Structure of *Vibrio cholerae* ToxT reveals a mechanism for fatty acid regulation of virulence genes

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Cholera is an acute intestinal infection caused by the bacterium *Vibrio cholerae*. In order for *V. cholerae* to cause disease, it must produce two virulence factors, the toxin-coregulated pilus (TCP) and cholera toxin (CT), whose expression is controlled by a transcriptional cascade culminating with the expression of the AraC-family regulator, ToxT. We have solved the 1.9 Å resolution crystal structure of ToxT, which reveals folds in the N- and C-terminal domains that share a number of features in common with AraC, MarA, and Rob as well as the unexpected presence of a buried 16-carbon fatty acid, *cis*-palmitoleate. The finding that *cis*-palmitoleic acid reduces TCP and CT expression in *V. cholerae* and prevents ToxT from binding to DNA in vitro provides a direct link between the host environment of *V. cholerae* and regulation of virulence gene expression.

AraC | crystal structure | pathogenesis | oleic acid | palmitoleic acid

Vibrio cholerae is a Gram-negative bacterium and the causative agent of the acute intestinal infection known as cholera. Upon entry into the host intestine, *V. cholerae* induces a transcriptional cascade resulting in the expression of the master virulence regulator, ToxT. ToxT directly activates the expression of the two primary virulence factors of *V. cholerae*, the toxin-coregulated pilus (TCP) and cholera toxin (CT) (1–3) and also autoregulates its own expression from the *tcp* promoter (4, 5). ToxT is a member of the AraC-family of transcriptional regulators which are defined by a 100 amino acid region of sequence similarity that forms an independently folding DNA-binding domain (DBD) containing two helix-turn-helix (HTH) motifs (6). Members fall into three functional groups depending on the types of genes that they regulate. Those members that regulate carbon metabolism, such as AraC of *E. coli*, are active as dimers and respond to small effector molecules that bind to the N-terminal domain of the protein. Those members that are involved in the stress response, such as SoxS, Rob, and MarA typically function as monomers. The third group is involved in regulating virulence gene expression and includes Rns of enterotoxigenic *E. coli* (7), BfpT (PerA) of enteropathogenic *E. coli* (8), and ExsA of *Pseudomonas aeruginosa* (9), amongst numerous others. These regulators may respond to physical cues such as temperature and pH and it is not yet known if they function primarily as monomers or dimers. To date, of the large number of known AraC-family proteins (PROSITE, PS01124 (6)), only two full-length structures, MarA (10) and Rob (11), have been solved.

ToxT activates the transcription of many promoters, including those that control the *tcp* and *ctx* operons, via binding to degenerate thirteen base-pair sequences called toxboxes. These sequences are organized either singly, or in direct or inverted repeat configurations (12) such that ToxT can function at promoters to positively regulate gene transcription as either a monomer or a dimer depending on the structure of the promoter (13, 14). The activity of ToxT is known to be sensitive to bile, a mixture of many molecules including saturated fatty acids (SFAs), unsaturated fatty acids (UFAs), salts, and cholesterol that is secreted into the intestine from the gall bladder (15, 16). In the presence of bile, and more specifically bile components oleic, linoleic acid,

or arachidonic acid, transcription of *ctxA* and *tcpA* by *V. cholerae* is drastically reduced, leading to the suggestion that ToxT function is inhibited by UFAs found in bile (16).

In this study, the full-length structure of ToxT is determined and shown to be similar in overall structure with the regulator AraC. Unexpectedly, the structure revealed the regulatory domain of ToxT was bound to a ligand, the sixteen-carbon fatty acid *cis*-palmitoleate. The addition of *cis*-palmitoleate to *V. cholerae* culture media reduced *ctx* and *tcp* expression by 6–8 fold similar to what was previously observed with oleic acid (16) and both UFAs prevented the sequence specific DNA-binding of ToxT in vitro. These results suggest a model in which the presence of UFAs reduce virulence gene expression by directly binding to ToxT and altering the conformation of the protein such that it is less competent to bind to DNA and less likely to form stable dimers.

Results and Discussion

Structure of Full-Length ToxT and Comparison with Other AraC-family Members. We have solved the 1.9 Å resolution crystal structure of ToxT (Fig. 1A, Table S1, PDB ID 3GBG). The crystal contained one monomer of ToxT per asymmetric unit, with each monomer containing two domains. The N-terminal domain (amino acids 1–160) is comprised of three α -helices (helix α 1– α 3) and a nine stranded β -sandwich (strand β 1– β 9) forming a “jelly roll” or “cupin-like” fold (17) containing a binding pocket enclosed by residues Y12, Y20, F22, L25, I27, K31, F33, L61, F69, L71, V81, and V83 from the N-terminal domain and residues I226, K230, M259, V261, Y266, and M269 from the C-terminal domain (Fig. S1). The volume of this predominantly hydrophobic pocket is 780.9 Å³ as calculated by the program CASTp (18). The pocket contains a sixteen-carbon fatty acid bound such that its negatively charged carboxylate head group forms salt bridges with both K31 from the N-terminal domain and K230 from the C-terminal domain (Fig. 1B; see discussion below). Following a short linker (amino acids 161–169), the C-terminal domain (170–276) is made up of two HTH DNA-binding motifs (the more N-terminal HTH1 and the more C-terminal HTH2) linked by a relatively long α -helix, helix α 7. The interface between the two domains has an area of ~2000 Å² and is very polar, with few hydrophobic interactions (Fig. S2).

Structures of other AraC-family members are limited to three members: AraC, in which the N- (19) and C-terminal (20) domain structures have been determined separately (Fig. 2), MarA, which contains only a DNA-binding domain (10), and Rob, which, in contrast to ToxT and AraC, contains an N-terminal DNA-binding domain and a C-terminal regulatory domain (11). Both the MarA and Rob structures have been cocrystallized with DNA. The

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Fig. 1. The structure of ToxT. (A) Ribbon diagram of ToxT showing α -helices (gold), β -strands (cyan), and loops (dark red). The bound *cis*-palmitoleate is shown in stick form with carbons in green and oxygens in red. The N and C termini are indicated. Helices and strands are numbered according to their topological connectivity in the full-length protein. Note that residues 101–110 are disordered in the structure, as indicated by the loop ends on the left side of the molecule. (B) Close up of the *cis*-palmitoleate binding region showing interactions of the carboxylate head group with side chains.

C-terminal domain of Rob, like the N-terminal domains of ToxT and AraC, is composed of several helices and β -sheets forming a binding pocket. While the structure of Rob contains no ligand, the N-terminal domain of AraC (PDB ID 2ARC) has been determined with arabinose bound in the β -sandwich in a position similar to the fatty acid in ToxT (Fig. 2).

A comparison of full-length ToxT with existing high resolution structures using Distance ALIGNment (DALI) (21) and Secondary Structure Matching (SSM) (22) gave no significantly similar hits over the entire 276 amino acids. However, when the two domains are taken separately, the N-terminal domain of ToxT most closely resembles the N-terminal domain of AraC (for 126 α -carbons, the RMSD is 3.63 Å; PDB ID 1XJA (23)), while the C-terminal domain is most similar to the DBD of AraC (RMSD 2.12 Å for 92 α -carbons; PDB ID 2K9S (20)). ToxT and AraC have a very similar N-terminal topology (Fig. S3A) and other than the N-terminal arm of AraC (residues 7–17), all of the other secondary structural elements of these two proteins can be aligned (Fig. S3B).

Fig. 2. Ribbon diagrams of ToxT and the N- and C-terminal domains of AraC. The models are colored by a rainbow effect with blue at the N terminus and red at the C terminus. The arabinose bound in the N-terminal domain of AraC is shown in stick form. PDB accession numbers are indicated for the three structures.

N-Terminal Domain. The fold of the N-terminal domain of ToxT is similar to AraC in that it contains eight antiparallel β -sheets (Fig. 1A) followed by helix $\alpha 1$ (19). However, ToxT is missing the N-terminal arm that is present in AraC that interacts with arabinose. Helix $\alpha 1$ and sheet $\beta 9$ are linked by a disordered region between residues 101 and 110. Childers et al. have shown that alanine substitutions of four of these residues (M103, R105, N106, and L107) show either greatly enhanced *ctxAp-lacZ* expression or $\leq 10\%$ expression of the *ctxAp-lacZ* and *acfA-phoA* fusions (24) demonstrating this region is important for virulence gene expression. Helix $\alpha 3$ of ToxT is analogous to the helix that allows for coiled-coil N-terminal dimerization in the AraC structure (19). Although ToxT is clearly a monomer in this structure and appears to bind to independent toxboxes as a monomer (13), certain promoters such as *tcp*, *ctx*, and *tagA* require ToxT dimerization on adjacent toxboxes for full activation (13, 14). In AraC, the coiled-coil is anchored at the ends by a triad of leucine residues providing stability (19). Although analogous leucine residues are not present in $\alpha 3$, if ToxT were to dimerize in a manner similar to that observed in AraC, complementary salt bridges would be formed between helix $\alpha 3$ residues such as D141, E142, K157, and K158 of one monomer and the same residues on the other monomer. In fact, Hsaio et al. have suggested that a D141G substitution is able to repress *msh* promoters as a monomer, but is unable to activate *tcp* (25).

A number of residues in the N-terminal domain have been shown to be important for ToxT mediated activation of virulence gene expression (24). As suggested by Childers et al., those involved in maintaining an N-terminal hydrophobic core (M32, W34, I35, L42, L60, L71, W117, L127, F147–148, and F151–152) are essential for protein folding and stability (24). Two surface exposed glutamates, E52 in $\beta 5$, and E129 in $\alpha 2$, as well as S140, which lies in the loop between $\alpha 2$ and $\alpha 3$, have also been shown important for function (24) for reasons not illuminated by the structure.

Hung et al. have identified a small molecule inhibitor of ToxT, virstatin (26), that interferes with its ability to dimerize and activate transcription of the *tcp* and *ctx* promoters (14). They also demonstrated that a L114P substitution is virstatin resistant and suggested that it may favor a conformation that allows the protein to dimerize more efficiently (14). It is interesting to note that L114 lies in the vicinity of the unresolved residues (residues 101 and 110) (Fig. 1A) and substitution to a proline may result in

a conformational change affecting the adjacent unresolved loop or N-terminal ligand binding pocket.

DNA-Binding Domain. The DBD of ToxT is composed of seven α -helices. HTH1 is comprised of $\alpha 5$ and $\alpha 6$, HTH2 is comprised of $\alpha 8$ and $\alpha 9$, and they are connected by a central helix $\alpha 7$ (Fig. 1A). Helix $\alpha 4$ and helix $\alpha 10$ are involved in scaffolding and stability of HTH1 and HTH2 respectively. Pairwise SSM alignments performed by WinCoot (27, 28) of the DBD's of ToxT (amino acids 170–273), AraC, and MarA, show consistently close alignments of HTH2, with greater variability in the orientation of HTH1 (Fig. 3). The DNA-bound structure of MarA demonstrates that it is possible for AraC-family members to utilize helices $\alpha 6$ and $\alpha 9$, oriented in a parallel manner, to bind consecutive major grooves on curved target DNA (10). This parallel arrangement is conserved in Rob; however the structure does not show both HTH motifs bound to major grooves (11). As suggested by Rodgers and Schleif (20), helix $\alpha 6$ of AraC, which is at a divergent angle with respect to helix $\alpha 9$, would likely have to undergo a conformational change in order to allow for consecutive major groove binding on target DNA. In ToxT, helix $\alpha 6$ is not only non-parallel with helix $\alpha 9$, but is also more distorted and bent when compared to what is observed in AraC (Fig. 3). Another difference in this domain is in the orientation of helix $\alpha 7$. In AraC and MarA, the orientation of helix $\alpha 7$ is virtually the same, whereas in ToxT helix $\alpha 7$ is orientated differently with respect to the other structures. As discussed below, the position of helix $\alpha 7$ is such that it could link the N-terminal binding pocket to conformational changes occurring in the DNA-binding domain.

Residues identified by Childers et al. in the C-terminal domain that are important for ToxT function include several in the cores of HTH1 and HTH2 (I174, V178, W186, W188, L206, V211, I217, F245, F251, and F255) (24) that are critical for proper folding and stability. There are also a number of surface exposed residues that could be involved in stabilizing the DBD (S175, R184, R221, S227, E233, K237, G244, and N260) (24). Finally, it appears that residues such as K203 ($\alpha 6$), R214 ($\alpha 7$), T253 ($\alpha 9$), and S257 ($\alpha 9$) are positioned to be directly involved in protein/DNA interactions.

A Fatty Acid is Present in ToxT and Influences its DNA-binding Activity.

UFAs such as arachidonic, linoleic, and oleic acid have previously been shown to strongly inhibit the expression of ToxT-activated genes, whereas SFAs such as palmitic and stearic acid do not (16). The structure of ToxT contains an almost completely buried and solvent inaccessible sixteen-carbon fatty acid bound to the pocket in the N-terminal domain (Fig. 1A and B, Fig. 2, Fig. S1A and B). The negative charge on the carboxylate head group hydrogen bonds with Y12 and forms salt bridges with K31 from the N-terminal domain and K230 from the C-terminal domain (Fig. 1B, Fig. S1D). NMR studies of chloroform/methanol extractions from pure ToxT samples indicate the presence of a long-chain, singly unsaturated fatty acid in a *cis* configuration (Fig. S4). Although the electron density ends after carbon sixteen of the hydrophobic chain, suggesting *cis*-palmitoleate, the ToxT structure could accommodate the two additional carbons of oleate (Fig. S1C). An $F_o - F_c$ difference map calculated after refinement with oleate placed into the pocket shows strong negative density after carbon sixteen, further indicating that the bound molecule is *cis*-palmitoleate.

To address whether *cis*-palmitoleate was capable of influencing the activity of ToxT, different UFAs and SFAs were added to cultures of *V. cholerae* strains carrying transcriptional fusions to the *tcp* and *ctx* operons. We found that the expression of these operons were reduced between 6–8 fold with *cis*-palmitoleic acid and between 10–15 fold with oleic acid, whereas only a twofold reduction was observed with palmitic acid (Fig. 4A and B). As previous

Fig. 3. Pairwise SSM alignments of the DBDs of ToxT (3GBG), AraC (2K95), and MarA (1BL0). ToxT is represented in silver, AraC in blue, and MarA in dark red. Helix $\alpha 7$ and the DNA-binding helices $\alpha 6$ and $\alpha 9$ are labeled. Arrows indicate the relative direction each DNA-binding helix is pointing. The asterisk indicates the distorted section of helix $\alpha 6$ on ToxT.

studies have shown that *toxT* transcription is unaffected by UFAs, it has been suggested that UFAs act on ToxT directly (16).

EMSAs were performed, and a 100-fold molar excess of protein was shown to bind to a 40 base-pair probe containing two toxboxes from the *tcp* promoter in vitro (Fig. 4C, lane 2). This interaction is specific since it was completely inhibited by a 70-fold molar excess of specific competitor DNA (lane 3) but not

Fig. 4. Effects of fatty acids on *tcp* and *ctx* expression. (A) and (B) β -galactosidase activity of *tcp-lacZ* and *ctx-lacZ* fusion constructs respectively. Cells were grown in LB pH 6.5 at 30 °C for 18 hours $\pm$ the indicated fatty acids at 0.02% dissolved in methanol. The inset shows TspA expression by Western blot under the same conditions in the corresponding lanes (C—control with methanol; PA—sodium palmitate; POA—palmitoleic acid; OA—oleic acid). (C) EMSA showing a specific interaction between ToxT and a 40-base-pair segment of the *tcp* promoter. All lanes contain 0.0025 μ M labeled probe. Lane 1, free DNA; lane 2, 0.2 μ M ToxT; lane 3, 0.2 μ M ToxT with a 70-fold molar excess of cold competing DNA; lane 4, 0.2 μ M ToxT with a 70-fold molar excess of a 42-base-pair nonspecific DNA cold competitor. (D) EMSA showing the effect of several fatty acids on ToxT/DNA interactions. All lanes contain 0.0025 μ M labeled probe. Lane 1, free DNA; lane 2, 0.125 μ M ToxT; lane 3, 0.2 μ M ToxT; lane 4, 0.25 μ M ToxT; lane 5, 0.25 μ M ToxT with methanol; lane 6, 0.25 μ M ToxT with 0.002% palmitic acid; lane 7, 0.25 μ M ToxT with 0.002% palmitoleic acid; lane 8, 0.25 μ M ToxT with 0.002% oleic acid.

by a 70-fold molar excess of nonspecific competitor DNA (lane 4). Addition of methanol or 0.002% palmitic acid to the reaction had no effect on ToxT binding (Fig. 4D, lanes 5 and 6). However, addition of 0.002% palmitoleic or oleic acid completely prevented ToxT from binding to DNA (Fig. 4D, lanes 7 and 8), consistent with the reduction of *tcp* and *ctx* transcription observed in the presence of these fatty acids (Fig. 4A and B). A control EMSA experiment with a different protein/DNA pair was also performed to show that unsaturated fatty acids do not block all protein/DNA interactions (Fig. S5).

As no fatty acids were added to any buffer or crystallization condition, the *cis*-palmitoleate most likely originated from the *E. coli* used as the protein expression strain. Indeed, *cis*-palmitoleic acid comprises 10.5% of the total fatty acid content in *E. coli* membranes, whereas oleic acid is absent (29). As it is expected that there would be very little free fatty acid in the cytoplasm of these bacteria, it is likely that ToxT bound *cis*-palmitoleate released from the membrane upon cell lysis. Other groups have seen similar phenomena such as the binding of *cis*-vaccenic acid by the pheromone-binding protein of *Bombyx mori* when purified from an *E. coli* expression system (29). Previous studies indicate that 23.5% of the fatty acid content of bile is oleic acid, and if *cis*-palmitoleic acid is present in bile, it is at a concentration of less than 0.5%. As both oleic and *cis*-palmitoleic acids are monounsaturated at the ninth carbon and as there is room in the ToxT structure to potentially accommodate the longer oleic acid, it is not surprising that both fatty acids can serve as a

ligand for ToxT. However, given the abundance of oleic acid in bile when compared to *cis*-palmitoleic acid, it seems that oleic acid would be the natural ligand responsible for altering ToxT function in vivo.

A Structural Model for ToxT Activation. The finding that UFAs reduce the expression of *tcp* and *ctx* expression in *V. cholerae* and that they significantly reduce the ability of ToxT to bind to DNA in vitro suggests a model for the regulation of ToxT function via fatty acid binding (Fig. 5). In this model, when the bacteria are in the lumen of the intestine in the presence of fatty acids, the position of the carboxylate head group of the fatty acid bridging K31 from the N-terminal domain with K230 from the C-terminal domain (Fig. 1B) keeps ToxT in a “closed” conformation that is not capable of binding DNA (5). Restraint of K230, which is located at the C-terminal end of helix $\alpha 7$, would cause helix $\alpha 7$ to assume a position that pulls and distorts helix $\alpha 6$ into an orientation that is unfavorable for DNA-binding (Fig. 3). Once the bacteria have penetrated the mucus of the intestine where the concentrations of fatty acids are presumably reduced (15), charge-charge repulsion between K31 and K230 destabilizes the closed conformation, leading to an opening of the N- and C-terminal domains. In this “open” conformation, K230, helix $\alpha 7$, and helix $\alpha 6$ would no longer be restrained, and reorient into a conformation that is competent for DNA-binding. The EMSA data support this model, in which an equilibrium exists between fatty acid bound “closed” ToxT that cannot bind to DNA and fatty acid free “open” ToxT that can bind to DNA. While a 50-fold molar excess of ToxT over the probe is not sufficient to drive the binding equilibrium in the direction of the DNA-bound state (Fig. 4D, lane 2), increasing the concentration of ToxT (Fig. 4D, lanes 3 and 4) shifts the equilibrium in the direction of a protein/DNA complex. Addition of 0.002% palmitoleic or oleic acid then disrupts the protein/DNA complex by shifting the equilibrium back to the “closed” state, containing a protein/fatty acid complex, releasing it from DNA (Fig. 4D, lanes 7 and 8). As discussed above, a number of studies have suggested that ToxT dimerizes upon binding to adjacent toxboxes (12, 14, 26, 30). It is clear from the structure that side-by-side dimerization of “closed” ToxT on adjacent toxboxes would be

Fig. 5. Model for the regulation of ToxT by monounsaturated fatty acids. Fatty acid bound ToxT is in a “closed” conformation, which cannot bind to DNA. Release of the fatty acid results in an “open” conformation that can bind to DNA. In the “open” conformation the N-terminal domain is free to move in relation to the C-terminal domain, and is able to dimerize with another ToxT at an adjacent toxbox. The linker is sufficiently flexible to allow dimerization on DNA in either direct or inverted orientations.

difficult if not impossible due to steric constraints. However, we predict the “open” form of ToxT would be able to dimerize on adjacent toxboxes in either the direct or inverted orientations (Fig. 5).

These studies describe the high resolution structure of ToxT and provide evidence that UFAs decrease the ability of ToxT to interact with DNA. The presence of a UFA in ToxT provides a direct link between the host environment of *V. cholerae* and the regulation of virulence gene expression. Further insights into the mechanisms by which UFAs influence ToxT will contribute to our understanding of this critical regulatory step in the disease process. In addition, this structure provides a foundation for studying the mechanisms by which small molecules such as virstatin (14, 26) are able to inhibit ToxT function as well as the therapeutic potential of UFAs in the treatment of cholera.

Materials and Methods

ToxT Expression. ToxT was purified using the IMPACT-CN fusion protein system (New England Biolabs). Full-length ToxT was cloned from *Vibrio cholerae* O395 and ligated into pTXB1 (New England Biolabs) to produce a *toxT-intein/CBD* (chitin binding domain) fusion construct. ToxT was expressed by autoinduction (31) in ZYM-5052 media using BL21-CodonPlus® (DE3)-RIL (Stratagene) *E. coli*. All Luria-Bertani broth (LB) agar plates and media contained 100 $\mu\text{g mL}^{-1}$ carbenicillin and 25 $\mu\text{g mL}^{-1}$ chloramphenicol. Selenomethionine ToxT was produced by growing the same *E. coli* strain in a minimal medium phosphate amino acids selenomethionine (PASM-5052) containing a mixture of 10 $\mu\text{g mL}^{-1}$ methionine, 125 $\mu\text{g mL}^{-1}$ selenomethionine, and 100 nanomolar (nM) vitamin B₁₂.

Purification of ToxT. Cells were harvested by centrifugation, resuspended in column buffer (20 mM Tris pH 8.0, 1 mM EDTA, and 500 mM NaCl), lysed via French press, and clarified by centrifugation. Chitin beads (New England Biolabs) were equilibrated with cold column buffer, mixed with the clarified supernatant, and incubated at 4 °C with gentle rocking. The chitin bead slurry was then loaded onto a gravity flow column, washed with 10 column volumes of column buffer, and equilibrated with five column volumes of cleavage buffer (20 mM Tris pH 8.0, 1 mM EDTA, and 150 mM NaCl). The intein with the CBD was cleaved from ToxT using cleavage buffer with 100 mM dithiothreitol (DTT) and left at 4 °C for 20 h. Eluant from the chitin column was then loaded onto a HiTrap sepharose packed (SP) fast flow (FF) cationic exchange column (GE) to separate the ToxT-intein/CBD fusion protein that coeluted with the native ToxT using a sodium chloride gradient. Pure fractions were pooled and concentrated to 1.75 mg mL⁻¹ for crystallization.

Crystallization of ToxT. ToxT was crystallized in hanging drops where 50% of the drop was ToxT in buffer from the cationic exchange column, 30% of the drop was 0.1 M Hepes pH 7.5 with 10% (w/v) PEG 8,000 (the mother liquor), and 20% of the drop was 36–40% 2-methyl-2,4-pentandiol (MPD) as an additive. ToxT crystals were transferred to a solution containing the mother liquor and 20% ethylene glycol as a cryoprotectant.

X-ray Data Collection. A multiple anomalous dispersion (MAD) dataset from selenomethionine ToxT was collected on X6A in the National Synchrotron Light Source at the Brookhaven National Laboratory, Long Island NY. A high resolution native data was collected on GM/CA-CAT in the Advanced Light Source at Argonne National Laboratory, Argonne IL. Data were indexed with X-ray Detector Software (XDS) (32), solved by Solve/Resolve (33, 34), refined with the Crystallography and NMR System (CNS) (35, 36), and the model was built using WinCoot (27, 28). A Ramachandran plot generated with Procheck (37, 38) shows 99.6% of residues in the most favored or additionally allowed regions and no residues in the disallowed regions.

Fatty Acid Extractions. Fatty acids were extracted from samples of aqueous ToxT by the method of Bligh and Dyer (39). Samples were resuspended in methanol-d₄ and used for NMR spectra. Positive controls of sodium palmitate (Sigma, P9767) and *cis*-palmitoleic acid (Fluka, 76169) were also dissolved in methanol-d₄.

NMR Experiments. All NMR experiments were acquired on a Bruker spectrometer operating at 600 MHz, utilizing a TCI cryoprobe. All data were collected at 25 °C. Spectral assignment utilized chemical shift comparison with values reported in the literature for fatty acids (40) and reference spectra obtained for samples of sodium palmitate and palmitoleic acid in the same experimental conditions. The assignment was confirmed by two dimensional homonuclear NMR experiments Total Correlation Spectroscopy (TOCSY) (41), mixing times of 60 and 120 ms, and Nuclear Overhauser Enhancement Spectroscopy (NOESY) (42), mixing time of 200 ms), and heteronuclear ¹H-¹³C HMQC (43) and HMBC (44) experiments.

Electrophoretic Mobility Shift Assays. Single-stranded, forty base-pair complementary oligos (Operon) from the *tcp* promoter (5'GTGTTATTAAAAAATAAAAAACACAGCAAAAAATGACA) were end labeled with a biotin-conjugated dUTP using the Biotin 3' End Labeling Kit (Pierce) following the manufacturer's instructions and then annealed to form double-stranded fragments. EMSA's were carried out using the LightShift Chemiluminescent EMSA Kit (Pierce) following the manufacturer's instructions. Briefly, 2.5 picomole (pmole), 4 pmole, 5 pmole of ToxT were mixed with 50 femtomole (fmole) of double-stranded labeled DNA in a binding buffer (10 mM Tris pH 7.5, 1 mM EDTA, 100 mM KCl, 5 mM MgCl₂, 1 mM DTT, 0.3 mg mL⁻¹ BSA, 150 μg herring sperm DNA, and 10% glycerol). Fatty acids were dissolved in methanol and added to a final concentration of 0.002% using the same volume of methanol as a control. To show specificity, a 70-fold molar excess of unlabeled double-stranded *tcp* fragment and a 70-fold molar excess of unlabeled nonspecific DNA (42 base pairs) were added as controls. Reactions were then incubated for 30 min at 30 °C and loaded on a 1x Tris Borate EDTA (TBE) 6% polyacrylamide gel at 4 °C then transferred onto a positively charged membrane (Hybond XL, GE Healthcare) and detected by chemiluminescence. An EMSA experiment was conducted using the control reagents from the LightShift Chemiluminescent EMSA Kit following the manufacturer's instructions while adding methanol and 0.02% fatty acids as indicated in Fig. S5.

β -galactosidase Assays. β -galactosidase activity was determined by the method of Miller (45). The *tcp-lacZ* and *ctx-lacZ* strains MBN135 (46) and KSK218 (47) were grown for 18 h in LB media pH 6.5 at 30 °C. Either methanol or the indicated fatty acids were added to 0.02%.

Immunoblot Analysis. Cell extracts from 18 h cultures grown as for the β -galactosidase assays were prepared and analyzed on 16% SDS-polyacrylamide slab gels. Proteins were visualized by transferring to nitrocellulose and probing with antiTcxA antibody (48) using the enhanced chemiluminescence (ECL) detection system (Amersham).

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