Regulation by ToxR-Like Proteins Converges on vttRB Expression To Control Type 3 Secretion System-Dependent Caco2-BBE Cytotoxicity in Vibrio cholerae

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ABSTRACT
Genes carried on the type 3 secretion system (T3SS) pathogenicity island of Vibrio cholerae non-O1/non-O139 serogroup strain AM-19226 must be precisely regulated in order for bacteria to cause disease. Previously reported results showed that both T3SS function and the presence of bile are required to cause Caco2-BBE cell cytotoxicity during coculture with strain AM-19226. We therefore investigated additional parameters affecting in vitro cell death, including bacterial load and the role of three transmembrane transcriptional regulatory proteins, VttRA, VttRB, and ToxR. VttRA and VttRB are encoded on the horizontally acquired T3SS genomic island, whereas ToxR is encoded on the ancestral chromosome. While strains carrying deletions in any one of the three transcriptional regulatory genes are unable to cause eukaryotic cell death, the results of complementation studies point to a hierarchy of regulatory control that converges on vttRB expression. The data suggest both that ToxR and VttRA act upstream of VttRB and that modifying the level of either vttRA or vttRB expression can strongly influence T3SS gene expression. We therefore propose a model whereby T3SS activity and, hence, in vitro cytotoxicity are ultimately regulated by vttRB expression.

IMPORTANCE
In contrast to O1 and O139 serogroup V. cholerae strains that cause cholera using two main virulence factors (toxin-coregulated pilus [TCP] and choler toxin [CT]), O39 serogroup strain AM-19226 uses a type 3 secretion system as its principal virulence mechanism. Although the regulatory network governing TCP and CT expression is well understood, the factors influencing T3SS-associated virulence are not. Using an in vitro mammalian cell model to investigate the role of three ToxR-like transmembrane transcriptional activators in causing T3SS-dependent cytotoxicity, we found that expression levels and a hierarchical organization were important for promoting T3SS gene expression. Furthermore, our results suggest that horizontally acquired, ToxR-like proteins act in concert with the ancestral ToxR protein to orchestrate T3SS-mediated pathogenicity.

Pathogenic bacteria must effectively control the expression of virulence factors in order to achieve productive infection. As a waterborne pathogen, Vibrio cholerae senses and responds to signals from two distinct environments, the aquatic reservoir and the human host, requiring appropriate temporospatial changes in gene expression. Epidemic-causing O1 and O139 serogroup strains use the ToxR-ToxT regulatory hierarchy to modulate the expression of genes encoding factors responsible for colonization (toxin-coregulated pilus [TCP]) and diarrhea (choler toxin [CT]) (1, 2). Whereas toxR is an ancestral gene found in all V. cholerae strains, toxT is carried on horizontally acquired Vibrio pathogenicity island 1 (VPI-1), which encodes the TCR. ToxR can directly activate transcription from the toxT promoter (3, 4). VPI-1 also encodes the TcpPH proteins, which can increase toxT transcription and are required for optimal virulence gene expression (5). In addition, the AphAB proteins positively regulate tcpPH expression and, like toxR, are encoded in ancestral genomic loci (6–8). Thus, a complex network of regulators is involved in modulating the expression of horizontally acquired virulence factors in TCP/CT-positive V. cholerae strains.

Pathogenic strains of other serogroups, collectively called non-O1/non-O139 serogroup strains, typically do not encode the TCP or CT and instead encode other virulence factors (9–19). Although the mechanisms resulting in non-O1/non-O139 serogroup-associated disease are less well understood, we now know that a subset of non-O1/non-O139 serogroup strains carries genes for a type 3 secretion system (T3SS) (20–23). Previous studies using O39 serogroup strain AM-19226, which lacks the TCP and CT, have shown that T3SS activity is required for colonization and disease in murine and rabbit animal models of infection (24, 25). T3SS genomic island-encoded, translocated proteins have been identified and named Vops, and several Vops have been assigned functions (25–30).

VttRA and VttRB are two transcriptional regulators encoded...
within the T3SS genomic island. Both proteins are integral membrane proteins with N-terminal, cyttoplasmic DNA binding domains and overall sequence similarity to ToxR (31). ΔvtrA and ΔvtrR strains are severely attenuated for colonization in vivo and deficient in bile-mediated enhancement of T3SS gene expression in vitro (31, 32). Consistent with other V. cholerae strains, AM-19226 also encodes ToxR. We previously reported that the AM-19226 ToxR protein contributes to T3SS-related in vitro and in vivo phenotypes but has a less prominent role than VttR and VttR in vitro versus in vivo phenotypes and the molecular regulatory events that shape virulence remain poorly defined.

Coculture of strain AM-19226 with the Caco2-BBE intestinal epithelial cell line results in rapid eukaryotic cell death in the presence of bile (32). The phenotype does not require the HlyA hemolysin or the putative thermostable direct-related hemolysin (TRH) but requires a functional T3SS. We therefore sought to further examine the bacterial requirements during coculture and the roles of VttR and VttR in bile-mediated Caco2-BBE cell death. We included ToxR in our studies since its role in the coculture assay had not previously been examined; we were interested in further understanding how ToxR influences horizontally acquired virulence gene expression in TCP/CT-negative strains. We present genetic data supporting a model that correlates levels of vttR and vttRgene expression with T3SS gene expression and mammalian cell cytotoxicity in vitro. Furthermore, our results suggest an important role for ToxR in modulating T3SS virulence phenotypes by modifying vttR expression levels.

**MATERIALS AND METHODS**

**Bacterial strains, growth conditions, and in silico analysis.** Strains (Table 1) were maintained and grown under standard conditions and as described below. Ampicillin (Amp) and streptomycin (Str) were used at 100 μg/ml for Escherichia coli and V. cholerae. Bile (bovine bile, catalog number B3883; Sigma) was prepared as previously described (31). Clone Manager Professional Suite, version 9 (Sci-Ed Software), was used for sequence analysis.

**Strain and plasmid construction.** Nucleic acid manipulations were performed by using standard molecular biology techniques (33). Strain MD992 was used as the wild-type (WT) parental strain for β-galactosidase assays and strain derivatives. For coculture assays, MD992 lacking hap, hlyA, and rtcA (AAC155) was used as the parental strain (26). toxR, vttR, vttR, or vopX deletions were constructed in AAC155 as previously described (26, 31). pBAD18-toxR was constructed by using restriction enzyme-based methods to insert the open reading frame downstream of the arabinose-inducible promoter (Table 2).

For VopX complementation studies, a wild-type copy of vopX, including ~500 bp of native sequences upstream of the translational start site, was integrated into the AM-19226 lacZ locus by using plasmid pMD86. The vopX’ truncation was constructed by using pCD442ΔvtrB, which contains the vopX coding sequence and sequences homologous to the AM-19226 genome, allowing for allelic exchange of an engineered nucleotide point mutation that introduced a stop codon (A157T, resulting in amino acid change K54X). DNA sequencing analyses confirmed genomic integration of the point mutation. Primer sequences are available upon request.

**β-Galactosidase assay.** Multicopy pAAC3 plasmids carrying transcriptional lacZ reporter fusions to vcsRTCNS2, vcsJ2, and vttR were previously described (31). Kinetic β-galactosidase assays were performed as previously described, where units equal micromoles o-nitrophenyl-β-D-galactopyranoside (ONPG) hydrolyzed per minute per optical density at 600 nm (OD600) (31).  

**Mammalian cell lines and culture conditions.** Caco2-BBE cells were routinely maintained in Dulbecco’s modified Eagle medium (DMEM) (4.5 mg/ml glucose, sodium pyruvate, and L-glutamine; Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Gemini Bio-Products) at 37°C with 5% CO₂. Coculture of Caco2-BBE cells and V. cholerae strain AM-19226 was performed in the presence of DMEM supplemented with 5% FBS as previously described, using DMEM containing 1 mg/ml glucose (low-glucose medium; Invitrogen) for arabinose induction experiments (32).

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**TABLE 2 Plasmids used in this study**

<table>
<thead>
<tr>
<th>Plasmid</th>
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<tr>
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<td>Suicide vector; Amp†</td>
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<td>pMD86</td>
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<td>pAAC3-vttR</td>
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<tr>
<td>pBAD18-toxR</td>
<td>ToxR expression vector; Amp†</td>
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† Amp†, ampicillin resistant.
Lactate dehydrogenase release assay. Percent cytotoxicity was determined as previously described, using the CytoTox 96 nonradioactive cytotoxicity assay (Promega) according to the manufacturer’s instructions (32). Briefly, Caco2-BBE cells were seeded into 96-well plates, grown for ~24 h (80% confluence), and infected at a multiplicity of infection (MOI) of ~10 (unless otherwise noted) using strains grown overnight at 37°C in LB medium. Bile and/or arabinose was added to the coculture medium as indicated. A total of 0.5 mg/ml gentamicin was added during coculture where indicated, which is a concentration previously confirmed to result in 100% bacterial killing.

Statistical analyses. Statistical analyses were conducted by using GraphPad Prism with 2-way analysis of variance (ANOVA) followed by Dunnett or Tukey post hoc tests as appropriate.

RESULTS

Bacterial MOI modulates cytotoxicity. To investigate how the bacterial MOI modulates the kinetics of cell death, we infected Caco2-BBE cells with AM-19226 at MOIs ranging from ~1 to 300. For reference, under our standard conditions of an MOI of ~10 in the presence of 0.2% bile, we observed ~30% cytotoxicity at 2 h postinfection (hpi) and ~80% cytotoxicity at 3 hpi, as measured by lactate dehydrogenase (LDH) release and consistent with previous results (Fig. 1) (32). Infection at an MOI of ~1 decreases cytotoxicity to <10% at 2 hpi and to ~45% at 3 hpi, while an increase of the MOI to 39 results in increased cytotoxicity at 2 hpi (~65%) but does not increase cell death above ~80% at 3 hpi. Infection at an MOI of ~75 results in ~80% cytotoxicity after 2 hpi, which was not increased at 3 hpi (Fig. 1 and data not shown). Therefore, increasing the MOI above 10 shortened the time to maximal LDH release and thus cytotoxicity but did not increase the percentage of cytotoxic cells after 3 h of coculture.

Cytotoxicity requires viable bacteria. Given that the bacterial burden can modulate the kinetics of cytotoxicity, we asked whether live bacteria are required throughout the 3 h of infection or whether a short incubation with bacteria was sufficient to initiate the process. Following the addition of bacteria in the presence of bile, we added gentamicin at 0.5-h intervals from 0 to 2.5 hpi and measured LDH release at 3 hpi. We did not observe cytotoxicity when gentamicin was added at the time of infection (0 h) or at 0.5, 1, or 1.5 hpi (Fig. 2). However, we measured ~40% cytotoxicity when gentamicin was added to the coculture medium at 2 hpi, a time point at which the bacterial count increased 2- to 4-fold (Fig. 2 and data not shown). Levels of replication were similar for T3SS-positive and T3SS-negative strains and increased 15- to 20-fold over that of the initial inoculum (MOI = 10) at 3 hpi (data not shown). Interestingly, the addition of gentamicin at 2.5 hpi did not alter the cytotoxicity levels compared to levels achieved in the absence of gentamicin (our standard infection conditions) (Fig. 2). The collective data therefore suggest that cytotoxicity requires viable bacteria for a period of at least 2 h postinfection.

VttRA and VttRB are required for AM-19226-mediated mammalian cell cytotoxicity. Previous results from our laboratory showed that VttRA and VttRB modulate T3SS island gene expression in vitro in the presence of bile (31, 34). Data also support a model for hierarchical regulation whereby VttRA regulates the expression of vttRB, since both a ΔvttRA single-deletion strain and a ΔvttRA ΔvttRB double-deletion strain can be complemented for T3SS gene expression by providing vttRB in trans (32).

We therefore assessed the contribution of each regulator to AM-19226-induced Caco2-BBE cell cytotoxicity. We used single- and double-deletion strains that were complemented by using pBAD-based plasmids expressing either vttRA or vttRB, grown in the presence of two different arabinose (inducing) concentrations. Figure 3 shows ~70 to 80% Caco2-BBE cell cytotoxicity resulting from infection with the AM-19226 T3SS WT strain carrying the pBAD18 vector alone when strains were grown under all conditions. Coculture with strains deleted for either vttRA or vttRB does not result in cytotoxicity in the absence of arabinose, similar to results obtained when a ΔvcsN2 (T3SS-deficient) strain was used for infection (Fig. 3, black bars) (32). For ΔΔvttRA (pBAD18-vttRA) complementation analyses, cytotoxicity was observed only when 0.5% arabinose was included during coculture (Fig. 3, gray bars). However, we observed increased cytotoxicity during infection with the AM-19226 ΔvttRA strain carrying pBAD18-vttRA when 0.05% or 0.5% arabinose was included during coculture (Fig. 3, checked and gray bars). Expression of vttRB with 0.05% or 0.5% arabinose in AM-19226 ΔΔvttRA cells also restored cytotoxicity, whereas vttRA expression in the ΔΔvttRA background did not. Notably, restoration of cytotoxicity in a ΔΔvttRA ΔvttRB strain

FIG 1 Multiplicity of infection influences kinetics of Caco2-BBE cell cytotoxicity. The AM-19226 T3SS WT strain was grown overnight in LB medium and used to infect Caco2-BBE cells at various MOIs in the presence of 0.2% bile. Percent cytotoxicity was determined by measuring LDH levels in the coculture supernatant 2 h (gray bars) or 3 h (black bars) after infection. The experiment was performed by using three AM-19226 colonies and was repeated, with similar results.

FIG 2 Addition of gentamicin halts progression of mammalian cell cytotoxicity. Three colonies of the AM-19226 T3SS WT strain were grown overnight in LB medium and used to infect Caco2-BBE cells at an MOI of ~10 in the presence of 0.2% bile. Gentamicin was added to individual coculture wells at 0.5-h intervals between 0 h and 2.5 h following infection to kill bacterial cells. After the addition of gentamicin, the coculture was continued until 3 h from initial infection, and percent cytotoxicity was determined. Data are representative of results from two independent experiments.
was achieved only by expression of \( \text{vttR} \) \( \text{B} \) (Fig. 3, checkered and gray bars). These results are consistent with data from studies measuring T3SS structural gene expression in deletion strains using \( \text{lacZ} \) transcriptional reporter fusions, in which expressing \( \text{vttR} \) \( \text{B} \) alone complemented a \( \text{vttR} \) \( \text{A} \) strain (31, 32). Together, our studies demonstrate that \( \text{vttR} \) \( \text{A} \) cannot replace \( \text{vttR} \) \( \text{B} \) and suggest that a hierarchy of T3SS gene expression exists where \( \text{vttR} \) \( \text{A} \) is epistatic to \( \text{vttR} \) \( \text{B} \).

ToxR activity has critical functions in modulating gene expression and cytotoxicity. T3SS structural gene-\( \text{lacZ} \) reporter fusion studies suggested that ToxR affects T3SS gene expression in vitro 2-fold, whereas more dramatic effects were observed for \( \text{vttR} \) \( \text{A} \) and \( \text{vttR} \) \( \text{B} \) strains (31). Similar trends were observed by using the infant mouse model, where \( \text{vttR} \) \( \text{A} \) and \( \text{vttR} \) \( \text{B} \) strains were severely attenuated for colonization (100- to 1,000-fold) and a \( \text{toxR} \) strain had an ~10-fold defect (31). We therefore determined whether ToxR has a role in effecting Caco2-BBE cell cytotoxicity. The results shown in Fig. 4, comparing cytotoxicity levels during coculture with a T3SS WT strain, a \( \text{toxR} \) deletion strain, and the \( \text{toxR} \) strain complemented with pBAD18-\( \text{toxR} \), clearly indicate that ToxR is absolutely required for mammalian cell death under the conditions of our assay (Fig. 4A, black bars). The pBAD18-\( \text{toxR} \) construct restored cytotoxicity in the \( \text{toxR} \) strain when levels of arabinose of up to 0.2% were included in the growth medium and even when no arabinose was present. Coculture assays performed with medium that contained a higher concentration of glucose (4.5 mg/ml) did not result in complementation in the absence of arabinose, supporting our conclusion that leaky expression of \( \text{toxR} \) from the arabinose-inducible promoter occurs in low-glucose tissue culture medium and provides sufficient levels of \( \text{toxR} \) to restore cytotoxicity (data not shown) (35). Interestingly, cocultures including higher levels of arabinose in the medium (0.5%) did not result in cytotoxicity during infection with the \( \text{toxR} \) (pBAD18-\( \text{toxR} \)) strain (Fig. 4A, gray bars).

To examine the possibility that ToxR modulates the cytotoxicity phenotype by interacting within the VttR\( \text{A} \)/VttR\( \text{B} \) hierarchy, we tested whether the expression of \( \text{vttR} \) \( \text{A} \) or \( \text{vttR} \) \( \text{B} \) from a pBAD-based arabinose-inducible promoter can complement a \( \text{toxR} \) deletion strain for the cytotoxicity phenotype. Based on the results

\[ \text{FIG 3 vttR}_\text{A} \text{ functions upstream of vttR}_\text{B} \text{ to tightly regulate Caco2-BBE cell cytotoxicity. The AM-19226 T3SS WT, } \Delta\text{vttR}_\text{A}, \Delta\text{vttR}_\text{B}, \text{ or } \Delta\text{vttR}_\text{A} \Delta\text{vttR}_\text{B} \text{ strain carrying pBAD18, pBAD18-\text{vttR}_\text{A}, or pBAD18-\text{vttR}_\text{B} \text{ was grown overnight in LB medium and used to infect Caco2-BBE cells at an MOI of } \sim 10 \text{ in the presence of } 0.2\% \text{ bile. Arabinose at a concentration of 0%, 0.05%, or 0.5% was included in the coculture medium to induce vttR}_\text{A} \text{ or vttR}_\text{B} \text{ expression. Percent cytotoxicity was determined after 3 h of coculture. Data shown are from one experiment using three colonies of each AM-19226 strain. ** indicates a P value of } <0.0001, \text{ and * indicates a P value of between 0.001 and 0.0001. The experiment was repeated, with similar results.} \]

\[ \text{FIG 4 ToxR activity is integrated into the regulatory network governing Caco2-BBE cell cytotoxicity. The AM-19226 T3SS WT or } \Delta\text{toxR} \text{ strain carrying pBAD18 (A and B), pBAD18-\text{toxR} (A and B), pBAD18-\text{vttR}_\text{A} \text{ (B), or } \text{pBAD18-\text{vttR}_\text{B}} \text{ (B) was grown overnight in LB medium and used to infect Caco2-BBE cells at an MOI of } \sim 10 \text{ in the presence of } 0.2\% \text{ bile. Arabinose was included in the coculture medium at the indicated concentrations to induce toxR, vttR}_\text{A}, \text{ or vttR}_\text{B} \text{ expression. Percent cytotoxicity was determined after 3 h of coculture. Data for each panel represent results from one experiment with three colonies of each AM-19226 strain. ** indicates a P value of } <0.0001. Each experiment was repeated, with similar results.} \]
shown in Fig. 3, we included 0%, 0.05%, or 0.5% arabinose in the coculture medium. As shown in Fig. 4B, induction of vttR_a expression with 0.5% arabinose or of vttR_b expression with 0.05% or 0.5% arabinose completely restored cytotoxicity in the ΔtoxR strain. Our data therefore suggest that ToxR acts upstream of VttR_a/VttR_b. This conclusion is supported by lacZ transcriptional fusion reporter data showing decreased vttR_b expression in a ΔtoxR strain and is consistent with our interpretation that a transcriptional hierarchy results in vttR_b expression and subsequent activity that is responsible for T3SS gene expression (31, 32).

vttR_a regulatory regions overlap the vopX coding sequence. During the course of parallel studies investigating the requirement for different T3SS-translocated proteins (Vops) for cytotoxicity, we found that a ΔvopX strain did not cause cytotoxicity (K. A. Miller, C. H. Seward, and M. Dziejman, unpublished results). Although we constructed the strain as an unmarked, in-frame deletion, we were unable to complement the vopX defect by providing a chromosomally encoded wild-type copy of vopX that included its endogenous promoter (Fig. 5A). The vopX and vttR_a genes are adjacent and divergently transcribed, with 238 bp between the translational start sites of the two coding sequences (Fig. 6A). We recognized that the ΔvopX deletion, which was constructed to remove almost the entire open reading frame, could impact sequences required for vttR_a expression and thus influence T3SS gene expression. To test our hypothesis, we conducted β-galactosidase assays using isogenic wild-type, ΔvttR_a, and ΔvopX strains carrying plasmid-based lacZ transcriptional reporter fusions to promoters previously shown to be VttR_a regulated: vcsRTCNS2, vcsJ2 (T3SS structural gene operons), and vttR_b (31). Consistent with previously reported results, the vttR_a deletion strain exhibited an ~5- to 15-fold decrease in structural gene expression compared to that of the wild-type strain (Fig. 5B, compare black and gray bars) (31). Interestingly, similar trends were found for the ΔvopX strain (Fig. 5B, compare black and light gray bars). vttR_b expression levels were also decreased, further suggesting that the T3SS regulatory network and, consequently, T3SS gene expression are altered in the vopX deletion strain.

We reasoned that the vopX deletion strain could not cause cytotoxicity due to decreased vttR_a expression rather than the lack of VopX. We thus predicted that expression of vttR_a or vttR_b would complement the cytotoxicity defect. Figure 5C shows the results of such complementation experiments. Coculture of Caco2-BBE cells with the AM-19226 ΔvopX strain expressing either vttR_a or vttR_b resulted in cytotoxicity levels similar those achieved by using the isogenic parental strain (Fig. 5C, compare black and gray bars). We therefore conclude that the ΔvopX phenotype can be complemented by the expression of vttR_a or vttR_b.

To provide additional evidence that cytotoxicity required wild-type levels of vttR_a expression, we constructed a point mutation in vopX that introduced a premature stop codon at amino acid 54 (vopX^*). The strain was therefore VopX null but VttR_a WT. Coculture of Caco2-BBE cells with the AM-19226 vopX^* strain caused cytotoxicity levels similar to those of the parental strain (Fig. 5D). Taken together, these data suggest that the VopX-coding region overlaps vttR_a regulatory sequences that are critical for producing levels of vttR_a expression that in turn result in wild-type T3SS gene expression levels, presumably through precise levels of vttR_b expression.

**DISCUSSION**

Transmembrane transcriptional regulatory proteins are a hallmark of gene regulation in *V. cholerae*, and T3SS-positive strains encode three such regulators: VttR_a, VttR_b, and ToxR. We previously demonstrated that each protein influences T3SS gene expression to different extents, but in the experiments reported here, we found that each protein is essential for bacteria to cause mammalian cell cytotoxicity during *in vitro* coculture. We therefore sought to better understand the bacterial parameters and regulatory network influencing cytotoxicity.

When complementing the vttR_a and vttR_b deletions, the re-
VtrB, based on data from complementation and electrophoretic sequence analyses, with 26 bp between the 238-bp intergenic region. Arrows indicate predicted transcriptional start sites (T3SS). Sequences absent in the transcriptome analyses, with 26 bp between the +1 site and the coding sequence for VtrR. (A) Model based on data presented here and by Alam et al. (31), whereby both positive regulation and negative regulation affect the expression of VtrR. A positive regulator of VtrR expression has not yet been identified, although transcriptional reporter data indicate that expression may be negatively regulated by VtrR itself and ToxR. In this model, each regulatory protein has the ability to act both positively and negatively to modulate transcription.

FIG 6 Schematic of the vopX-vttR locus and model for T3SS gene regulation. (A) vttR and vopX gene positions are indicated relative to the 238-bp intergenic region. Arrows indicate predicted transcriptional start sites (+1) based on transcriptome analyses, with 26 bp between the vopX +1 site and the VopX coding sequence and 60 bp between the +1 site and the coding sequence for VttR, (34). Sequences absent in the vopX deletion strain are indicated by the triangle above the gene. (B) Model based on data presented here and by Alam et al. (31), whereby both positive regulation and negative regulation affect the expression of VttR, a positive regulator of vttR expression has not yet been identified, although transcriptional reporter data indicate that expression may be negatively regulated by VttR itself and ToxR. In this model, each regulatory protein has the ability to act both positively and negatively to modulate transcription.

Results of inducible expression studies suggest that different minimum levels of gene expression are required to achieve wild-type levels of cytotoxicity (Fig. 3). For example, complementation of the ΔvttR strain with the pBAD18-vttR construct occurred only with 0.5% arabinose, whereas a 10-fold lower level of arabinose was sufficient to complement the ΔvttR strain phenotype (Fig. 3). Our results are consistent with previously reported data showing that, in comparison to vttRΔ or lower levels of vttR expression are sufficient to promote T3SS gene expression (32). Adding 0.01% to 0.2% arabinose both in the overnight culture of AM-19226 ΔvttR(pBAD18-vttR) and during coculture did not restore cytotoxicity, again suggesting either that vttR expression was insufficient or, consistent with previously reported results, that vttR overexpression produces a T3SS-null phenotype (32) (data not shown). Although it is unclear at this time whether posttranscriptional regulation is a contributing factor, it is possible that the amounts of protein required for wild-type regulation differ among target promoters. However, we are unable to definitively assess protein levels at this time since efforts aimed at antibody production and the production of tagged proteins have not provided reagents sufficient to conduct comparisons of protein levels from chromosomal versus episomally induced expression.

Previously reported results of studies using transcriptional reporter fusions suggested that VttR acted downstream of VttRΔ and the results of the present study indicate that a similar transcriptional hierarchy likely functions during mammalian cell culture (32). Similar conclusions were drawn for the homologous T3SS regulatory proteins in Vibrio paraehemolyticus, VtrA and VtrB, based on data from complementation and electrophoretic mobility shift assays (36, 37). Interestingly, when the V. cholerae ΔvttR strain was complemented by using vttR, we observed cytotoxicity using a lower arabinose concentration than that for complementation with pBAD18-vttR, (Fig. 3). Our data indicate that dysregulation of vttR also interferes with T3SS gene expression via vttR, since the vopX deletion strain decreased both vttR and structural gene expression levels and was not cytotoxic (Fig. 5). Results of transcriptional reporter fusion assays further support the conclusion that regulatory sequences governing vttR expression extend beyond the vopX-vttR intergenic regions (Fig. 6A) and overlap the vopX open reading frame (Y. Patel, S. Kim, and M. Dziejman, unpublished data).

If we conclude that vttR expression requires tight regulation to produce a WT T3SS phenotype, one interpretation of our collective data is that a single factor regulates vttR expression to fine-tune levels or, alternatively, that ToxR, VtrRΔ, and VttRΔ, itself are all required to appropriately modulate vttR expression levels (Fig. 6B). The two interpretations are not mutually exclusive, and additional data indicate that negative autoregulation may influence both vttR and vttR expression levels and that ToxR negatively regulates vttR expression while positively influencing vttR expression (31) (Fig. 6B). Our collective evidence therefore points to protein levels as an important facet of how VttR and VttR regulate T3SS gene expression, although as discussed above, definitive experiments await the development of sufficient reagents to detect protein levels.

We were surprised to find an absolute requirement for ToxR in the cytotoxicity assay, since T3SS gene expression in the ΔtoxR strain was moderately affected compared to that in the absence of VttR or VttR (31). In addition, a toxR deletion strain is moderately attenuated for colonization in the infant mouse model (~10-fold) compared to strains deleted for vttR or vttR, which show more dramatic colonization defects (31). Our results suggest that the cytotoxicity assay is a very sensitive assay for T3SS function and that results can uncover even small perturbations in gene expression that affect activity. Regardless of a role for ToxR, our current data suggest several possibilities: T3SS gene promoters may respond directly via ToxR interactions, indirectly through modulation of vttR or vttR, or indirectly by altering the expression of a currently unknown factor that contributes to T3SS regulation. Combined with previously reported results and our finding that vttR expression levels are critical for T3SS regulation, we propose that ToxR is acting through vttR to influence T3SS gene expression and activity (Fig. 6B).

We found that toxR expression at the highest arabinose concentration (Fig. 4) did not complement the toxR deletion, consistent with previously reported results showing that increased toxR expression in the wild-type AM-19226 strain leads to decreased trh-lacZ transcriptional reporter fusion expression (32). One interpretation is that overexpression simply results in protein agglomeration or mislocalization. Alternatively, ToxR may act as a negative regulator of T3SS gene expression when expression levels exceed a certain threshold. In epidemic strains, plasmid-based ToxR overexpression can restore virulence gene expression, suggesting that the protein retains activity when produced at elevated transcriptional levels (38, 39). Again, however, we cannot make definitive conclusions about protein levels at this time.

In our current assay, Caco2-BBE cell cytotoxicity requires bile in the medium during V. cholerae coculture. Although bile could be acting on the V. cholerae cells to increase the expression of T3SS genes, expression of the vcsRTCS and T3SS structural gene operon during coculture with 0.2% bile is similar to that in the absence of bile (32). Several other possible explanations remain to be explored. We interpret the results of the gentamicin killing assay as consistent with the MOI data, which further suggest that Caco2-BBE cell death is due to bacterial cell-host cell interactions rather
that a “domino effect” triggered early during coculture by the death of a subset of neighboring Caco2-BBE cells whose cytoplasmic contents are released into the coculture medium. This result is consistent with an increased bacterial burden but also with data indicating that adherence to Caco2-BBE cells requires ~1.5 h of coculture (K. A. Miller, S. Gregoire, and M. Dziejman, unpublished data).

In summary, our collective results support the hypothesis that precise levels of vttRα and vttRβ expression are an important component for wild-type regulation of T3SS gene expression. Furthermore, the ancestral toxR gene product contributes to the regulation of the horizontally acquired T3SS sequences, likely by regulating the expression of one or both of the T3SS genomic island-encoded transcriptional regulators. ToxR has a longstanding role as a critical component of virulence gene regulation in epidemic strains, where it controls hierarchical gene expression that governs the expression of the toxin-coregulated pilus and cholera toxin, which, like the T3SS, are laterally acquired virulence factors (1). It is interesting, then, that proteins encoded on the ancestral chromosome and proteins encoded on laterally acquired islands coordinate regulate virulence gene expression in strains carrying different virulence factors. The conserved topology and sequence similarity of Vttrα, Vttrβ, and ToxR also suggest that the unique features of V. cholerae regulation may provide an advantage for this marine organism in its ability to respond to different environments encountered during its transient residence in the human host.

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REFERENCES


