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MOLECULAR PATHOGENESIS

ToxR Mediates the Antivirulence Activity of Phenyl-Arginine-β-Naphthylamide To Attenuate *Vibrio cholerae* Virulence

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ABSTRACT Multidrug efflux systems belonging to the resistance-nodulation-cell division (RND) family are ubiquitous in Gram-negative bacteria and are critical for antimicrobial resistance. This realization has led to efforts to develop efflux pump inhibitors (EPI) for use as adjuvants for antibiotic treatment of resistant organisms. However, the functions of RND transporters extend beyond antimicrobial resistance to include physiological functions that are critical for pathogenesis, suggesting that EPIs could also be used as antivirulence therapeutics. This was documented in the enteric pathogen *Vibrio cholerae*, in which EPIs were shown to attenuate the production of the critical virulence factors cholera toxin (CT) and the toxin-coregulated pilus (TCP). In this study, we investigated the antivirulence mechanism of action of the EPI phenyl-arginine-β-naphthylamide (PAβN) on *V. cholerae*. Using bioassays, we documented that PAβN inhibited virulence factor production in three epidemic *V. cholerae* isolates. Transcriptional reporter studies and mutant analysis indicated that PAβN initiated a ToxR-dependent regulatory circuit to activate *leuO* expression and that LeuO repressed the expression of the critical virulence activator *aphA* to attenuate CT and TCP production. The antivirulence activity of PAβN was found to be dependent on the ToxR periplasmic sensing domain (PPD), suggesting that a feedback mechanism was involved in its activity. Collectively, the data indicated that PAβN inhibited *V. cholerae* virulence factor production by activating a ToxR-dependent metabolic feedback mechanism to repress the expression of the ToxR virulence regulon. This suggests that efflux pump inhibitors could be used as antivirulence therapeutics for the treatment of cholera and perhaps that of other Gram-negative pathogens.

KEYWORDS efflux pump inhibitors, virulence inhibition, RND, efflux, cholera, pathogenesis, virulence

Vibrio cholerae is an enteric Gram-negative bacterium that causes the severe diarrheal disease cholera (1, 2). Cholera is an epidemic disease that affects 2 to 3 million people each year, particularly in places with poor sanitation. *V. cholerae* is a common inhabitant of aquatic ecosystems across the globe and can be found in both fresh (3, 4) and brackish waters (5), from which people acquire cholera through the consumption of contaminated food or water. Following ingestion, *V. cholerae* passes through the gastric acid barrier of the stomach before colonizing the crypts of the small intestine to cause disease. As this pathogen must traverse a wide variety of environments to colonize the small intestine and cause disease in its human host, its pathogenicity hinges on its ability to coordinate the expression of multiple virulence genes in response to environmental stimuli. This process is orchestrated by the ToxR virulence regulon.

The ToxR virulence regulon is a hierarchical regulatory cascade that activates the expression of critical virulence genes following host entry that code for the production of cholera toxin (CT) and the toxin-coregulated pilus (TCP) (6). The regulon is named after the first regulator identified, ToxR, which is a membrane-associated transcription

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factor with a periplasmic sensing domain (PPD) that is thought to modulate ToxR activity and the expression of the regulon in response to environmental cues. ToxR functions in conjunction with another membrane protein, ToxS, which stabilizes the ToxR PPD in response to environmental cues (7–9). CT is an enterotoxin that is responsible for the secretory diarrhea that is the hallmark of cholera, and the TCP is a type IV pilus that is essential for intestinal colonization. The ToxR regulon consists of five main transcription factors that respond to environmental cues in the host to activate virulence gene expression (10). AphA and AphB are cytoplasmic transcription factors that respond to quorum sensing molecules and oxygen, respectively. Together, they bind to the *tcpP* promoter to activate its expression. TcpP then binds with the *toxT* promoter to activate its expression. ToxT then directly activates the expression of the genes responsible for CT and TCP production. Several other regulatory genes function to modulate the expression of the ToxR regulon in response to environmental cues. This includes the LysR family transcriptional regulator *leuO* and the two-component regulator *ompR*. The expression of *leuO* is positively regulated by ToxR in response to bile salts and is predominantly expressed at high cell density during growth *in vitro* (11, 12). LeuO negatively regulates the ToxR regulon by repressing *aphA* transcription. OmpR is activated in response to membrane intercalating agents and represses the ToxR regulon by repressing *aphB* transcription (13).

The ability of *V. cholerae* to colonize the human gastrointestinal tract is not only dependent on virulence factor production but also requires the expression of efflux systems belonging to the resistance-nodulation-cell division (RND) family. RND efflux systems are ubiquitous tripartite transporters that are found in Gram-negative bacteria (14). *V. cholerae* encodes six RND transporters that share TolC as their outer membrane pore protein (15). These systems have been shown to be essential for intestinal colonization and for the intrinsic resistance of *V. cholerae* to antibiotics, detergents, bile acids, and antimicrobial peptides (16). In addition, the RND efflux systems were also required to produce CT and the TCP (16). The absence of RND-mediated efflux was shown to result in increased *leuO* transcription, leading to repression of the ToxR regulon and attenuated CT and TCP production (11). These studies suggested that impaired efflux resulted in the initiation of a metabolite feedback loop via the periplasmic sensing domain of ToxR to effect virulence repression (11). Collectively, these findings indicate that RND-mediated efflux has dual functions in *V. cholerae* pathogenesis, i.e., providing resistance to antimicrobial compounds in the host and modulating virulence gene expression.

The observation that RND-mediated efflux was required for *V. cholerae* virulence gene expression suggested that efflux could be a target for the development of antivirulence therapeutics. Indeed, we previously found that the RND efflux pump inhibitors (EPIs) phenylalanine-arginine-β-naphthylamide (PAβN) and 1-(1-naphthylmethyl)-piperazine (NMP) attenuated *V. cholerae* CT and TCP production (17). However, the mechanism by which EPIs attenuated virulence factor production was unresolved. In this work, we used PAβN to investigate the mechanism of EPI inhibition of *V. cholerae* virulence factor production. We found that PAβN activated *leuO* expression by a process that was dependent on the presence of the periplasmic sensing domain of ToxR. LeuO then repressed *aphA* transcription, leading to downregulation of the ToxR regulon and attenuated CT and TCP production. The data supported a model where PAβN activated an efflux-dependent regulatory feedback circuit by inhibiting the efflux of native substrates of the RND transporters to repress virulence gene expression. Our results suggest that the use of EPIs could extend beyond antibiotic potentiation and be used as antivirulence therapeutics for treatment of cholera and, likely, that of other bacterial diseases.

RESULTS

PAβN inhibits virulence factor production in pandemic *V. cholerae* strains. A previous study suggested that EPIs inhibited virulence factor production in *V. cholerae* O1 El Tor strain N16961. To confirm and extend this finding, we tested the effect of PAβN on CT and TCP production in three epidemic strains of *V. cholerae*, O1 El Tor

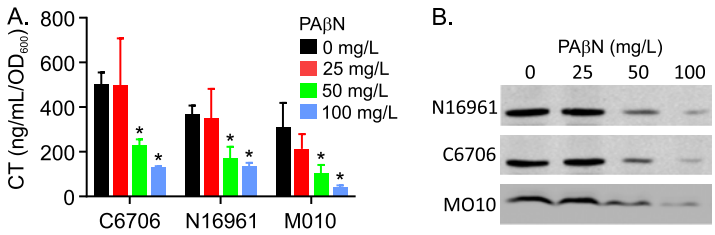

FIG 1 PAβN inhibits virulence factor production in *V. cholerae*. The indicated *V. cholerae* strains were cultured for 15 h under AKI conditions in AKI medium containing the indicated concentrations of PAβN. Cholera toxin (CT) production in each culture was then determined by GM1 enzyme-limited immunosorbent assay (ELISA) (A), and toxin-coregulated pilus (TCP) production was assessed by TcpA Western blotting (B). The results are representative of at least three independent experiments. Statistical significance was determined by analysis of variance (ANOVA) with the Tukey-Kramer multiple-comparison test. *, $P \leq 0.05$ relative to the no-PAβN control.

strains N16961 and C6706 and O139 strain MO10. The three *V. cholerae* strains were cultured under virulence gene-inducing conditions (i.e., AKI conditions) in the presence of 0, 25, 50 or 100 mg/liter of PAβN. The MIC for PAβN in *V. cholerae* was previously shown to be 300 mg/liter (17). Consistent with this, PAβN at the test concentrations did not affect the growth of any of the *V. cholerae* strains. The following day, culture aliquots were collected, normalized by optical density, and assayed for CT production and TcpA production as described in Materials and Methods. The results showed a PAβN concentration-dependent inhibition of both CT (Fig. 1A) and TcpA (Fig. 1B) production in each strain beginning at 50 mg/liter PAβN. The PAβN-dependent decrease in CT and TcpA production in N16961 confirmed the results of a previous study (17). The observation that PAβN exhibited similar virulence-repressing activity in strains C6706 and MO10 showed that the virulence inhibitor activity of PAβN was not strain specific and likely extends to all currently circulating epidemic and pandemic *V. cholerae* strains.

PAβN inhibits ToxR regulon expression. The results described above confirmed that PAβN inhibited *V. cholerae* virulence factor production, but the mechanism by which PAβN affected this process was undetermined. The production of CT and TCP in *V. cholerae* is positively regulated by the ToxR regulon. We therefore tested if PAβN was affecting the expression of the primary regulatory genes in the ToxR regulon. We cultured *V. cholerae* strains N16961 (Fig. 2A) and C6706 (Fig. 2B), bearing *lux*-based transcriptional reporters for the primary regulatory genes (i.e., *aphA*, *aphB*, *toxT*, *tcpP*, and *toxR*), and CT and TCP (i.e., *ctxA* and *tcpA*) under AKI conditions in AKI broth plus and minus PAβN (75 mg/liter) for 6 h, at which time we assessed gene expression as relative light units (RLU) divided by the optical density at 600 nm (OD_{600}). An RND-deficient *V. cholerae* strain (ΔRND) cultured in AKI broth without PAβN was included as a control. The results showed that PAβN inhibited the expression of *ctxA* and *tcpA* (Fig. 2A) in both N16961 and C6706, confirming the CT enzyme-limited immunosorbent assay (ELISA) and TcpA Western blotting results presented above. The expression of *aphB* and *toxR* was not decreased in the ΔRND mutant or in PaβN-treated N16961 or C6706, confirming previous reports (11, 16). In contrast, the expression of *aphA*, *tcpP*, and *toxT* was repressed by PAβN in both N16961 and C6706 to a level that was similar to what was observed in the ΔRND mutant. As *aphA* is one of the most upstream regulators in the ToxR regulon, and it directly activates *tcpP* transcription and indirectly activates *toxT* (via *tcpP*), this result indicates that PAβN inhibition of *aphA* transcription was likely responsible for downregulation of the ToxR regulon and the attenuated CT and TCP production. The fact that the effects of PAβN on the ToxR regulon were conserved between N16961 and C6706 further confirms that the antivirulence activity of PAβN is not strain specific.

PAβN represses virulence factor production via ToxR-dependent activation of *leuO*. We then set out to further determine the mechanism by which PAβN repressed *aphA* transcription. The expression of *aphA* is negatively regulated by the main quorum

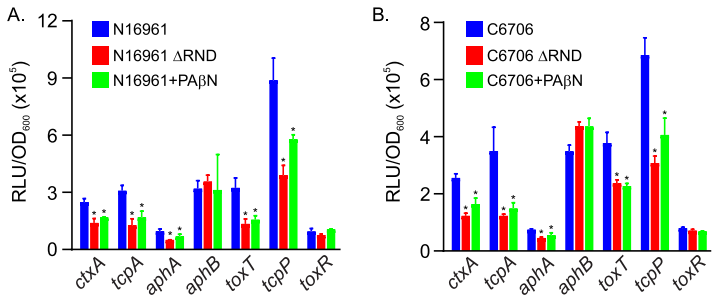

FIG 2 Effect of PAβN on the expression of genes in the ToxR virulence regulon. The indicated *V. cholerae* N16961 JB58 (A) or C6706 (B) strains bearing plasmids with *lux*-based promoter fusions for the indicated genes were cultured under AKI conditions in the presence or absence of PAβN (75 mg/liter) for 5 h, at which time gene expression was quantified as relative light units (RLU) divided by the optical density at 600 nm (OD_{600}). RND efflux pump-negative mutants (ΔRND) for both strains were included as a comparator. The results represent the average RLU/OD and standard deviation of three independent experiments. Statistical significance was determined by ANOVA with the Tukey-Kramer multiple-comparison test. *, $P \leq 0.05$ relative to the wild type (WT) cultured in AKI broth.

sensing (QS) regulator HapR, with maximal expression occurring at low cell density (28). However, the fact that N16961, like most pandemic strains, contains a frameshift mutation in *hapR* and is QS negative suggested that another mechanism must be involved in *aphA* repression (18, 29). ToxR also indirectly regulates *aphA* via *leuO*. ToxR activates *leuO* transcription in response to environmental cues, including *V. cholerae* metabolites such as indole, cyclo(phe-pro), and malate (11, 21, 30). LeuO can directly bind to the *aphA* promoter to repress its expression (21). We therefore tested if PAβN was repressing *aphA* through *leuO* by repeating the above-described experiments using N16961 bearing a *leuO-lux* reporter. The results showed a PAβN-dependent increase in *leuO* expression (Fig. 3, black bars) in the wild type (WT). The increase in *leuO* expression in the PaβN-treated cells was similar to what was observed in an isogenic RND-deficient mutant (ΔRND), confirming that treatment of WT with PAβN phenocopied a ΔRND mutant for *leuO* expression.

Previous studies documented that ToxR activation of *leuO* expression in response to environmental cues was dependent on the presence of the ToxR periplasmic domain (PPD) (11, 21, 30). We therefore tested if the PAβN-dependent induction of *leuO* was also dependent on the ToxR PPD. We cultured *leuO-lux* reporter bearing derivatives of N16961 and the ΔRND mutant that expressed a truncated ToxR allele that lacked the periplasmic sensing domain (*toxR*$^{\Delta PPD}$). The *toxR*$^{\Delta PPD}$ allele was previously shown to support virulence factor production and porin regulation (11, 21). The results showed that *leuO* was not expressed in the strains expressing the *toxR*$^{\Delta PPD}$ allele and that the addition of PAβN to the *toxR*$^{\Delta PPD}$-expressing mutants did not significantly affect *leuO* expression (Fig. 3, gray bars). This confirmed that the PAβN induction of *leuO* was dependent on the presence of the periplasmic sensing domain of ToxR and suggested that *leuO* may modulate the PAβN-dependent repression of virulence factor production.

PAβN antivirulence activity is mediated by LeuO. The results described above indicated that PAβN indirectly activated *leuO* via ToxR. As *leuO* was previously reported to repress *aphA* (21), and *aphA* expression was reduced by the addition of PAβN (Fig. 2), we hypothesized that PAβN-dependent virulence repression was mediated by *leuO*. If this was true, then deletion of *leuO* should abrogate the antivirulence activity of PAβN. To test this, we cultured N16961 and isogenic *leuO* and *toxR*$^{\Delta PPD}$ mutants under AKI conditions in the presence and absence of PAβN and then assayed for CT and TcpA production. The results showed that *leuO* deletion restored near-WT-level production of CT and TcpA in the cultures grown in the presence of PAβN (Fig. 4A and B). In contrast, PAβN did not have any effect on *ctxA* or *tcpA* expression in N16961 expressing the truncated *toxR*$^{\Delta PPD}$ allele, which is

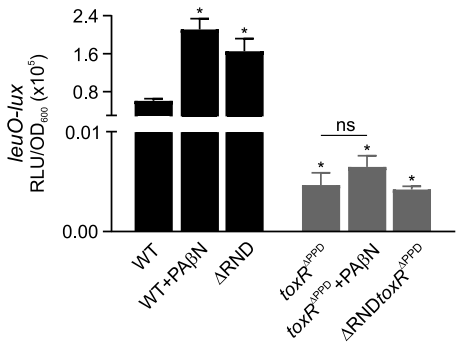

FIG 3 PAβN activates *leuO* expression. *V. cholerae* strain N16961 and isogenic mutants lacking the ToxR periplasmic domain (ΔPPD) and all six RND efflux pump proteins (ΔRND) bearing a *leuO-lux* promoter fusion reporter were cultured under AKI conditions in AKI medium in the presence or absence of PAβN (75 mg/liter) for 5 h, at which time gene expression was quantified as relative light units (RLU) divided by the optical density at 600 nm. The results represent the average RLU/OD and standard deviation of three independent experiments. Statistical relevance to the WT was calculated using a one-way ANOVA with Dunnett's *post hoc* test. Statistical significance for the effects of PAβN on *leuO* expression in $toxR^{\Delta PPD}$ and $toxR^{\Delta PPD}$ was determined using Student's *t* test. *, $P \leq 0.05$ relative to the WT. NS, not significant.

consistent with the inability of this mutant to activate *leuO* transcription. Collectively, these results, combined with the above-described data, suggested that the antivirulence activity of PAβN resulted from the activation of a ToxR-dependent signaling cascaded that resulted in the increased expression of *leuO*, with LeuO then repressing *aphA* transcription to downregulate the ToxR regulon to attenuate CT and TcpA production (Fig. 5).

DISCUSSION

Here, we documented that exposure of *V. cholerae* to PAβN resulted in the activation of a negative regulatory circuit that attenuated production of the two most critical *V. cholerae* virulence factors, CT and the TCP. In this regulatory cascade, PAβN initiated signaling through the periplasmic signaling domain of ToxR (Fig. 5), which led to the activation of *leuO* transcription. LeuO then repressed the expression of *aphA*. As AphA is one of the most upstream regulators in the ToxR regulon, its repression results in downregulation of the ToxR regulon and attenuated CT and TCP production. The fact that PAβN activation of this regulatory circuit was dependent upon the periplasmic sensing domain of ToxR is consistent with a previous study suggesting that impaired RND-mediated efflux leads to intracellular metabolite accumulation to initiate adaptive responses via periplasmic sensors such as ToxR and other two-component regulatory systems (11, 30–33). The fact that production of CT and TCP are essential for the development of cholera suggests that EPIs could be used to treat cholera. These results also have potential implications for the application of EPIs to other Gram-negative pathogens in which RND-mediated efflux is critical for virulence.

While the present studies document that PAβN activated a putative metabolite-dependent negative regulatory circuit to suppress *V. cholerae* virulence gene expression, the environmental cue(s) that initiated this regulatory cascade remains unresolved. The dependence of this regulatory circuit on the ToxR PPD suggests that the activating stimuli is localized to the periplasm, a conclusion that is consistent with the function of RND transporters in capturing substrates from the periplasmic compartment. This conclusion is further supported by multiple studies showing that the ToxR PPD senses and responds to extracellular chemical cues, which include indole (30), cyclic di-peptides (21), malate (11), and bile salts (12). It is noteworthy that all of these compounds have been shown to induce the ToxR-dependent transcription of *leuO* and that indole, malate, and bile salts are substrates of the RND transporters.

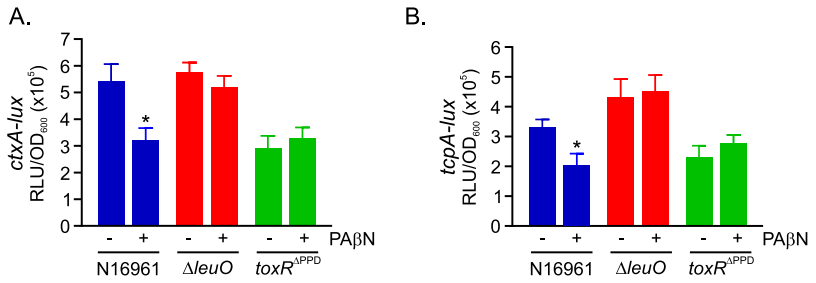

FIG 4 Deletion of *leuO* restores virulence factor production. The indicated *Vibrio cholerae* N16961 strains bearing *lux*-based promoter fusions for (A) *ctxA* and (B) *tcpA* were cultured under AKI conditions in the presence or absence of PAβN (75 mg/liter) for 5 h, at which time gene expression was quantified as relative light units (RLU) divided by the optical density at 600 nm. The results represent the average RLU/OD and standard deviation of three independent experiments. Statistical analysis was determined using Student's *t* test. *, $P \leq 0.05$.

Based on this, we propose that treatment of *V. cholerae* with PAβN resulted in the periplasmic accumulation of metabolites. Once the metabolites reached a critical concentration, they interact with the ToxR PPD to activate *leuO* expression to attenuate virulence factor production. The fact that PAβN treatment of wild-type *V. cholerae* phenocopies an RND efflux pump null mutant provides additional support for this model (16). Furthermore, metabolite efflux was proposed to explain dysregulated virulence gene expression in a *V. cholerae* classical biotype strain lacking *tolC* (the outer membrane pore protein for the *V. cholerae* RND systems) (34, 35). The identity of the specific metabolite (or metabolites) responsible for activating the ToxR-dependent regulatory circuit remain unknown and will require additional studies.

The studies described here suggest that EPIs could also be used as antivirulence therapeutics in cholera. However, the contributions of RND efflux to virulence is not limited to *V. cholerae*. RND systems have been shown to be important for virulence in a large number of pathogens, including pathogens that are rapidly becoming refractory to antibiotics (e.g., *Acinetobacter*, *Burkholderia*, *Escherichia*, *Klebsiella*, *Moraxella*, *Mycobacterium*, *Pseudomonas*, *Salmonella*, and *Stenotrophomonas*) (14, 36–38) and for which new therapeutic approaches are needed. While the mechanism linking RND efflux to virulence in these pathogens is largely unknown, the metabolite feedback circuits described in *V. cholerae* appear to be conserved in other Gram-negative bacteria. For example, AcrAB in *Escherichia coli* has been shown to be feedback regulated in response to at least four metabolic pathways, namely, enterobactin, cysteine and purine biosynthesis, and gluconeogenesis (39). Consistent with this, additional studies have linked TolC to metabolite feedback in *E. coli* (40). In *Salmonella*, mutation of the AcrD efflux pump resulted in dramatic transcriptional changes that were consistent

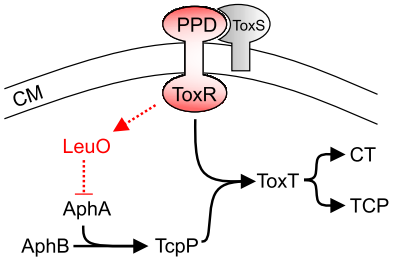

FIG 5 Model for the antivirulence activity of PAβN in *V. cholerae*. Exposure of *V. cholerae* to PAβN results in the activation of ToxR by a process that is dependent upon its periplasmic signaling domain (PPD). Activation of ToxR results in increased *leuO* transcription. LeuO then represses *aphA* transcription, leading to downregulation of the ToxR virulence regulon and attenuated CT and TCP production. PAβN, phenyl-arginine-β-naphthylamide; CM, cytoplasmic membrane; CT, cholera toxin; TCP, toxin-coregulated pilus.

with metabolite accumulation initiating feedback mechanisms (41). Furthermore, RND systems have been linked to efflux of quorum sensing molecules and siderophores in multiple organisms with impaired efflux, affecting feedback responses for both classes of molecules (32, 42–46). Although not well studied, there is accumulating evidence to suggest that EPIs negatively impact virulence in other organisms. For example, EPIs were shown to inhibit quorum sensing and virulence factor production in *Pseudomonas aeruginosa* (47). EPIs were also shown in a cell culture model to inhibit *P. aeruginosa* invasion (48), and EPIs were also shown affect biofilm production in *Klebsiella pneumoniae*. *P. aeruginosa*, and *E. coli* (49). Thus, we conclude that EPIs may have utility beyond antibiotic potentiation and suggest that EPIs represent potential antivirulence therapeutics for Gram-negative pathogens and warrant further studies. If efficacious, EPIs could help to combat the global pandemic of antimicrobial resistance that threatens human health.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *V. cholerae* O1 El Tor strains N16961 (18) and C6706 (19) and serogroup O139 strain MO10 (20) were obtained from our laboratory collection. N16961 JB58 (Δ*lacZ* Sm^r), JB58 *toxR*$^{\Delta PPD}$, ΔRND (Δ*vexB* Δ*vexD* Δ*vexF* Δ*vexH* Δ*vexK* Δ*vexM*), and ΔRND *toxR*$^{\Delta PPD}$ mutants have been previously described (11, 12, 21). N16961 JB58 was used as the WT in this study. *Escherichia coli* strain EC100pir (Epicentre; Madison, WI) was used as a host for DNA cloning experiments. *E. coli* and *V. cholerae* strains were routinely grown in lysogeny broth (LB) or on LB agar at 37°C. Induction of the ToxR virulence regulon was accomplished by culturing *V. cholerae* strains under AKI conditions as follows. Overnight LB broth cultures of the test strains were individually diluted (10^{-3}) into 10 ml of AKI broth (15 g Bacto peptone, 4 g Difco yeast extract and 5 g of NaCl per liter [pH 7.4]) in 150- × 15-mm glass test tubes (22). The inoculated test tubes were then incubated statically for 4 h at 37°C or until the OD_{600} reached ≥0.08 before the cultures were transferred to 125-ml Erlenmeyer flasks and incubated with shaking at 37°C for 1 h for the *lux* reporter assays or overnight for CT and TCP quantification. Carbenicillin and streptomycin were used at 100 μg/ml as necessary.

Chemicals and reagents. Chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO). PAβN stock solutions were made in ultrapure water and filter sterilized before being aliquoted and stored at −20°C until needed. Enzymes for cloning were purchased from New England Biolabs (Beverly, MA), and oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA) and designed based on the *V. cholerae* C6706 genome (23).

Strain and plasmid construction. The promoter probe vector pBBR-lux (24), which codes for chloramphenicol resistance, was used to generate transcriptional reporter plasmids for the *V. cholerae* ToxR regulon genes as follows. We amplified the ampicillin resistance gene from pBAD18 (25) using the AmpR-F (gcccgcctgatgaatgctcatccgggaattc-TGACGGATGGCCTTTTTGCGTTTCT) and AmpR-R (ctcaccgtc tttcattgccatacgggaattc-TACAGGGCGCGTAAATCAATCTAAAG) PCR primers. These primers were tailed with homology (lowercase letters) to the DNA flanking the EcoRI site in pBBR-lux that intersected the chloramphenicol resistance gene to render the resulting plasmids ampicillin (Amp) resistant and chloramphenicol sensitive. The resulting PCR amplicon was then recombined by Gibson cloning into EcoRI-restricted pBBR-lux or pBBR-lux that already contained ToxR regulon promoter reporters to generate the following plasmids (26, 27): pTB17 (*toxT-lux*), pTB18 (pBBR-lux-Amp^r; empty vector control), pTB19 (*aphA-lux*), pTB20 (*tcpP-lux*), pTB21 (*toxR-lux*), pTB22 (*tcpA-lux*), pTB23 (*ctxA-lux*), and pTB25 (*aphB-lux*). pTB32 (*leuO-lux*) was constructed by PCR amplifying the *leuO* promoters from pXB266 (21) using the pTL61T-F-SacI (ATGAGCTCGTTTGACAGCTTATCATCGGAGCTC) and pTL61T-R-BamHI (TTGGATCCGT CGGGATCGCTAGTTAGTTAGG) PCR primers. The resulting PCR amplicon was restricted with SacI and BamHI and cloned into similarly restricted pTB18 to generate pTB31 (*leuO-lux*). The reporter plasmids were sequence verified prior to use.

Virulence factor production. Virulence factor production was assessed in all strains following overnight growth under AKI conditions in AKI medium (22). All cultures were normalized by optical density prior to assessing CT and TCP production. AKI culture supernatants were collected to quantify CT production using a GM1 enzyme-linked immunosorbent assay as previously described, with purified CT being used as a standard for quantitation (11). Cell pellets from the overnight AKI cultures were used to assess TCP production by Western immunoblotting for TcpA as previously described (11).

Transcriptional reporter assays. *V. cholerae* strains bearing the indicated reporter plasmids were cultured under AKI growth conditions in AKI medium, with PAβN being added as indicated. Triplicate culture aliquots (200 μl) were collected at the indicated time points and transferred to the wells of a white microtiter plate with a clear bottom. Luminescence production and the optical density at 600 nm were then determined using a BioTek Synergy HT plate reader, with the results being reported as relative light units (RLU) divided by the optical density. The reported results are the average and standard deviation of at least three independent experiments.

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The content is solely the responsibility of the authors.

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