAGTAAAGGTTTATTAAAAAAAATTTAAAACACAGGAAAAATGAGATCTGTC-3′. The double-stranded probe was labeled utilizing polynucleotide kinase and [γ-32P]ATP and then purified via a NucTrap column (Stratagene). Purified ToxT protein was mixed with labeled probe (20,000 cpm) andpoly(dI-dC) (0.3 ng) in LSB (10 mM phosphate, 30 mM NaCl, 1 mM azide, 10 mM β-mercaptoethanol, 1 mM EGTA, pH 7.0), incubated for 10 min at 37 °C, and loaded into a 6% (w/v) acrylamide 0.06% (w/v) bisacylamide gel. Gels were electrophoresed in a Tris-glycine buffer (50 mM Tris base, 100 mM glycine, pH 8.5) and subsequently visualized by autoradiography. For specific competitor DNA, unlabeled annealed probe was used, whereas an unlabeled PCR product of the cat gene was used for nonspecific competitor DNA, as described previously (8).
ToxT\textsuperscript{N}-fusion partner (Fig. 1). As a control, we also included the L114P mutation that was previously shown to disrupt ToxT\textsuperscript{N} dimerization (17) (this mutation was erroneously referred to as "L113P" in this study). These assays demonstrated that, like the L114P mutation, 19 of the 20 Ala substitutions within the N terminus disrupt dimerization and that these mutations are cis-acting, i.e. the Ala substitution only needs to be in one of the two interacting subunits to prevent dimerization. We also measured dimerization of ToxT\textsuperscript{N} with a D141G mutation; this mutation was previously suggested to also disrupt dimerization (13), and our analysis confirmed that the D141G mutation disrupts ToxT\textsuperscript{N} dimerization. These results identify 20 N-terminal residues (including Asp-141) that contribute to the dimerization and activity of ToxT.

The E52A mutation was the only Ala substitution that disrupted ToxT activity but still allowed for high levels of dimerization in the bacterial two-hybrid assay. To determine whether this might be a trans-acting mutation (i.e. requires that the mutation be in both subunits to inhibit dimerization), we measured the ability of ToxT\textsuperscript{N}E52A-Zif and ToxT\textsuperscript{N}-\omega to interact in this assay (supplemental Fig. S1). The results demonstrated that even with the E52A mutation in both monomers, the N termini are still able to interact and stimulate ~50% WT β-galactosidase activity, suggesting that the lack of transcriptional activity of the E52A mutant is not due to a major defect in dimerization.

F151A ToxT Protein Is a Monomer—The F151A mutation was previously shown to inhibit ToxT dimerization in a LexA-based assay (11) and was confirmed to inhibit ToxT dimerization in the bacterial two-hybrid system above. To substantiate that dimerization of ToxT is altered with the introduction of the F151A mutation, we performed sedimentation velocity analysis on purified wild type and F151A full-length ToxT proteins (Fig. 2).

The enhanced van Holde-Weischet analysis of wild type ToxT revealed a broad distribution of species with sedimentation coefficients ranging between 5 and 30 S. This suggests that the wild type protein aggregates readily to form large oligomeric structures. The F151A mutant, however, displayed a very different pattern as follows: 60% of the high concentration sample and 80% of the low concentration sample sedimented with a value centered only around 3 S, showing a vertical distribution, suggesting that the majority of the sample was not associated and instead was homogeneous. The remaining 20 – 40% showed a similar aggregation pattern as the wild type, however, with a much reduced partial concentration (Fig. 2). To further identify the oligomeric status of the major component found in the mutant samples, we performed genetic algorithm-Monte Carlo analysis. The results confirm that the F151A mutant protein remains largely monomeric with a molecular mass of 31.3 kDa (95% confidence intervals of 29.8 and 32.7 kDa), which is in excellent agreement with the known monomer molecular weight of ToxT (32.26 kDa). Furthermore, the monomer appears to be present in multiple conformations, giving rise to multiple frictional ratios centered at 1.27 with a fairly broad 95% confidence interval of 1.22 to 1.31 for this species (supplemental Fig. S2). This result suggests that the F151A mutant protein may be destabilized and has a significantly decreased ability to self-associate and form functional dimers in comparison with the wild type.

Monomeric ToxT (F151A) Shows Decreased Binding at the tcpA Promoter—ToxT binds to virulence gene promoters to activate their transcription, and it has been hypothesized that dimerization is required for DNA binding (8–10), at least at the promoters positively activated by ToxT. Because we have shown by three different assays that the F151A ToxT protein fails to dimerize, we utilized this protein along with the wild type protein in a gel mobility shift assay to determine whether dimerization is required for DNA binding at the tcpA promoter. Wild type ToxT bound the tcpA-binding site and caused a mobility shift that could be competed with unlabeled tcpA promoter DNA, but not with unlabeled nonspecific DNA, demonstrating specific DNA binding (Fig. 3). F151A ToxT was also able to bind the tcpA promoter and cause a specific mobility.
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**FIGURE 3. Mobility shift assay indicates weaker binding of ToxT F151A to tcpA promoter.** A, mobility shift assay was performed as described under “Experimental Procedures” with wild type and F151A ToxT proteins and 32P-labeled tcpA promoter fragment. Lane 1 contains no protein (−), followed by identical concentrations (150, 75, 37.5, and 18.75 pM) of ToxT wild type (lanes 2–5) or F151A protein (lanes 6–11) added to these lanes. An excess of unlabeled tcpA fragment (+S, lanes 6 and 12) or unlabeled nonspecific competitor (+NS, lanes 7 and 13) was added to reactions that also contained 150 nM wild type (lanes 6 and 7) or F151A protein (lanes 12 and 13) as specific and nonspecific competitor, respectively. B, percent shifted species from A was quantified using a Typhoon imaging system.

shift, although at a lower affinity (half-maximal concentration of binding ~118 pm versus ~48 pm for the wild type protein). Moreover, the shifted species with the F151A protein appeared at a slightly lower mobility shift than that caused by the wild type protein bound to this fragment (arrows, Fig. 3). The tcpA-binding site contains two toxbox sequences (42), suggesting that the shifted species caused by F151A may represent a single ToxT monomer bound, whereas the predominant shifted species caused by the wild type protein may represent a dimer of ToxT bound. Minimally, these results indicate that dimerization enhances ToxT binding at the tcpA promoter, consistent with dimerization enhancing transcriptional activation.

V. cholerae toxTF151A Mutant Is Unable to Colonize Intestine—To determine the importance of ToxT dimerization on V. cholerae virulence in vivo, the toxTF151A allele was recombined onto the V. cholerae chromosome, and the resultant toxTF151A V. cholerae strain was evaluated for intestinal colonization in the infant mouse competition assay (Fig. 4). The toxTF151A mutant was unable to colonize the intestine (C. I. < 10⁻⁴); no toxTF151A mutant bacteria were recovered from the intestine, indicating a high degree of virulence attenuation. In contrast, a wild type toxT allele allows for equal levels of colonization as the coinoculated wild type strain (C. I. = 0.85). The toxTF151A mutant strain also expressed no detectable CT or TCP under in vitro inducing conditions, in contrast to the high levels of CT and TCP expression by the wild type V. cholerae strain (Fig. 4B). The high level of virulence attenuation of the toxTF151A mutant in vivo and in vitro emphasizes the importance of ToxT dimerization to cholera pathogenesis.

**FIGURE 4. V. cholerae toxTF151A is defective for intestinal colonization.** A, V. cholerae strains KKV2289 (WT; Lac⁺) and KKV2293 (toxTF151A; Lac⁻) were coinoculated with the isogenic wild type strain KKV2290 (Lac⁺) perorally into infant mice at a ratio of 1:1; intestinal homogenates were recovered at 22 h postinoculation, and numbers of colony-forming units of wild type and mutant strains were determined. The competitive index is given as the ratio of mutant/wild type bacteria in the output divided by the ratio of mutant/wild type bacteria in the input; each value shown is from an individual mouse. The detection limit is estimated at 10⁻⁸, noted by dotted line; no toxTF151A colonies were recovered in any mice (C.I. < 10⁻⁴). B, KKV2289 (WT) and KKV2293 (toxTF151A) were grown under in vitro virulence factor-inducing conditions. TcpA was detected by Western immunoblot with anti-TcpA antiserum, and CT in the supernatant was measured by GM₁-ganglioside ELISA.

embedded tightly within the monomer (9). These results suggest that bile, POA, and virstatin modulate ToxT activity by the common mechanism of regulating dimerization.

We screened the scanning Ala ToxT library in a V. cholerae ΔtoxT strain to identify Ala substitution mutants that exhibit enhanced CT expression in the presence of 0.4% bile. Of 162 Ala substitution mutants within the N terminus screened, six (D44A, Y51A, M103A, N106A, L114A, and K154A) exhibited higher levels of CT expression than the wild type protein.
assayed under identical conditions in the presence of bile (Fig. 5). Interestingly, a proline substitution at Leu-114 (L114P) was previously identified as providing enhanced activity in the presence of virstatin (17). We also assayed ToxT with an L114P mutation for activity in the presence of 0.4% bile, and like the L114A protein, it also exhibited enhanced CT expression in the presence of bile.

To determine whether POA and virstatin inhibit ToxT activity by a similar mechanism, we measured CT and TCP expression by the *V. cholerae* H9004 toxT strain carrying these seven ToxT alleles in the presence of inhibitory concentrations of POA and virstatin, as was done in the presence of bile above. The results demonstrate that all six Ala substitution mutants, as well as the L114P mutant, identified as having enhanced activity in the presence of inhibitory concentrations of bile, also exhibit enhanced CT and TCP expression in the presence of inhibitory concentrations of POA and virstatin (Fig. 5). Our results suggest that bile, POA, and virstatin inhibit ToxT activity via a common mechanism.

Resolution of the monomeric ToxT-POA crystal structure (9) indicates that extensive interactions of the N and C termini with POA cause these two domains to form a “closed conformation,” resulting in the N and C termini in close proximity to each other. The close proximity of Ser-223 in α7 of the C terminus to residues in β2 within the N terminus in this closed conformation suggested to us that substitution of positively charged long chain residues at position 223 might force the N and C termini apart and counteract the effect of binding to POA, thus allowing the protein to adopt a more “open” conformation. We created ToxT proteins with Lys and Arg substitutions at position 223 (S223K and S223R), and we assayed these proteins for transcriptional activity in the presence of inhibitory concentrations of bile, POA, and virstatin, as described above. The S223K and S223R proteins exhibited enhanced CT and TCP expression in the presence of bile, POA, and virstatin, similar to the Ala substitution mutants within the N terminus (Fig. 5), suggesting that one of the outcomes of ToxT binding to these inhibitory substances is adoption of this closed conformation that brings α7 and β2 together and that these are further apart in the “open (i.e. active) conformation.”

**ToxT N-terminal mutants with higher virulence factor expression in the presence of bile, virstatin, and POA.** Plasmid-borne ToxT proteins were expressed from the araBAD promoter in a *V. cholerae* toxT strain in the presence of 0.1% arabinose and 0.4% bile (black bars), or 0.01% virstatin (white bars), or 0.1% POA (gray bars), and strains were assayed for A. CT expression by GM1-ganglioside ELISA and TcpA (B) expression by Western immunoblot with anti-TcpA antiserum. CT values are normalized to % CT expression by the wild type *V. cholerae* strain grown in the absence of inhibitory compound and represent samples assayed in triplicate. Wild type *V. cholerae* TcpA expression in the absence of added compound (−) is included in B.

**N-terminal Ala Substitutions That Enhance Activity Enhance Dimerization in the Presence of Fatty Acids—** Virstatin has been shown to disrupt dimerization of the ToxT N terminus (10). Because the Ala substitution mutations identified above enhance ToxT activity in the presence of bile, POA, and virstatin, it suggests that these mutations may enhance dimerization of the N terminus in the presence of these compounds. To determine the effect of bile, POA, and virstatin on ToxT N-terminal dimerization in *V. cholerae*, we adapted the bacterial two-hybrid system used to study ToxT dimerization in *E. coli* (Fig. 1) for use in *V. cholerae* (Fig. 6). This was accomplished by inserting the Zif-dependent promoter into the *V. cholerae* chromosome so it drives transcription of the *V. cholerae* lacZ gene. We additionally inactivated the *V. cholerae* rpoZ (encodes ω) and toxT genes in the reporter strain to prevent any potential interactions with the fusion proteins that might interfere with the assay, resulting in strain KKV2297.

Interactions between the wild type ToxT N-Zif and ToxT N-ω proteins could be detected in the *V. cholerae* reporter strain KKV2297 (Fig. 6) by increased β-galactosidase activity. The control assays utilizing F151AToxTN-Zif and F151AToxTN-ω (monomeric forms), or using just one ToxTN fusion protein paired with empty vector, did not stimulate β-galactosidase activity above background levels as anticipated (data not shown).
shown). The level of β-galactosidase activity induced by interaction of the wild type ToxTN was ~10-fold lower in the V. cholerae reporter strain (Fig. 6) than in the E. coli reporter strain (Fig. 1); this lower activity may be due to the absence of a lacY gene in V. cholerae or to poor heterologous interaction of E. coli with V. cholerae RNA polymerase. Still, significant levels of β-galactosidase activity stimulated by ToxTN dimerization in this reporter strain allowed us to use this system to investigate the effect of bile, virstatin, and POA on ToxTN dimerization in V. cholerae.

Dimerization of the wild type ToxTN is disrupted by the addition of bile, POA, or virstatin, as evidenced by the loss of β-galactosidase activity when these compounds are added to the V. cholerae reporter strain carrying the two ToxTN fusion proteins (Fig. 6). These results are consistent with a previous study showing that virstatin could inhibit ToxT dimerization in E. coli, utilizing the same two-hybrid system (10), and extend this observation to show that bile and POA have the same effect on ToxTN dimerization and in the native V. cholerae background.

We then introduced the six N-terminal Ala substitution mutations identified above as providing enhanced activity in the presence of bile, POA, and virstatin (D44A, Y51A, M103A, N106A, L114A, and K154A) into both the ToxTN-Zif and ToxTN-ω fusion proteins, and we assayed these mutant ToxTN proteins for their ability to dimerize in the presence of these compounds, utilizing the same assay conditions. We also included the L114P mutation identified in a previous study (10) as enhancing dimerization in the presence of virstatin. We were unable to detect any enhanced dimerization by the D44A, Y51A, and M103A mutant ToxTN fusion proteins in the presence of bile, POA, or virstatin, in comparison with the wild type ToxTN proteins (Fig. 6). Either these mutations act through a different mechanism to enhance ToxT activity in the presence of these compounds or the V. cholerae two-hybrid assay is not sensitive enough to detect low levels of enhanced dimerization.

In contrast, the N106A, L114A, L114P, and K154A ToxTN fusion proteins stimulated enhanced β-galactosidase activity compared with the wild type ToxTN proteins in the presence of bile, POA, and virstatin, consistent with enhanced dimerization by these mutant forms in the presence of these inhibitory compounds (Fig. 6). Moreover, the N106A mutation in just one of the ToxTN fusion proteins enhances dimerization with the wild type ToxTN fusion protein, in the presence of these inhibitory compounds ("N106A/WT"), indicating that N106A is a cis-acting mutation (i.e. this mutation only needs to be in one of the monomers to enhance dimerization). Our results demonstrate the involvement of residues Asn-106, Leu-114, and Lys-154 in the modulation of ToxTN dimerization in response to fatty acids.

Effect of Chain Length, Saturation, on Fatty Acid Inhibition of ToxT Activity—We examined the ability of a number of additional fatty acids for their ability to inhibit ToxT activity and dimerization. We examined CT expression from V. cholerae carrying either the wild type or L114A ToxT proteins (as in Fig. 5), as well as ToxTN N-terminal dimerization in V. cholerae using the two-hybrid assay (as in Fig. 6), in the presence of various fatty acids (Fig. 7). POA is a 16-carbon, ω7 monounsaturated fatty acid that is able to inhibit the wild type ToxT dimerization and activity, whereas the L114A mutant protein exhibits enhanced dimerization and activity in the presence of POA, as shown above. As demonstrated previously (9), oleic acid (18 carbons, ω9 monounsaturated) was also able to inhibit ToxT activity, and we found that it inhibits ToxT N terminus dimerization, whereas the L114A mutant exhibited enhanced activity in both assays. We found that shorter monounsaturated fatty acids, myristoleic (14 carbons), 9-decanoic (10 carbons), and 7-octenoic (8 carbons) acids, were also able to inhibit ToxT dimerization and activity, as was the shorter saturated fatty acid, decanoic acid (10 carbons); the L114A protein showed enhanced dimerization and activity in the presence of all of these compounds (Fig. 7). These results demonstrate that multiple fatty acids, both saturated and unsaturated, in a range of lengths, can inhibit ToxT dimerization and activity. Also, as shown above, virstatin is also able to inhibit ToxT dimerization and activity, and L114A exhibits enhanced dimerization and activity in the presence of virstatin. Capsaicin, which is synthesized by the addition of a branched chain fatty acid to vanillylamine, has been reported to inhibit CT expression in V. cholerae (16); we found that capsaicin inhibits wild type ToxT dimerization and activity, whereas the L114A protein showed enhanced activity in the presence of capsaicin. Interestingly, the trans-isomer of oleic acid, elaidic acid, was the only fatty acid tested that showed little ability to inhibit ToxT dimerization and activity (Fig. 7). This finding illuminates that conformations of unsaturated fatty acids exist that may be unable to bind and inhibit ToxT activity.

Previous studies have shown that POA disrupts the binding of ToxT to the tcpA promoter in a gel mobility shift assay (9). We measured the ability of WT and N106A ToxT to bind the

FIGURE 6. Dimerization of ToxTN mutants in the presence of bile, virstatin, and POA determined by two-hybrid assay in V. cholerae. The V. cholerae two-hybrid reporter strain KKV2297 ("Experimental Procedures") expressing the ToxTN-Zif and ToxTN-ω fusion proteins indicated was grown in the presence of 0.4% bile (striped bars), 0.01% virstatin (white bars), or 0.1% POA (gray bars) in 0.1 mM IPTG. Both −Zif and −ω fusion partners contained the indicated mutation, with the exception of the ToxTN/N106A-Zif protein, which was also paired with both wild type ToxTN (N106A/WT) and ToxTLN114A-ω (N106A/L114A). The activity of the wild type ToxTN fusion proteins in the absence of inhibitory compounds, as well as background activity from empty vectors (−), is indicated (black bars). Strains were assayed for β-galactosidase activity, and values represent triplicate samples normalized to % wild type ToxTN fusion protein activity. Asterisks mark significant difference to the wild type ToxTN fusion in the presence of the same compound (p value < 0.01).
 tcpA promoter in the presence of increasing amounts of POA in a gel mobility shift assay (supplemental Fig. S3). Binding to the tcpA promoter by the wild type protein was disrupted by increasing concentrations of POA, as shown previously (9), but we were unable to detect any enhanced binding by the N106A protein in the presence of POA. This may be due to either differences between the in vitro and in vivo DNA binding activity of N106A or to subtle changes in DNA binding activity unable to be detected by this assay.

**Enhanced ToxT Dimerization Facilitates Enhanced Intestinal Colonization**—To determine whether the enhanced dimerization and transcriptional activity of the N106A and L114A ToxT proteins in the presence of fatty acids would enhance *V. cholerae* intestinal colonization, we first constructed *V. cholerae* strains with toxTN0106A and toxTL114A alleles in the chromosome in place of native wild type toxT. The toxTN0106A and toxTL114A *V. cholerae* strains were then assayed for their ability to colonize the infant mouse intestine utilizing a competition assay with a coinfecting wild type *V. cholerae* strain. The toxTN0106A and toxTL114A *V. cholerae* strains showed significantly higher levels of intestinal colonization than the wild type strain (p value <0.0001), approximately 14- and 4-fold higher than the wild type strain, respectively (Fig. 8A). Bile was also added to the inoculum, to determine whether bile might enhance colonization by the toxTN0106A and toxTL114A strains; however, the colonization levels by these strains in the presence of added bile was not significantly different from the absence of bile. This result suggests that bile is already present at sufficient amounts within the infant mouse intestine to distinguish between the activities of wild type and the N106A/L114A ToxT proteins. Our results indicate that enhanced dimerization by ToxT in the presence of fatty acids leads to enhanced intestinal colonization.

The toxTN0106A, and toxTL114A strains were also measured for CT and TCP expression under *in vitro* virulence factor inducing conditions in the presence of inhibitory concentrations of bile, POA, and virstatin. In contrast to the wild type *V. cholerae* strain, which showed reduced expression of CT and TCP in the presence of bile, POA, and virstatin, the toxTN0106A and toxTL114A strains expressed enhanced amounts of CT and TCP in the presence of all three inhibitory compounds (Fig. 8B). Our results are consistent with fatty acids modulating dimerization of the ToxT N terminus, and thereby ToxT transcriptional activity.

We also examined the effect of the ToxT C-terminal S223K mutation on the ability of *V. cholerae* to colonize the intestine. Because this mutation was designed to force the N and C termini apart (i.e. for ToxT to adopt an open conformation), and the ToxTS223K protein exhibited enhanced transcriptional activity in the presence of bile, virstatin, and POA (Fig. 5), we anticipated that the *V. cholerae* toxTS223K mutant might colonize the intestine at higher levels than the wild type strain, similar to the toxTL114A and toxTN0106A strains. However, the toxTS223K strain exhibited an approximate 5-fold defect for
intestinal colonization in comparison with the wild type V. cholerae strain (C.I. = 0.18), whether or not bile was added to the inoculum (supplemental Fig. S4). This indicates that the C-terminal S223K mutation, designed to force ToxT into an open conformation, does not enhance intestinal colonization.

**DISCUSSION**

ToxT is a key regulatory factor in the disease progression of cholera, because it directly activates transcription of the two most important virulence factors, CT and TCP. Previous studies have suggested a relationship between dimerization of the ToxT N terminus and transcriptional activity. The crystal structure of ToxT revealed a monomer tightly bound to POA, and this is presumably the inactive form of ToxT (9); thus it is still unclear exactly how the ToxT N terminus dimerizes. We utilized a comprehensive scanning alanine library to identify 20 residues within the N terminus fall into this category. Notably, α3 contains eight of these critical residues for dimerization, including Phe-151 (discussed below); the hydrophobic nature of these residues is predicted to hold this helix in place in relation to the rest of the N terminus.

The three additional N-terminal residues that contribute to dimerization determined by Ala scanning mutagenesis may be more directly involved in dimerization. Ser-140 lies immediately adjacent to Asp-141, which was suggested previously to be involved in dimerization (D141G (13)), and which we have confirmed here. These residues lie at one end of α3, which contains five additional hydrophobic (buried) residues critical for dimerization, and Val-146, which is solvent-exposed and also critical for dimerization. The high concentration of residues critical for dimerization in this region suggests that it represents a dimerization interface. Moreover, a linker separates this helix from α2, where the solvent-exposed Glu-129 lies. These surface-exposed residues may represent contact points between adjacent monomers; based on the ToxT structure, a monomer oriented with the recognition helices bound to DNA, and with some reorientation of α3, it might be able to make contact with α3 in an adjacent monomer bound in the opposite orientation (i.e. α6 of one monomer bound to DNA in close proximity to α6 of adjacent monomer bound to DNA).

We showed that the F151A mutation within α3 causes ToxT to behave as a monomer in solution, and the F151A protein binds poorly to the tcpA promoter, indicating that dimerization contributes to DNA binding. Interestingly, F151A was still able to specifically bind the tcpA-binding site in vitro, albeit at lower affinity and at an apparent lower mobility, consistent with DNase footprinting data suggesting that a ToxT monomer can bind DNA (43). We would suggest that the cooperative interactions mediated by N-terminal dimerization allow ToxT to not only occupy promoters at a lower concentration but also alter the conformation to a form more able to interact with RNA polymerase and stimulate transcription. Importantly, a V. cholerae toxTF151A strain expresses no CT or TCP in vitro and is completely unable to colonize the intestine within an animal model for cholera. These results emphasize the importance of ToxT N-terminal dimerization to cholera pathogenesis.

Of particular interest is the solvent-exposed charged residue Glu-52. An Ala substitution at this position prevents transcriptional activation by ToxT, but it has little effect on ToxT N-terminal dimerization. This indicates that the transcription defect of this mutant is not due to a lack of dimerization; we hypothesize that this may represent a contact site with RNA polymerase, given the surface-exposed nature of this residue.

Bile, virstatin, and POA inhibit the transcriptional activity of ToxT (9, 14, 17), and virstatin was shown to inhibit dimerization of the ToxT N terminus (10). The recent crystal structure of ToxT revealed a monomer tightly bound to POA (9), a component found in bile. We have extended these previous studies to show that additional monounsaturated and saturated fatty acids with shorter chain length, as well as capsaicin, can also inhibit ToxT dimerization and activity. The only monounsaturated fatty acid that failed to inhibit ToxT dimerization and transcriptional activity was elaidic acid, the trans-isomer of...
oleic acid. Within the ToxT structure, the cis-conformation of POA (and presumably OA) allows the fatty acid to fold into the binding pocket between the N and C termini (Fig. 9), and it is anticipated that the trans-conformation of elaidic acid would prevent the fatty acid from being able to fit into this binding pocket. This also suggests that other trans-isomers of unsaturated fatty acids may also fail to inhibit ToxT activity, which will be tested experimentally in the future.

We screened the entire scanning Ala ToxT library for proteins with enhanced activity in the presence of inhibitory concentrations of bile, and we found six mutants with substitutions in the N terminus that enhance transcriptional activity in the presence of bile, viristatin, and POA. Two of the residues (Met-103 and Asn-106) lie within the flexible region (amino acids 101–110) that is unstructured within the crystal structure (Fig. 9, dashed line). Interestingly, three of the residues lie in close proximity to the N terminus, which suggests that these residues may play a role in modulating the activity of ToxT in response to fatty acids.
proximity to this unstructured region and appear to interact with each other (Asp-44, Tyr-51, and Leu-114; Fig. 9 highlighted in cyan), thus defining a region of the N terminus involved in modulating ToxT activity in response to fatty acids. The last residue identified (Lys-154) lies solvent-exposed in α3, where a number of residues critical for dimerization are also located, as described above. Further analysis showed that the L114A mutation identified here behaves similar to the L114P mutation identified previously as being insensitive to the inhibitory activity of virstatin (10). Measurement of dimerization via two-hybrid assay in V. cholerae demonstrated that the N106A, L114A, and K154A mutations enhance N-terminal dimerization in the presence of bile, virstatin, and POA, whereas no enhanced dimerization could be detected with the other three substitution mutations. These mutant proteins (D44A, Y51A, and M103A) induced lower levels of CT expression than N106A and L114A in the presence of inhibitory compounds, indicating that they either cause weaker enhancement of dimerization that cannot be detected by this assay or enhance activity through another mechanism besides dimerization. It is not yet clear whether these mutations alter the conformation of the N terminus to inhibit fatty acid binding or make the dimerization region more insensitive to the inhibitory effect of fatty acid binding; we favor the latter explanation, given the extensive contacts between ToxT and POA evident in the crystal structure, which are unlikely to be dramatically altered by modest conformational changes.

To determine whether enhanced N-terminal dimerization and transcriptional activity in the presence of inhibitory fatty acids might enhance V. cholerae virulence, we first created V. cholerae strains with chromosomal toxTN106A and toxTL114A alleles, and we then measured these strains for virulence in vivo and in vitro. The toxTN106A and toxTL114A strains expressed higher levels of CT and TCP than the wild type strain in the presence of bile, virstatin, and POA under in vitro virulence factor-inducing conditions. Importantly, the toxTN106A and toxTL114A strains colonized the intestine at significantly higher levels than the wild type strain, whether or not bile was included in the inoculum. These results demonstrate that enhanced dimerization of ToxT in the presence of fatty acids enhances intestinal colonization, indicating that fatty acids normally modulate V. cholerae colonization. It also indicates that native ToxT is not maximally active during the colonization process. Thus, negative regulation of ToxT activity during colonization must be evolutionarily advantageous, because ToxT is not normally synthesized in a fatty acid-insensitive state. Our animal model only measures intestinal colonization, so perhaps ToxT modulation enhances other aspects of cholera infection, such as escape and dissemination. Thus, toxTN106A and toxTL114A would also be predicted to enhance intestinal colonization in humans and therefore vaccine efficacy in live attenuated cholera vaccine strains.

The monomeric form of ToxT bound to POA is predicted to be in a closed conformation with the N and C termini clamped onto the POA and unable to dimerize and activate transcription (9). The Ser-223 side chain in the C terminus is located at the interface between the ligand-binding/dimerization (N-terminal) domain and the DNA-binding (C-terminal) domain, where it makes a hydrogen bond to the carbonyl oxygen of Tyr-26 (Fig. 9). Substitution of Ser-223 with Lys or Arg is predicted to decouple the interactions between the N- and C-terminal domains of the protein, permitting ToxT dimerization, DNA binding, and transcriptional activation. We introduced Lys and Arg substitutions at residue Ser-223 in the C terminus to drive the N and C termini apart (Fig. 9B), and we found that S223K and S223R proteins exhibited enhanced expression of CT and TCP in the presence of bile, virstatin, and POA, similar to the Ala substitutions in the N terminus described above (e.g. L114A). However, a toxTS223K V. cholerae strain colonized the intestine at lower levels than the wild type strain, indicating that this mutant form of ToxT is deleterious to the colonization process. This may be due to the inability of this protein to adopt a closed conformation, in contrast to the N-terminal mutations that exhibit enhanced dimerization in the presence of fatty acids but not necessarily unable to adopt a closed conformation.

Recently, bicarbonate has been shown to enhance ToxT transcriptional activity, and bicarbonate is found in the intestinal environment (44). A possible scenario is that bicarbonate and the fatty acids in bile in differing amounts are present within the intestinal environment traversed and ultimately colonized by V. cholerae, and the competing effects of these modulatory compounds on ToxT allow for optimal spatial and temporal ToxT transcriptional activity to facilitate successful colonization and virulence.

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