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Citation

Shakhnovich, E. A., D. T. Hung, E. Pierson, K. Lee, and J. J. Mekalanos. 2007. "Virstatin Inhibits Dimerization of the Transcriptional Activator ToxT." *Proceedings of the National Academy of Sciences* 104 (7): 2372–77. doi:10.1073/pnas.0611643104.

Published version

<https://doi.org/10.1073/pnas.0611643104>

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Virstatin inhibits dimerization of the transcriptional activator ToxT

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Contributed by John J. Mekalanos, December 28, 2006 (sent for review December 19, 2006)

The development of antimicrobials is critical in this time of increasing antibiotic resistance of most clinically relevant bacteria. To date, all current antibiotics focus on inhibiting crucial enzymatic activities of their protein targets (i.e., trimethoprim for dihydrofolate reductase), thus disrupting *in vitro* essential gene functions. In contrast, we have previously reported the identification of virstatin, a small molecule that inhibits virulence regulation in *Vibrio cholerae*, thereby preventing intestinal colonization in an infant mouse model for cholera. Virstatin prevents expression of the two major *V. cholerae* virulence factors, cholera toxin (CT) and the toxin coregulated pilus, by inhibiting the virulence transcriptional activator ToxT. It has previously been described that the N-terminal domain of ToxT has the ability to form homodimers. We now demonstrate that virstatin inhibits ToxT dimerization, thus demonstrating that it further falls into a unique class of inhibitors that works by disrupting protein-protein interactions, particularly homodimerization. Using virstatin, truncation mutants of ToxT, and a virstatin-resistant mutant, we show that dimerization is required for ToxT activation of the *ctx* promoter. In contrast, ToxT dimerization does not appear to be required at all of the other ToxT-regulated promoters, suggesting multiple mechanisms may exist for its transcriptional activity.

antibiotics | cholera | pharmacology | regulation | virulence

Over the past several decades, much effort has been invested in the elucidation of virulence mechanisms used by many bacterial species to cause disease. These efforts have laid the groundwork for a new class of antibiotics that target regulation of these virulence mechanisms. Several recent reports have described the identification of many such drugs, including inhibitors of type III secretion, quorum sensing, and toxin activity (1–7).

We previously reported the identification of virstatin, a small molecule that inhibits *Vibrio cholerae* virulence regulation (2). *V. cholerae* is a Gram-negative, facultatively anaerobic pathogen that causes the diarrheal disease cholera by elaboration of two major virulence factors, cholera toxin (CT) and the toxin-coregulated pilus (8). In response to unknown stimuli in the host, a transcriptional cascade results in the expression of both virulence factors. The transcriptional factor ToxT is the most downstream regulator, directly activating *ctxAB* and the *tcp* genes. We previously identified ToxT to be the target of virstatin and isolated a virstatin-resistant mutant that contains a single leucine to proline point mutation at position 113 in the N-terminal domain of ToxT.

ToxT is a member of the large AraC/XylS family of transcriptional regulators defined by two conserved helix–turn–helix DNA-binding domains (9). In ToxT, these domains are located in the C-terminal portion of the protein and have been defined as the domains critical for both binding and activation of transcription by ToxT (10). The N-terminal portion of AraC/XylS transcriptional regulators displays a large amount of sequence diversity; however, it functions in oligomerization and binding of cofactors in some members of the family (11, 12). By analogy, it has long been

speculated that the N-terminal domain of ToxT may result in dimerization as a prerequisite to activity. Prouty *et al.* (13) recently demonstrated the ability of the N-terminal domain to dimerize in heterologous systems; however, Withey *et al.* (14) raised the possibility that ToxT can bind and activate transcription as a monomer based on mapping of putative ToxT-binding sites at various ToxT-regulated promoters.

Based on the location of the mutation in the virstatin-resistant mutant of ToxT (L113P), we hypothesized that virstatin may inhibit dimerization of ToxT. In this study, we demonstrate that there is a direct correlation between the activity of ToxT at the *ctx* promoter and its ability to dimerize as determined by studies with ToxT truncation mutants. Furthermore, we show that virstatin inhibits dimerization of the N-terminal domains in a bacterial-2-hybrid system and by gel filtration chromatography. Finally, we use virstatin as a tool to regulate dimerization of ToxT to identify differences in the mechanism of ToxT at the various promoters that cannot be explained solely by the location and orientation of the ToxT-binding sites.

Results

The Transcriptional Activity of ToxT at the *ctx* Promoter Requires Amino Acids 10–276. To characterize the N-terminal domain of ToxT with respect to activity, we determined the minimal sequence necessary for activation of the *ctx* promoter. We constructed truncation mutants of the ToxT N terminus, deleting the first five amino acids as well as the first nine amino acids and examined the ability of these mutants to complement a *toxT* deletion in *V. cholerae* classical strain O395. We induced expression of ToxT variants from a plasmid as C-terminal His₆-tag fusions in strain O395Δ*toxT* and assayed CT production by CT ELISA.

Expression of full-length ToxT_{WT} (aa_{1–276}), virstatin-resistant mutant ToxT_{L113P} (aa_{1–276}), and truncation mutant ToxT_{WT6} (aa_{6–276}) produced similar quantities of CT, whereas expression of truncation mutant ToxT_{WT10} (aa_{10–276}) did not complement the *toxT* deletion (Fig. 1A). Expression of all constructs was confirmed to be equal by Western blot analysis by using an anti-His antibody. These results suggest that the N-terminal amino acids 6–9 are necessary for ToxT activation of the *ctx* promoter.

The N-Terminal Domain of ToxT Is Able to Dimerize. On the basis of an analogy to other AraC/XylS family transcriptional regulators, it has been proposed that dimeric ToxT is required to activate

Author contributions: E.A.S. and D.T.H. contributed equally to this work; J.J.M. designed research; and E.A.S., D.T.H., E.P., and K.L. performed research.

The authors declare no conflict of interest.

Abbreviations: CT, cholera toxin; Zif, zinc-finger DNA-binding protein; MBP, maltose-binding protein.

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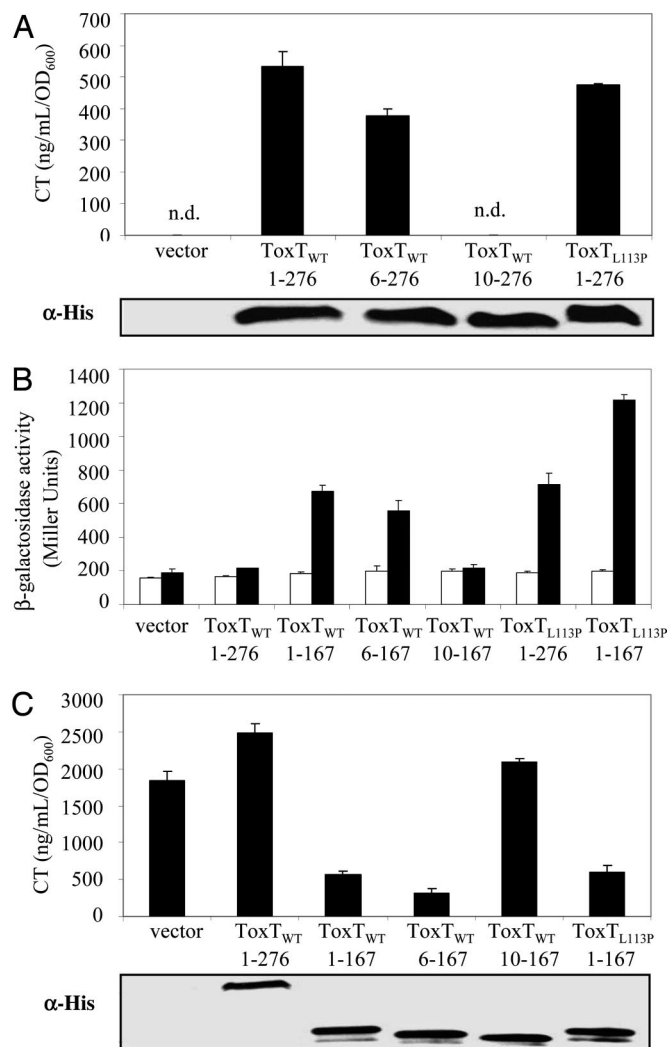


Fig. 1. The N-terminal domain of ToxT dimerizes. (A) (Upper) Activity of ToxT at the *ctx* promoter when it is expressed from a plasmid in O395 Δ *toxT* as measured by CT ELISA. ToxT_{WT6} (aa_{6–276}) is active, whereas ToxT_{WT10} (aa_{10–267}) does not complement the *toxT* deletion. Full-length ToxT is 276 aa. (Lower) Western blot with α -His demonstrates that all portions of ToxT were expressed to equal levels. (B) Portions of, but not full-length, ToxT drive a protein–protein interaction in a bacterial two-hybrid system in *E. coli* when induced with IPTG as measured by β -galactosidase assay. Truncation of the first five amino acids allows *lacZ* transcription, whereas truncation of the first nine abolishes activity. Full-length and N-terminal portion of ToxT_{L113P} point mutant (2) are able to dimerize in this system. Vector control is a transformant containing empty pActRap-Zif and pBRG_{pw} plasmids. White, no IPTG; black, 10 μ M IPTG. (C) (Upper) Only N-terminal portions of ToxT that can induce a protein–protein interaction can exert a dominant negative effect on CT production. ToxT truncations were expressed from a plasmid in wild-type O395. (Lower) Western blot with α -His demonstrates that all N-terminal portions were expressed to equal levels.

transcription and that the capacity to form dimers is encoded in the N-terminal domain (13). Using a bacterial two-hybrid system in *Escherichia coli*, we confirmed the ability of the N-terminal domain to dimerize. We constructed various translational fusions of ToxT with a zinc-finger DNA-binding protein (Zif) and with the ω subunit of the *E. coli* RNA polymerase. The ability of the various ToxT variants to recruit RNA polymerase to Zif was assayed by using the transcriptional *lacZ* reporter gene (15).

The N-terminal domain of ToxT (aa₁₋₁₆₇, chosen based on an analogy to the N-terminal domain of AraC), when fused to both Zif and the ω subunit of the *E. coli* RNA polymerase, activated

lacZ transcription strongly, thus demonstrating its ability to homodimerize (Fig. 1B). We also examined ToxT_{L113P}, previously identified in a screen for virastatin-resistant ToxT mutants (2), in the two-hybrid system and discovered that its N-terminal domain (aa₁₋₁₆₇) also homodimerized and, in fact, resulted in increased β -galactosidase relative to wild-type. Translational fusions of full-length ToxT_{WT} were unable to dimerize, whereas the full-length ToxT_{L113P} dimerized effectively although not as well as its N-terminal domain alone. The fact that full-length ToxT_{L113P} dimerizes but not ToxT_{WT} suggests that the conformations of the fusion proteins differ. It seems less likely that the inability of the full-length wild-type fusion to dimerize is an artifact of the two-hybrid system. It is possible that a conformational change may be required for full-length ToxT to dimerize after some activation step such as binding a small molecule activator. Even in the absence of an inducer, ToxT_{L113P} may be in a conformation more conducive to dimerization than ToxT_{WT}, although the increased β -galactosidase activity of the ToxT_{L113P} N-terminal domain over full-length suggests that a small conformational change may still be necessary.

Finally, to examine the sequence requirements for transcriptional activity in *V. cholerae* compared with those for dimerization, we tested the ability of the truncation mutants in both the N-terminal and C-terminal ends to activate transcription in the two-hybrid system. Truncation of ToxT from the C-terminal end before amino acid 167 results in a gradual loss of dimerization ability (data not shown). Although truncating the first five amino acids at the N terminus (aa₆₋₁₆₇) led to only a slight decrease in β -galactosidase activity, truncating the first nine amino acids (aa₁₀₋₁₆₇) completely abolished the interaction (Fig. 1B). These results directly correlate with those obtained in the CT ELISA for ToxT activity. Thus, the ability of the N-terminal domain of ToxT to dimerize in the two-hybrid system requires the same amino acids (6–9) that are required for ToxT activation of the *ctx* promoter in *V. cholerae*.

The N-Terminal Domain of ToxT Behaves as a Dominant Negative for Cholera Toxin Expression. Having demonstrated the ability of the N-terminal domain of ToxT to form homodimers in a heterologous system, we examined the ability of the N-terminal domain of ToxT to behave as a dominant negative in *V. cholerae* strain O395. We expressed various ToxT mutants from a plasmid in O395 and measured activity by CT ELISA. Although expression of the empty plasmid or full-length ToxT resulted in normal CT production, expression of the N-terminal domains of both ToxT_{WT} and the ToxT_{L113P} mutant significantly reduced CT production (Fig. 1C). This result demonstrates that the N-terminal domain is able to form a heterodimer with and sequester the wild-type, full-length ToxT expressed by O395 and thus prevent normal transcriptional activation.

In concordance with the observations made in the bacterial-2-hybrid system, expression of the aa₆₋₁₆₇ fragment of ToxT produced the dominant negative effect, whereas expression of the aa₁₀₋₁₆₇ fragment had no effect on CT production. Again, the same amino acids (6-9) that were required for dimerization in our heterologous *E. coli* system and the activation of *ctx* transcription in *V. cholerae* are also required for the dominant negative effect in *V. cholerae*. We confirmed that all N-terminal constructs were expressed equally by performing Western blot analysis against a C-terminal His₆-tag (Fig. 1C).

Virstatin Inhibits the Ability of ToxT to Form Dimers. As a result of the position of the virstatin-resistant mutation at position 113 in the N-terminal domain, we hypothesized that virstatin may inhibit the dimerization of ToxT. We adapted the bacterial-2-hybrid system to test the effect of virstatin on dimerization by deleting *tolC* in the *E. coli* reporter strain KDZif1Z to increase the cellular permeability of virstatin. We compared the effects

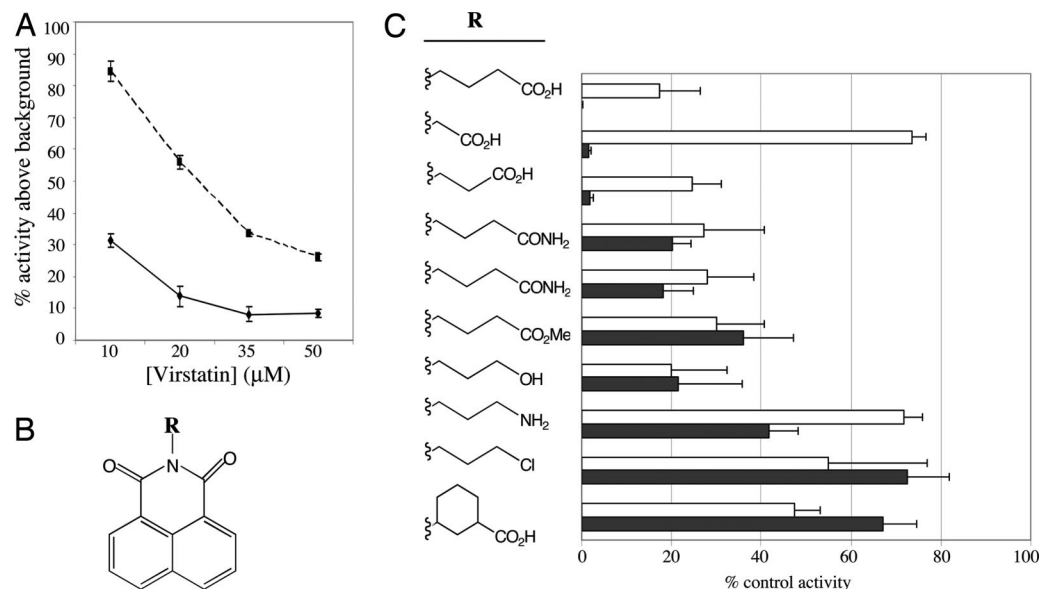


Fig. 2. (A) Virstatin inhibits ToxT dimerization in bacterial two-hybrid system in *E. coli*. The addition of increasing concentrations of virstatin inhibited the protein–protein interaction of the ToxT N terminus with itself as measured by β -galactosidase assay. Point mutant ToxT_{L113P} was more resistant to virstatin. Activity is presented as a percentage of reporter activity above background in the presence of virstatin compared with no virstatin. Solid line, ToxT; dashed line, ToxT_{L113P}. (B) Chemical structure of virstatin base with the R group labeled in bold. (C) Inhibition of ToxT dimerization in the bacterial two-hybrid system and CT production in *V. cholerae* by analogs of virstatin is correlated. Each compound is designated by its R group and inhibition is graphed as the percentage of control activity. Virstatin is the first compound listed. White, β -galactosidase production; black, CT.

of virstatin on dimerization of the N-terminal domains of ToxT_{WT} and ToxT_{L113P}. KDZif1ZΔ*tolC* carrying plasmids that express N-terminal domain fusions of either ToxT_{WT} or the ToxT_{L113P} mutant were grown overnight in the presence of varying concentrations of virstatin. These samples were then subcultured into media containing virstatin and IPTG to induce expression of the chimeric proteins and assayed for ability to induce expression of the β -galactosidase reporter.

Virstatin inhibited dimerization of the ToxT_{WT} N-terminal domain fusion even at 10 μ M, and at higher concentrations (20, 35, and 50 μ M), it reduced transcriptional activity to baseline levels (Fig. 2A). In contrast, the ToxT_{L113P} N-terminal domain fusion was relatively more resistant to virstatin than wild-type. At 10 μ M, virstatin had no effect on the amount of β -galactosidase activity of the ToxT_{L113P} mutant. Even at higher concentrations, virstatin only partially reduced the β -galactosidase activity of the mutant. Because the mutant also appears to dimerize more efficiently than wild-type, this stronger interaction could compete out virstatin binding, thus resulting in its relative resistance to virstatin. These results further demonstrate a strong correlation between the ability to dimerize and the previously reported transcriptional activity of ToxT_{WT} and ToxT_{L113P} in the presence and absence of virstatin.

To further define the relationship among virstatin, ToxT dimerization, and ToxT transcriptional activity, we analyzed the ability of several virstatin structural variants to inhibit dimerization and ToxT activity at the *ctx* promoter. Reporter strains KDZif1ZΔ*tolC* containing wild-type N-terminal domain fusions were grown in the presence or absence of 50 μ M virstatin or its structural variants and assayed for β -galactosidase activity. The amount of N-terminal domain dimerization in the presence and absence of the structural variants correlates for the most part with the transcriptional activity of ToxT in the presence of 20 μ M virstatin and its variants as assayed by CT ELISA (Fig. 2B). A few virstatin variants (e.g., the carboxylate series) showed more activity in inhibiting ToxT-mediated transcription in *V. cholerae* than in blocking ToxT dimerization in the *E. coli*-based two-hybrid system; however, these exceptions may be the result of

differences in the permeability or metabolism of molecules with different chemical structures in the two different bacterial species.

Virstatin Favors the Monomeric Form of ToxT. We determined the oligomeric state of ToxT in the presence and absence of virstatin by gel filtration chromatography. We constructed a 74-kDa fusion protein of an N-terminal maltose-binding protein (MBP) and a C-terminally His₆-tagged ToxT_{WT} or ToxT_{L113P} (Fig. 3A). The fusion was more soluble than ToxT alone. Both the ToxT_{WT} and ToxT_{L113P} fusions complemented a *toxT* deletion in *V. cholerae* (O395Δ*toxT*) as well as ToxT wild-type and could be inhibited by virstatin as shown by CT ELISA (Fig. 3B).

The wild-type and mutant fusions were expressed in O395 from a plasmid driven by a *P*_{tac}-inducible promoter and induced with IPTG in the presence or absence of virstatin. All subsequent purification steps were carried out in the presence or absence of virstatin (100 μ M) corresponding to their respective expression conditions. The ToxT fusions were purified from O395 on a Ni²⁺ column and protein concentrations of the eluted proteins were normalized by Bradford assay. The Ni²⁺ purified fusion proteins were then run on a Superdex 75 10/300 column (Amersham, Biosciences, Piscataway, NJ). The amount of the MBP–ToxT–His₆ fusion in each fraction was assayed by sandwich ELISA with a Ni²⁺-coated plate and an α -MBP antibody. We also performed Western blot analysis of individual fractions by using an α -MBP antibody that demonstrated an isolated 74-kDa band at varying molecular weights suggestive of varying oligomerization states.

Both ToxT_{WT} and ToxT_{L113P} fusion proteins expressed and isolated in the absence of virstatin ran at relatively high molecular weights by gel filtration, likely representing higher oligomers. SDS/PAGE and Western analysis confirmed that these higher molecular weight fractions in fact consisted of the 74-kDa ToxT fusion. In contrast, the ToxT_{WT} fusion protein in the presence of virstatin (100 μ M) resulted in an $\approx$ 74-kDa peak corresponding to the monomer. Only a very minor peak at $\approx$ 74 kDa was produced with the ToxT_{L113P} fusion protein in the

Materials and Methods

Bacterial Strains and Plasmids. Classical biotype strain O395 was used in all experiments. Virulence-inducing growth conditions were obtained by 1:1,000 dilution of an overnight culture into LB pH 6.5 and growth at 30°C shaking 260 rpm (Gyratory Shaker; New Brunswick Scientific, Edison, NJ). The method of Skorupski and Taylor (22) was used to construct in-frame deletions of all mutants in *V. cholerae*. Laboratory stocks of *E. coli* DH5 α pir and SM10 λ pir were used for cloning and mating into *V. cholerae*, respectively.

Bacterial Two-Hybrid System. Experiments were carried out as described (15). Plasmids and strains were a gift of Dr. Simon Dove. Briefly, identical portions of *toxT* were cloned between the NdeI and NotI sites of pACTrap-Zif and pBRGp ω . All cloning was done in strain DH5 α F'IQ. The constructs were then co-transformed into the *E. coli* strain KDZif1Z Δ tolC. Cultures were grown overnight in the absence or presence of 10 μ M IPTG and/or 50 μ M virstatin and subcultured into identical conditions in the morning. Activation of the reporter was assayed in triplicate by β -galactosidase assay. A representative experiment is shown.

Detection of Cholera Toxin Expression in O395/O395 Δ *toxT* Expressing ToxT Variants Under Control of the Heterologous pBAD Promoter. All portions of *toxT* were C-terminally His₆-tagged and cloned into pBAD18. The resulting plasmids were transformed into O395 (dominant negative experiments) or O395 Δ *toxT* (complementation experiments) (23). The strains were inoculated into LB pH 6.5 and grown at 30°C shaking in the presence of either 0.01% (dominant negative experiments) or 0.005% (complementation experiments) arabinose and GM1 ganglioside enzyme-linked immunosorbent CT assays (24) were performed as previously described on the resulting supernatants. CT expression was normalized for OD₆₀₀ of the growing culture and is the result of samples done at least in triplicate. A representative experiment is shown.

α -His Western Detection of ToxT Variants. Cells were grown in LB at 37°C shaking overnight in the presence of 0.1% arabinose. Cell extracts were subjected to SDS/PAGE, transferred to PVDF membrane, probed with anti-tetra-His antibody (Qiagen, Valencia, CA), and visualized by enhanced chemiluminescence (Amersham Pharmacia).

Virstatin Structural Variant Synthesis. A typical procedure for the synthesis of the virstatin analogs is as follows (25). A mixture of 1,8-naphthalic anhydride (9.7 mmol) and the functionalized amines (9.7 mmol) in DMF (25 ml) was heated at 120°C for 2–6 h. On completion of the reaction (by TLC), the reaction mixture was cooled to room temperature and a half of DMF was evaporated *in vacuo*; then Et₂O (50 ml) was added, resulting in

a white precipitate, which was collected by filtration. The crude product was recrystallized from EtOAc to afford the desired virstatin analogs (47–93% yield).

Gel Filtration Studies. To construct the MBP–ToxT fusion proteins, full-length (aa_{1–276}) *toxT*_{WT} and *toxT*_{L113P} were C-terminally His₆-tagged and cloned between XbaI and PstI of pMALc2x (Invitrogen, Carlsbad, CA). The resulting plasmids were transformed into O395 Δ *toxT*. An overnight culture of the ToxT fusions (wild-type and L113P mutant) was inoculated at a 1:500 dilution into 500 ml of LB in the presence or absence of 100 μ M virstatin. The cultures were grown with shaking at 37°C for 4 h and then cooled to 18°C. The cultures were induced with IPTG (0.5 mM) and grown for 12 h at 18°C with shaking. The cultures were pelleted by centrifugation, resuspended in 10 ml of lysis buffer (500 mM NaCl/50 mM Tris, pH 7.5/15 mM imidazole/10% glycerol/40 μ g/ml lysozyme/1 mM PMSF/protease inhibitors/2 mM β -mercaptoethanol) with or without 100 μ M virstatin, and flash-frozen at –80°C overnight. Extracts were thawed, sonicated, and pelleted at 12,000 rpm (Sorvall SS34 rotor; Thermo Fisher Scientific, Waltham, MA) for 30 min. The fusion proteins were purified over a nickel column (Invitrogen) and eluted with 250 mM imidazole with or without 100 μ M virstatin. The purity of the proteins was confirmed by SDS/PAGE electrophoresis with Coomassie staining and Western blot analysis by using an α -MBP antibody (Invitrogen). The amounts of each fusion were normalized by using the Bradford assay (Bio-Rad, Hercules, CA). The aggregation states of the fusions were analyzed by gel filtration chromatography on a Superdex 75 10/300 column (Amersham).

The amount of fusion protein in each 1-ml fraction was determined by sandwich ELISA by using Ni-coated 96-well plates and an α -MBP primary antibody (Invitrogen), followed by an alkaline phosphatase conjugated goat-anti-rabbit secondary antibody. Western blot analysis was also performed on each of the 1-ml fractions by using an α -MBP antibody.

Transcriptional Reporter Assays. Transcriptional fusions of *tcpA*, *tcpI*, *acfD*, *acfA*, *tagA*, and *aldA* promoters to *lacZ* in pTL61T were a gift of J. Withey and V. DiRita (University of Michigan Medical School, Ann Arbor, MI). The *ctx* reporter was constructed by cloning a 500-bp region upstream of *ctxA* from O395 between the HindIII and XbaI sites of pTL61T. All plasmids were transformed into O395 Δ *lacZ*. The strains were inoculated into LB (pH 6.5) and grown at 30°C shaking overnight in the presence or absence of 100 μ M virstatin. The resulting cultures were assayed in triplicate by β -galactosidase assay. A representative experiment is shown.

E.A.S. was supported by a National Science Foundation predoctoral fellowship. D.T.H. was supported by National Institutes of Health (NIH) Grant K08 AI060708-01. This work was supported by NIH Grant AI26289 (to J.J.M.).

- Hentz MWH, Andersen JB, Riedel K, Rasmussen TB, Bagge N, Kumar N, Schembri MA, Song Z, Kristoffersen P, Manefield M, et al. (2003) *EMBO J* 22:3803–3815.
- Hung DT, Shakhnovich EA, Pierson E, Mekalanos JJ (2005) *Science* 310:670–674.
- Nordfelth R, Kauppi AM, Norberg HA, Wolf-Watz H, Elofsson M, Hagglund U, Uvell H (2005) *Infect Immun* 73:3104–3114.
- Panchal RG, Hermone AR, Nguyen TL, Wong TY, Schwarzenbacher R, Schmidt J, Lane D, McGrath C, Turk BE, Burnett J, et al. (2004) *Nat Struct Mol Biol* 11:67–72.
- Roychoudhury SZ, NA, Ninfa AJ, Allen NE, Jungheim LN, Nicas TI, Chakrabarty AM (1993) *Proc Natl Acad Sci USA* 90:965–969.
- Wright JJR, III, Novick RP (2005) *Proc Natl Acad Sci USA* 102:1691–1696.
- Muschol S, Bailey L, Gylfe A, Sundin C, Hultenby K, Bergstrom S, Elofsson M, Wolf-Watz H, Normark S, Henriques-Normark B (2006) *Proc Natl Acad Sci USA* 103:14566–14571.
- Waldor MM, Mekalanos JJ (1996) in *Enteric Infections and Immunity*, ed Paradise L (Plenum, New York), pp 37–55.
- Higgins DE, Nazareno E, DiRita VJ (1992) *J Bacteriol* 174:6974–6980.
- Hulbert RR, Taylor RK (2002) *J Bacteriol* 184:5533–5544.
- Gallegos MT, Schleif R, Bairoch A, Hofmann K, Ramos JL (1997) *Microbiol Mol Biol Rev* 61:393–410.
- Martin RG, Rosner JL (2001) *Curr Opin Microbiol* 4:132–137.
- Prouty MG, Osorio CR, Klose KE (2005) *Mol Microbiol* 58:1143–1156.
- Withey JH, DiRita VJ (2006) *Mol Microbiol* 59:1779–1789.
- Vallet-Gely I, Donovan KE, Fang R, Joung JK, Dove SL (2005) *Proc Natl Acad Sci USA* 102:11082–11087.
- Yu RR, DiRita VJ (2002) *Mol Microbiol* 43:119–134.
- Withey JH, DiRita VJ (2005) *J Bacteriol* 187:7890–7900.
- Menon KP, Lee NL (1990) *Proc Natl Acad Sci USA* 87:3708–3712.
- Fry DC (2006) *Biopolymers* 84:535–552.
- Arkin MR, Wells JA (2004) *Nat Rev Drug Discov* 3:301–317.
- Toogood PL (2002) *J Med Chem* 45:1543–1558.
- Skorupski K, Taylor RK (1996) *Gene* 169:47–52.
- Hase CC, Mekalanos JJ (1998) *Proc Natl Acad Sci USA* 95:730–734.
- Gardel CL, Mekalanos JJ (1994) *Methods Enzymol* 235:517–526.
- Adam W, Beck AK, Pichota A, Saha-Moller CR, Seebach D, Vogl N, Zhang R (2003) *Tetrahedron Asymmetry* 14:1355–1361.