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Direct Regulation by the *Vibrio cholerae* Regulator ToxT To Modulate Colonization and Anticolonization Pilus Expression▽

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The pathogen *Vibrio cholerae* uses a large number of coordinated transcriptional regulatory events to transition from its environmental reservoir to the host and establish itself at its preferred colonization site at the host intestinal mucosa. The key regulator in this process is the AraC/XylS family transcription factor, ToxT, which plays critical roles in pathogenesis, including the regulation of two type IV pili, the anticolonization factor mannose-sensitive hemagglutinin and the toxin-coregulated pilus. Previously, it was thought ToxT required dimerization in order to effect transcriptional regulation at its cognate promoters. Here, we present evidence that ToxT directly represses transcription of the *msh* operon by binding to three promoters within this operon and that dimerization may not be required for transcriptional repression of target promoters by ToxT, suggesting that this regulator uses different mechanisms to modulate the transcriptional repertoire of *V. cholerae*.

Vibrio cholerae is a gram-negative bacterium normally found in aquatic reservoirs. It is also the etiologic agent of cholera, a severe dehydrating diarrhea that affects hundreds of thousands of people each year. Cholera is characterized by voluminous diarrhea caused by the action of the cholera toxin (CT), produced by vibrios colonizing the mucosa of the small intestine. The transitions between the stages of the infectious cycle of this pathogen, between colonization, toxin production, and dissemination back into the aquatic reservoir, is governed by a transcriptional cascade that regulates the production of virulence factors such as CT and the biogenesis of the colonization determinant the toxin-coregulated pilus (TCP). In addition to these provirulence factors, some elements, including the mannose-sensitive hemagglutinin (MSHA) pilus and quorum-sensing systems, which as "anticolonization factors" can have a negative influence on pathogenesis, are downregulated during infection (12, 16).

The keystone of the transcriptional regulatory system that governs the phenotypic shifts in this transition from aquatic organism to human pathogen is the AraC/XylS family transcription factor ToxT, which is the major regulator of pathogenesis activated in response to entry into the intestine (5, 11). At the end of this cascade are the biogenesis systems for two related but functionally antagonistic type IV pili, the TCP, and MSHA pili. The tight regulation of pili biogenesis is critical for *V. cholerae* infection, as TCP is necessary for colonization of the host intestinal mucosa (10, 26). The presence of MSHA pili, on the other hand, is actively detrimental to survival in the host, since it binds secretory immunoglobulin A antibodies that, synergistically with the mucin glycocalyx of the intestinal mucosa (17), excludes pathogens such as *V. cholerae* from the epithelium. We have previously demonstrated that ToxT plays an important part in *V. cholerae* fitness in infection by resolving the conflicting pressures of these two type IV pili functions by downregulating the production of MSHA, which if present act as potent anticolonization factors (12). In addition, ToxT activates elements of the TCP biogenesis pathway, including the prepilin leader peptidase TcpJ, which then posttranslationally degrades MshA prepilin, further suppressing MSHA function during infection (13). Although the mechanism of ToxT-mediated transcriptional activation has been extensively studied, very little is known about the requirements for anticolonization factor suppression, the latest documented role played by this versatile virulence regulator in *V. cholerae*.

In the present study, we demonstrate that ToxT directly regulates the expression of the MSHA biosynthesis gene cluster (*msh* genes) by binding to three different promoters within the locus. Interestingly, ToxT is able to repress the transcription of *msh* genes in the presence of the ToxT-dimerization inhibitor virstatin. Repression is maintained even by a truncated ToxT variant completely lacking the N-terminal dimerization/environmental sensing domain. Both virstatin and dimerization domain deletion abolish transcriptional activation at the TCP operon *tcpA* promoter, suggesting that ToxT uses different mechanisms in the transcriptional regulation of pro- and anticolonization factors.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *Escherichia coli* and *V. cholerae* strains were all propagated in LB with appropriate antibiotics at 37°C unless otherwise stated. To induce virulence gene expression in *V. cholerae*, cells were propagated in AKI media and growth conditions (15). All plasmids and

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oligonucleotides used in the present study are available upon request. The virstatin concentration used here was 100 μM.

ToxT mutant screens. pBAD-toxT plasmid (9) was transformed into the mutator XL1-Red *E. coli* by electroporation according to the manufacturer's instructions (Stratagene). Pools were then made of transformants, and plasmids were purified from each pool for transformation into a P_{tcpA}-*lacZ E. coli* reporter strain. These were then plated on selective minimal agar containing triphenyltetrazolium chloride (TTC) to a final concentration of 0.0025% and lactose as a carbon source. Lac^- colonies were then identified, and plasmids were extracted and transformed individually into *E. coli* containing a P_{mshH}-*lacZ* plasmid. These cells were then plated again on TTC-lactose agar, and Lac^- bacteria were identified. pBAD-toxT mutants that could not activate P_{tcpA} but could still repress P_{mshH} were then isolated and sequenced, and their activity was confirmed in P_{tcpA} and P_{mshH} reporter strains by β-galactosidase activity assays (20).

Lux activity assays. For Lux reporter assays, cells grown to late logarithmic phase and then withdrawn were normalized for growth using the optical density at 600 nm (OD_{600}), and the luminescence was determined by using a Bio-Tek Synergy HT spectrophotometer. Lux expression is reported as light units/OD_{600}.

Gel retardation assays. *E. coli* cells containing a plasmid overexpressing maltose-binding protein fused to the N terminus of ToxT (MBP-ToxT) were grown at 37°C and induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at 16°C. MBP-ToxT protein was purified through amylose columns according to the manufacturer's instructions (New England Biolabs). PCR products of ~400 bp containing *mshH*, *mshI*, *mshB*, and *tcpA* promoter regions were digested with EcoRI and end labeled using [α-^{32}P]dATP and the Klenow fragment of DNA polymerase I. Binding reactions contained 0.1 ng of DNA and MBP-ToxT proteins in a buffer consisting of 10 mM Tris-HCl (pH 7.9), 1 mM EDTA, 1 mM dithiothreitol, 60 mM KCl, and 30 mg of calf thymus DNA/ml. After 20 min of incubation at 37°C, samples were size fractionated using 5% polyacrylamide gels in 0.5× TAE buffer (20 mM Tris-acetate, 1 mM EDTA; pH 8.5). Where indicated, the competitive nonradiolabeled PCR products (1 μg) were included in the reaction. The radioactivity of free DNA and ToxT-DNA complexes was visualized by using a Storm B840 PhosphorImager (Molecular Dynamics).

Hemagglutination assays. *V. cholerae* mannose-sensitive hemagglutination was assayed as described previously (6). Briefly, defibrinated sheep erythrocytes washed in phosphate-buffered saline (PBS) were resuspended in Krebs-Ringer Tris (KRT) buffer (7.5 g of NaCl, 0.383 g of KCl, 0.318 g of $MgSO_4 \cdot 7H_2O$, and 0.305 g of $CaCl_2$ in 1 liter of distilled H_2O) to a final concentration of 10% (vol/vol). Erythrocyte suspensions were then mixed with mid-logarithmic-phase cultures of *V. cholerae* that had been serially diluted in KRT buffer. Samples checked for hemagglutination after 2 h.

Western blotting for TcpA and MshA. Relevant *V. cholerae* strains were grown under inducing AKI and noninducing LB conditions at 37°C as previously described (13). Cultures were then normalized by using the OD_{600}, and proteins were fractionated by size using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were then transferred to a nitrocellulose membrane and immunoblotted using polyclonal rabbit αTcpA antibody or αMshA antibody.

RESULTS AND DISCUSSION

Identification of ToxT regulatory sites at the *msh* locus. ToxT is the central regulator of a well-studied transcriptional activation cascade whereby environmental cues are transmitted by the TcpPH and ToxRS membrane complexes to activate *toxT* transcription (3–5, 9, 10, 19, 21). ToxT is then produced and is able to activate transcription of the *ctxAB* genes responsible for CT production and the *tcp* genes responsible for TCP biogenesis, as well as the genes responsible for accessory colonization factors, while at the same time repressing the transcription of the anticolonization MSHA pilus (5, 12, 30). ToxT comprises two domains: a C-terminal DNA-binding domain and an N-terminal domain that has been implicated in dimerization and sensing on environmental stimuli (11, 23). Several studies have examined the mechanism of transcriptional activation by ToxT. ToxT is able to mediate transcriptional activation at some target promoters, such as that for the *tcp* operon, by binding individually at AT-rich "toxbox" motifs (29). Interactions between the N-terminal domains of ToxT are required for efficient activation of many virulence genes in the ToxT regulon; chimeric proteins consisting of ToxT DNA-binding domains fused to heterologous dimerization domains, as well as those with the ToxT dimerization domain fused to different DNA-binding domains, are able to bind the DNA corresponding to the appropriate DNA-binding domain but not the individual truncated forms (23). However, little is known about the requirements for ToxT-mediated repression of *msh* genes, the only set of genes ToxT is currently known to transcriptionally repress.

Previously, we have shown that ToxT represses a transcriptional *msh-lacZ* fusion in *E. coli*, suggesting that ToxT may directly regulate the expression of *msh* operon. To further study the relationship between ToxT and *msh* gene expression, we searched for sequences homologous to previously identified ToxT binding sites in the *msh* locus (Fig. 1A) by using the PATSER program (27), taking into account known toxbox consensus sequences, as well as published results of mutagenesis of nucleotides within them (29). We identified potential ToxT binding sites in the *mshI* promoter and *mshB* promoters (Fig. 1A), which were reported previously as regulating *msh* gene expression (18). The putative ToxT binding sites of *mshI* and *mshB* promoters are similar to the toxbox sequences in the *aldA* and *acfD* promoters, respectively (29). In addition, we also identified tandem ToxT binding sites located upstream of *mshH* similar to the binding sites upstream of *tcpA* (Fig. 1A). Although *mshH* gene product may not be required for MSHA synthesis (18), it is predicted to form an operon with the downstream *msh* genes (22).

To test whether ToxT regulates these promoters containing putative ToxT binding sites, we constructed transcriptional Lux fusions to *mshH*, *mshI*, and *mshB* promoters and tested the effect of ToxT on Lux expression in a heterologous *E. coli* system. Figure 1A shows that ToxT was able to repress all of these promoters, suggesting that ToxT is capable of direct regulation at these sites. We then examined whether purified ToxT could bind to these putative regulatory regions in a gel retardation assay. Purified MBP-ToxT was able to shift DNA fragments containing each of the putative toxbox sequences identified by using PATSER (Fig. 1B), as well as control DNA containing the toxbox found within the *tcpA* promoter. All of these mobility shifts could be abolished by the addition of specific unlabeled DNA, indicating that the binding of ToxT to these DNA sequences is specific. These data demonstrate that ToxT is able to bind several regulatory elements in order to negatively regulate *msh* gene transcription. We currently do not know the exact physiological significance of ToxT binding of three separate promoters within *msh* locus, but it is possible that repression at multiple regulatory regions may be exploited by *V. cholerae* to ensure that no or very little MSHA is produced in the early stage of infection.

Effect of dimerization inhibitors on regulation by ToxT. To examine whether dimerization played a role in the transcriptional regulation of *msh* genes by ToxT, we examined the effects of a known inhibitor of ToxT dimerization, virstatin (14, 24), on the transcription of *msh* genes in *E. coli* (Fig. 1A). Using Lux transcriptional fusion reporters (see Fig. 1A), we observed that P_{mshH}-*lux* was repressed and that P_{tcpA}-*lux* was activated when *toxT* was expressed via an arabinose-inducible P_{BAD} promoter on a plasmid (pBAD-toxT) (9) (Fig. 2A). Re-

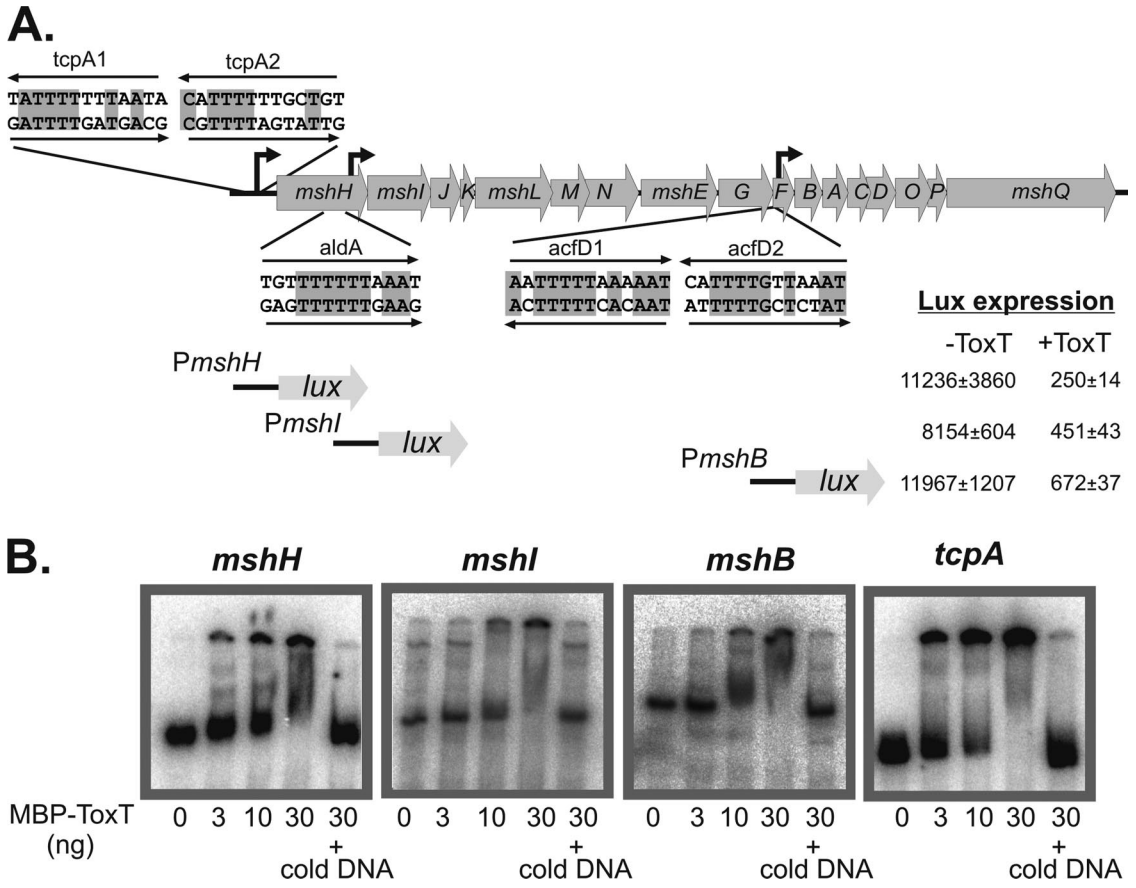

FIG. 1. ToxT binds to multiple toxbox-like sequences to regulate the *msh* gene expression. (A) *msh* locus and toxbox binding site sequences in the three promoters were identified using PATSER. Labels identify similar toxbox binding sites from other ToxT-regulated promoters (indicated in the upper strand). The binding site sequence of the corresponding *msh* promoter is given in the lower strand. The orientation of each is represented by the corresponding arrows. Shaded boxes correspond to identical base-pairs. Three promoter regions indicated were PCR amplified and cloned into pBBRlux containing a transcriptional Lux reporter (8). The Lux expression with pBAD24 (7) or with pBAD-toxT (9) in the presence of 0.1% arabinose is given in the table. Units are arbitrary light units/OD_{600}. The results represent the average of three experiments ± the standard deviation (SD). (B) Gel shift assays using purified MBP-ToxT and DNA containing ~400 bp of the regulatory regions identified in *msh* genes and *tcpA* promoter.

pression of P_{mshH}-*lux* was maintained upon the addition of the ToxT dimerization inhibitor virstatin, but P_{tcpA}-*lux* activity was completely abrogated in the presence of virstatin. The virstatin effects on expression of *mshI* and *mshB* were similar to that of *mshH* (data not shown).

ToxT represses the transcription of *msh* genes in *V. cholerae* after growth under *toxT*-inducing AKI conditions (15). To examine the effects of virstatin on ToxT-mediated regulation in *V. cholerae*, we immunoblotted for major pilin subunits TcpA and MshA using wild-type *V. cholerae* and a *toxT* mutant, both grown in AKI (Fig. 2B). After virulence-inducing growth in AKI, wild-type *V. cholerae* activated production of TcpA and repressed production of MshA, while *toxT* mutants continued to express MshA even under *toxT*-inducing conditions. The addition of virstatin led to a loss of detectable TcpA production as reported previously (14, 24) but had no effect on ToxT repression of MshA production, suggesting that ToxT does not require dimerization in order to mediate transcriptional repression. Moreover, MSHA-dependent *V. cholerae* hemagglutination was not affected by growth with virstatin (Table 1).

Mutagenesis of ToxT domains necessary for transcriptional regulation. To examine which regions of the ToxT protein were important for mediating transcriptional activation and repression, we generated a library of mutagenized *toxT* by passage of pBAD-toxT in *E. coli* XL1-Red mutator cells. We then screened for ToxT mutants that could no longer activate transcription but could still act as a repressor. Briefly, mutated pBAD-toxT plasmids were isolated from XL1-Red and used to transform an *E. coli* MC4100 containing a P_{tcpA}-*lacZ* reporter plasmid. Transformants that caused a Lac^- phenotype after growth on TTC-lactose indicator agar (25) (indicating that they contained mutated ToxT that lost the ability to activate the expression of *tcpA*) were then selected, and plasmids were isolated for transformation into P_{mshH}-*lacZ* plasmid reporter-containing *E. coli* (12). Most of colonies were blue on the X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) plates, suggesting that mutations in *toxT* abolished ToxT activity completely. However, we were able to obtain five $LacZ^-$ mutants in which mutated ToxT could still function as an *msh* repressor. The effects of these mutations in *toxT* on transcriptional

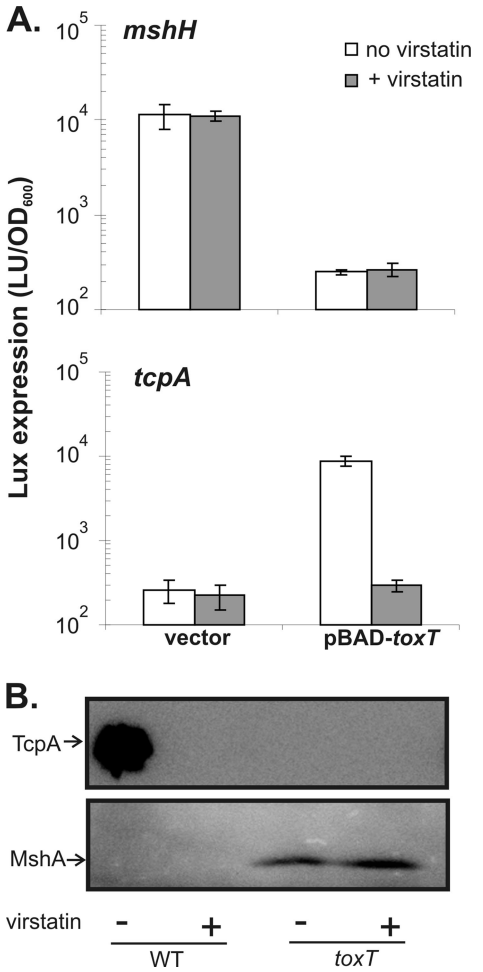

FIG. 2. The inhibitor of ToxT dimerization virstatin has no effect on ToxT regulation of MSHA. (A) Activity of indicated *lux* reporter constructs in *E. coli* containing either pBAD24 (7) as a vector control or pBAD-toxT (9), in the absence (□) or in the presence (▨) of 100 μM virstatin. Arabinose (0.1%) was used to induce P_{BAD} promoters. Units are arbitrary light units/OD_{600}. The results are average of three experiments ± the SD. (B) Western blot for TcpA (top panel) and MshA (bottom panel) from wild-type *V. cholerae* and *toxT* mutants (1) cultured with or without 100 μM virstatin under AKI conditions (15).

regulation were then confirmed by β-galactosidase reporter assays (20; data not shown). Sequence analysis revealed that all five ToxT mutants had an aspartate to glycine mutation at residue 141. This amino acid is within the first N-terminal amino acid region of ToxT that is proposed to be a dimerization domain.

When expressed from a P_{BAD} promoter on a plasmid, $ToxT^{D141G}$ was able to still repress *mshH*, *mshI*, and *mshB* promoter activities in *E. coli* (Fig. 3B and data not shown) and to repress MshA protein synthesis (Fig. 3C) and MSHA production (Table 1) in *V. cholerae*, but it was unable to activate *tcpA* expression in *E. coli* (Fig. 3B) and complement production of TcpA in *V. cholerae* (Fig. 3C). Further studies are required to test whether this D141G mutation affects the dimerization of ToxT.

The dimerization domain of ToxT is not required for repression. ToxT seems to be able to bind independently to toxbox motifs upstream of most known ToxT-regulated promoters (28), either dimerizing after binding or interacting with other bound monomers to interact with RNA polymerase and activate transcription. The N-terminal region of ToxT seems to be critical for both dimerization and modulation of ToxT activity by environmental signals (2, 23, 24). D141 lies within this putative N-terminal dimerization domain.

To examine the role of the putative dimerization domain of ToxT in transcriptional regulation of *msh* genes, we constructed a variant of ToxT, $ToxT^{\Delta N}$, that lacked the 2 to 144 N-terminal amino acid residues (Fig. 3A). When expressed from a plasmid in *E. coli*, this variant of ToxT could not activate transcription of P_{tcpA}-*lux* reporters but was still able to repress transcription of P_{msh}-*lux* (including *mshH*, *mshI*, and *mshB*), further suggesting that negative regulation of the *msh* locus by ToxT is independent of dimerization (Fig. 3B and data not shown). In *V. cholerae*, $ToxT^{\Delta N}$ was unable to complement an in-frame *toxT* mutation for production of TCP (Fig. 3C, top panel) but remained able to repress MshA expression (Fig. 3C, bottom panel) and attenuate hemagglutination (Table 1).

These data suggest that ToxT is capable of direct negative regulation of the *msh* genes involved in the biogenesis of the anticolonization factor MSHA in both *V. cholerae* and a heterologous *E. coli* reporter system. Furthermore, ToxT is able to bind to the *msh* operon in up to three toxbox-like regulatory regions (Fig. 1). Transcriptional repression by ToxT is not affected by a known inhibitor of dimerization and transcriptional activation, virstatin. Mutations in the putative N-terminal dimerization domain are can abrogate activation at the ToxT-regulated *tcpA* promoter, but these ToxT alleles remain capable of repressing *msh* gene transcription. A complete N-terminal deletion mutant lacking the first 2 to 144 amino acids shows the same phenotype, further suggesting that ToxT-mediated transcriptional repression is independent of dimerization. Although monomeric ToxT-binding to cognate promoters has been reported previously in the case of activated promoters (28, 29), the data presented here offer the first indications of a mechanism for ToxT-mediated transcriptional repression. It is possible that ToxT may bind as indepen-

TABLE 1. Effect of ToxT activity on MSHA production

Growth condition and strain[a]	Hemagglutination activity[b]
LB	
WT	16
Δ*msh* mutant	<2*
Δ*toxT* mutant	16
Δ*toxT* mutant (pBAD-toxT)	4
Δ*toxT* mutant (pBAD-$toxT^{\Delta N}$)	<2*
Δ*toxT* mutant (pBAD-$toxT^{D141G}$)	2
AKI	
WT	4
Δ*msh* mutant	<2*
Δ*toxT* mutant	16
WT + virstatin	4

[a] Arabinose was added to 0.1% for all pBAD plasmid-containing strains, and virstatin was used at a final concentration of 100 μM. Virstatin or arabinose alone had no effect on hemagglutination.

[b] Hemagglutination is reported as the highest reciprocal titer of bacteria still exhibiting mannose-dependent hemagglutination. The results are representative of three experiments. *, No hemagglutination was observed at the highest concentration of bacteria used for this experiment.

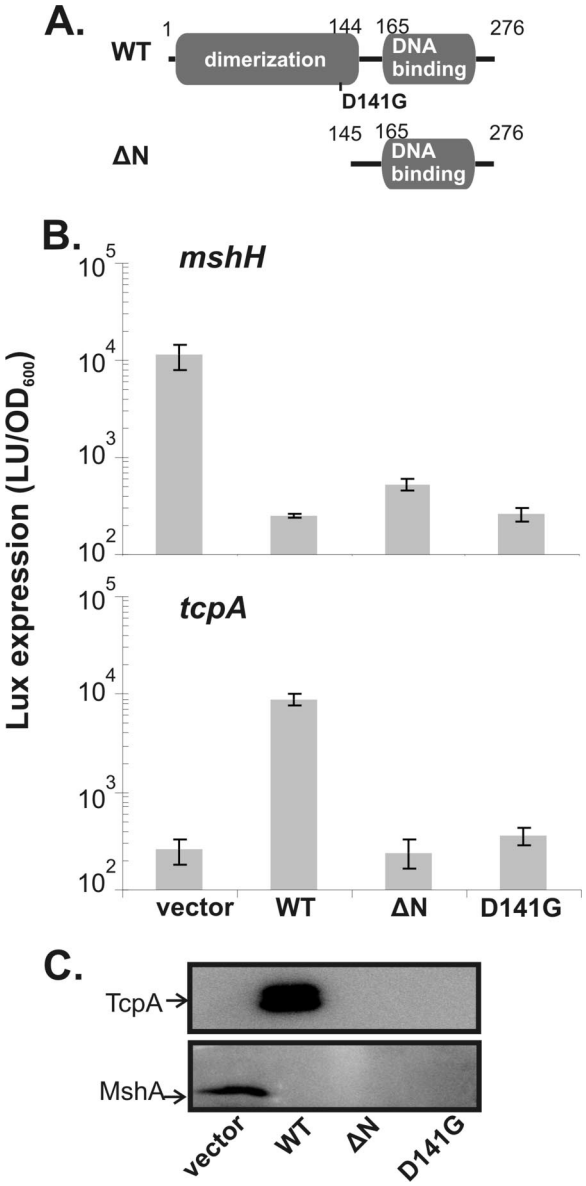

FIG. 3. The dimerization domain of ToxT is dispensable for repression of *msh* genes. (A) Schematic of the domains of ToxT, with the location of the D141G point mutation, and a comparison of wild-type and the truncated ΔN ToxT lacking the N-terminal dimerization domain. (B) Activity of Lux reporter constructs in *E. coli*, without ToxT, with ToxT, and with either D141G or ΔN ToxT. Arabinose (0.1%) was used to induce P_{BAD} promoters. Units are arbitrary light units/OD_{600}. The results are average of three experiments ± the SD. (C) Western blot for TcpA (top panel) and MshA (bottom panel) from *V. cholerae toxT* mutant complemented with vector pBAD24, wild type, ΔN, or D141G ToxT.

dent monomers to a number of toxbox or toxbox-like motifs within the regulatory region of the *msh* operon, which may then occlude RNA polymerase and repress transcription. Additional in vitro and in vivo biochemical studies may yet yield insights into how and where ToxT can bind to the *msh* operon regulatory region. Studies of ToxT regulation at this locus thus offer fertile ground for future investigations into how this versatile regulator resolves the contradiction between the effects of colonization and anticolonization factor production during *V. cholerae* infection.

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