Vibrio cholerae leuO Transcription Is Positively Regulated by ToxR and Contributes to Bile Resistance

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ABSTRACT

Vibrio cholerae is an aquatic organism and facultative human pathogen that colonizes the small intestine. In the small intestine, *V. cholerae* is exposed to a variety of antimicrobial compounds, including bile. *V. cholerae* resistance to bile is multifactorial and includes alterations in the membrane permeability barrier that are mediated by ToxR, a membrane-associated transcription factor. ToxR has also been shown to be required for activation of the LysR family transcription factor *leuO* in response to cyclic dipeptides. LeuO has been implicated in the regulation of multiple *V. cholerae* phenotypes, including biofilm production and virulence. In this study, we investigated the effects of bile on *leuO* expression. We show that *leuO* transcription increased in response to bile and bile salts but not in response to other detergents. The bile-dependent increase in *leuO* expression was dependent on ToxR, which was found to bind directly to the *leuO* promoter. The periplasmic domain of ToxR was required for basal *leuO* expression and for the bile-dependent induction of both *leuO* and *ompU* transcription. *V. cholerae* mutants that did not express *leuO* exhibited increased bile susceptibility, suggesting that LeuO contributes to bile resistance. Our collective results demonstrate that ToxR activates *leuO* expression in response to bile and that LeuO is a component of the ToxR-dependent responses that contribute to bile resistance.

IMPORTANCE

The success of *Vibrio cholerae* as a human pathogen is dependent upon its ability to rapidly adapt to changes in its growth environment. Growth in the human gastrointestinal tract requires the expression of genes that provide resistance to host antimicrobial compounds, including bile. In this work, we show for the first time that the LysR family regulator LeuO mediates responses in *V. cholerae* that contribute to bile resistance.

*V*ibrio cholerae* is a Gram-negative bacterial pathogen and the causal agent of the severe diarrheal disease cholera. *V. cholerae* exists naturally in aquatic reservoirs and is capable of colonizing the human small intestine. The transition of *V. cholerae* from the aquatic ecosystem to growth in the human gastrointestinal tract is mediated by transcriptional responses that are required for colonization and disease development. Many of the genes that contribute to intestinal colonization are under the control of the membrane-associated regulatory protein ToxR, which functions as one of the primary regulators in the ToxR regulon (reviewed in reference [1]). The ToxR regulon is divided into two branches, a ToxT-dependent branch, which controls the expression of virulence factors, and a ToxT-independent branch, which reciprocally regulates the production of the outer membrane porins OmpU and OmpT. The ToxT-dependent branch of the ToxR regulon is a hierarchical regulatory cascade that regulates the expression of genes encoding the production of cholera toxin (CT) and the toxin-coregulated pilus (TCP) in response to environmental cues in the host.

ToxR is a membrane-associated regulatory protein that belongs to the winged-helix family of transcriptional regulators [2, 3]. ToxR is a one-component signal-transducing protein that is composed of a periplasmic signaling domain and a cytoplasmic DNA binding domain that are linked by a single transmembrane-spanning domain [4]. *toxR* is borne on an operon along with *toxS*, which is located downstream of *toxR*. ToxS is an inner membrane protein, which is thought to interact with ToxR to facilitate its transcriptional activity [5, 6]. The ToxR periplasmic signaling domain is thought to sense and transduce environmental stimuli to

affect the activity of the cytoplasmic DNA binding domain at its target genes. ToxR has been shown to respond to a variety of environmental stimuli, including acidity, nutrient availability, salinity, small molecules, and bile [4, 7–9].

It has been shown that ToxR plays an essential role in modulating adaptive responses that contribute to bile resistance. Bile is produced by the liver and secreted at high concentrations into the small intestine to aid in the digestion of lipids. Bile is composed primarily of bile salts but also contains significant amounts of phospholipids, cholesterol, protein, and bilirubin. While bile is important in digestion, bile also provides a barrier against intestinal colonization by restricting bacterial growth in the small intestine, presumably through its detergent-like effects on bacterial cell membranes [10]. As such, enteric pathogens have evolved methods to overcome this barrier. This includes the modulation of outer membrane porin proteins to decrease the rate of diffusion of toxic molecules across the outer membrane and the expression of active efflux systems that remove bile salts from within the cell

envelope ([11]–[15]). In *V. cholerae*, resistance to the antimicrobial effects of bile is due to the combined actions of multiple factors, including active efflux- and ToxR-regulated genes. This is evident from the observation that *toxR* mutant strains exhibit greatly increased susceptibility to bile and bile salts ([16]).

The elevated susceptibility of *toxR* mutant strains to bile salts has been linked to the expression of the *ompU* and *ompT* porins ([16]). OmpU and OmpT are general diffusion porins located in the outer membrane. Porins are responsible for allowing the diffusion of nutrients, metabolites, and signaling molecules into and out of the cell ([17]). OmpU and OmpT have been found to be regulated in response to environmental stimuli, including bile, osmolarity, organic acids, cyclic dipeptides, and amino acids ([4], [7]–[9]). The genes encoding OmpU and OmpT are reciprocally regulated by ToxR, which binds to conserved direct repeat elements that are located in the *ompU* and *ompT* promoters ([18], [19]). ToxR positively regulates *ompU* and negatively regulates *ompT*. OmpU is preferentially produced during growth in rich medium or in minimal medium containing certain amino acids, bile, or mucin ([20]). In nutrient-poor environments or in a *toxR* mutant strain, the porin profile is reversed, and OmpT becomes predominant, while OmpU is no longer produced. Consistently with the activation of *ompU* expression by bile salts, the production of OmpU is associated with bile salt resistance, while the production of OmpT is associated with bile salt susceptibility ([21]). This phenotype is presumably related to the fact that OmpU, in contrast to OmpT, is an anion-selective porin, which restricts the passage of negatively charged compounds ([21], [22]).

Previous studies in our laboratory suggested that in *V. cholerae*, ToxR activated *leuO* expression in response to cyclo(Phe-Pro) (cFP) ([23]). Increased *leuO* transcription was linked to downregulation of the ToxR regulon and attenuated CT and TCP production. These results suggested that *leuO* functioned downstream of ToxR to modulate gene expression in response to environmental cues. LeuO is a LysR family transcription factor that was first identified as a regulator of leucine biosynthetic genes in *Salmonella enterica* serovar Typhimurium ([24]). Subsequent studies have shown LeuO to be a global regulator of diverse and unrelated phenotypes in the *Enterobacteriaceae*. For example, in *S. enterica*, LeuO has been shown to regulate outer membrane proteins, virulence genes, transport genes, biofilm production, and quorum sensing ([25]). Likewise, LeuO has been shown to be involved in the regulation of genes involved in carbohydrate utilization, phage resistance, acid shock, temperature adaptation, and biofilm production in *Escherichia coli* ([26]). LeuO has also been associated with virulence gene regulation in *Yersinia enterocolitica* ([27]). The function of LeuO as a global regulator appears to be conserved in the *Vibrionaceae*, in which LeuO has been shown to contribute to biofilm production, cell wall degradation, and virulence gene regulation ([23], [28]–[30]).

Since bile acids are an important environmental cue during *V. cholerae* pathogenesis, and since ToxR regulates the expression of many of its target genes in response to bile salts, we tested the hypothesis that *leuO* expression was also modulated in response to bile via ToxR. The results of our experiments showed that *leuO* expression was activated upon exposure to bile salts by a mechanism that was dependent on ToxR. Multiple approaches were used to show that ToxR acted directly at the *leuO* promoter and that the ToxR periplasmic domain was required for basal *leuO* expression and the bile-dependent induction of *leuO* and *ompU* expression.

Mutants that failed to express *leuO* exhibited reduced survival upon exposure to lethal concentrations of bile, indicating that LeuO contributed to bile resistance. Collectively, our results indicated that ToxR activated *leuO* and *ompU* expression in response to bile salts by a mechanism that was dependent on the ToxR periplasmic signaling domain and that LeuO contributed to the ToxR-mediated bile resistance.

MATERIALS AND METHODS

Strains, media, and chemicals. The bacterial strains used in this study are listed in Table 1. *E. coli* strain EC100λ*pir* was used for cloning and strain SM10λ*pir* for plasmid mobilization. The *V. cholerae* strains used in this study were derivatives of O1 El Tor strain N16961 ([31]). *V. cholerae* strain N16961 Δ*lacZ* Sm^r (Sm^r, streptomycin resistant) was used as the wild-type (WT) control for all experiments. Bacterial strains were grown at 37°C in lysogeny broth (LB) or on LB agar. Modified T medium was prepared as previously described ([20]). Bacterial stocks were maintained in LB containing 25% glycerol at −80°C. Antibiotics were added to the growth medium at 100 μg/ml for carbenicillin (Cb) and streptomycin (Sm) and 25 μg/ml for chloramphenicol (Cm), as required. Arabinose was added to the growth medium to induce expression from the arabinose-regulated promoter in pBAD18 and pBAD33. Stock solutions of the detergents and bile (Oxgall; Difco) were made in water and filter sterilized before use.

Plasmid and mutant construction. The plasmids and oligonucleotides used in this study are listed in Table 1. Genomic DNA from N16961 Δ*lacZ* Sm^r was used as a PCR template for cloning. Plasmid reporters containing derivatives of the *leuO* promoter lacking one or both ToxR binding sites were constructed as follows. pVA258 (P$_{leuO}$ lacking the distal ToxR binding site) was generated by PCR using the P$_{leuO1}$-F and P$_{leuO}$-R oligonucleotide primers. The resulting amplicon was digested with BamHI and XmaI restriction endonucleases and ligated into similarly digested pTL61T. pVA261 (P$_{leuO}$ lacking both ToxR binding sites) was generated by PCR using the P$_{leuO2}$-F and P$_{leuO}$-R primers. The resulting PCR amplicon was digested with BamHI and XmaI restriction endonucleases and ligated into similarly digested pTL61T. The *leuO* expression vector pVA94 (pBAD18::*leuO*) was constructed by moving *leuO* from pXB298 as a NheI and XbaI restriction fragment into the same sites in pBAD18. pXB302 (pBAD33::*toxRS*) was made by moving the *toxRS* genes from pXB289 as a SacI and SphI restriction fragment into the same sites in pBAD33. pDT1391 (pBAD33::*toxR*$^{\Delta ppd}$*S*) (ppd, periplasmic domain) was made by moving the *toxR*$^{\Delta ppd}$*S* genes from pXB286 as a SacI and SmaI restriction fragment into the same sites in pBAD33. pWM91::Δ*toxR*^{ppd}, which contains a 94-amino-acid C-terminal deletion of the ToxR periplasmic domain, was made by crossover PCR, as previously described ([32], [33]). Briefly, primer pairs *toxR*$^{\Delta ppd}$-F1/*toxR*$^{\Delta ppd}$-R2 and *toxR*$^{\Delta ppd}$-F2/*toxR*$^{\Delta ppd}$-R1 were used in separate PCRs with N16961 genomic DNA. The resulting ~1-kb amplicons were collected and used as the template for second-round PCR amplification with the flanking *toxR*$^{\Delta ppd}$-F1/*toxR*$^{\Delta ppd}$-R1 PCR primers. The resulting ~2-kb amplicon was then digested with SacI and SmaI restriction endonucleases before being ligated into similarly digested pWM91.

Deletion of the ToxR periplasmic domain in *V. cholerae* strain SS4 was accomplished as follows. pWM91::Δ*toxR*^{ppd} was conjugated into *V. cholerae* strain JB58, and plasmid cointegrants were selected for Sm and Cb resistance. Sm- and Cb-resistant cointegrants were then plated onto LB agar plates containing 5% sucrose and no NaCl. Sucrose-resistant and Cb-sensitive colonies were then screened by PCR using the *toxR*$^{\Delta ppd}$-F1/*toxR*$^{\Delta ppd}$-R1 primers to confirm the deletion of the ToxR periplasmic domain. Verification of *toxR*$^{\Delta ppd}$ in SS4 was accomplished by DNA sequencing of the *toxR* locus.

Reporter assays. β-Galactosidase assays were performed as follows. *V. cholerae* strains carrying the indicated *leuO-lacZ* reporters were cultured overnight in LB at 37°C with shaking. The cultures were then diluted 1:100 into fresh LB and incubated at 37°C with shaking. Culture aliquots were

TABLE 1 Strains, plasmids, and oligonucleotides used in this study

Strain, plasmid, or oligonucleotide	Relevant characteristics or sequence (5′ to 3′)[a]	Source or reference
Strains		
E. coli		
EC100λ*pir*	*supE44 ΔlacU169 (φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 (λpirR6K)*	Epicentre
SM10λ*pir*	*thi-1 thr leu tonA lacY supE recA::RP4-2-Tc::mu* Kmʳ *(λpirR6K)*	Lab collection
V. cholerae		
JB58	O1 El Tor strain N16961 Δ*lacZ* Smʳ	Lab collection
XBV222	JB58 Δ*leuO*	23
DT733	JB58 Δ*toxRS*	23
SS4	JB58 *toxR*^Δppd	This study
Plasmids		
pCM10	*luxCDABE* reporter plasmid, Kmʳ	51
pJB906	pCM10 containing *leuO* promoter	23
pTL61T	*lacZ* transcriptional reporter plasmid, Cbʳ	52
pXB266	pTL61T containing *leuO* promoter region with two ToxR binding sites	
pVA258	pTL61T containing *leuO* promoter region with one ToxR binding site	This study
pVA261	pTL61T containing *leuO* promoter region with no ToxR binding sites	This study
pXB233	pTL61T containing *vexRAB* promoter	53
pXB228	pTL61T containing *vexEF* promoter	54
pXB229	pTL61T containing *vexGH* promoter	54
pXB230	pTL61T containing *vexIJK* promoter	54
pXB231	pTL61T containing *vexCD* promoter	54
pXB232	pTL61T containing *vexLM* promoter	54
pBAD18	Arabinose-regulated expression plasmid, Cbʳ	55
pBAD33	Arabinose-regulated expression plasmid, Cmʳ	55
pXB298	pBAD18Km expressing *leuO*	23
pVA94	pBAD18 expressing *leuO*	This study
pXB289	pBAD18 expressing *toxRS*	23
pXB286	pBAD18 expressing *toxR*^Δppd*S*	23
pXB302	pBAD33 expressing *toxRS*	This study
pDT1391	pBAD33 expressing *toxR*^Δppd*S*	This study
pWM91	Suicide plasmid vector used for allelic exchange	41
pWM91::Δ*toxR*^ppd	pWM91 containing a fragment of *toxR* harboring a deletion of periplasmic domain	This study
Oligonucleotides		
P*leuO1*-F	CGCCCGGGAAATGCATTTTTATAGATTTTT	
P*leuO2*-F	CGCCCGGGAATCGTATTGATTATTAAGGCT	
P*leuO*-R	GGGGATCCGCGTCTTTTTTATCTAACATTTGCATGCCT	
toxR^Δppd-F1	GGGGAGCTCGGTCCTCAAAAGAGATAT	
toxR^Δppd-F2	CTGCTCACTAACTAGGATCTTGCTAT	
toxR^Δppd-R1	AACCCGGGCATGCCGCTCAGTCAGG	
toxR^Δppd-R2	AGCAAGATCCTAGTTAGTGAGCAGTA	
5′BIO	GCGGGAGTCGGCAGCG	
leuO-F-EMSA	GCGGGAGTCGGCAGCGGTTAAAACATTTTTGACGTGAATATTAGTG	
leuO-R-EMSA	GCGGGAGTCGGCAGCGCGTCACTAGCGATAAATATGCATAAATC	
ompU-F-EMSA	GCGGGAGTCGGCAGCGCAATTAGATTGCGTGCATTT	
ompU-R-EMSA	GCGGGAGTCGGCAGCGTTTTTTTTACTCCCAAAGTTC	
vexR-F-EMSA	GCGGGAGTCGGCAGCGTGCAAAACAGGGGGTATTAG	
vexR-R-EMSA	GCGGGAGTCGGCAGCGGCCGTACACTATTTCAGACA	
leuO-qPCR-F	GACCACTTCGCCACAAATCACCA	
leuO-qPCR-R	CGTTGGATGGCGGAAAATGCG	
ompU-qPCR-F	ACACCGTATAGGCTGTCATTG	
ompU-qPCR-R	GTGCTGAAGCTCGCCTATCTC	
gyrA-qPCR-F	CAATGCCGGTACACTGGTACG	
gyrA-qPCR-R	AAGTACGGATCAGGGTCACG	

[a] Kmʳ, kanamycin resistant; Cbʳ, carbenicillin resistant; Cmʳ, chloramphenicol resistant.

collected in triplicate at mid-exponential phase (optical density at 600 nm [OD₆₀₀], ~0.5) to quantify β-galactosidase activity, as previously described (34). The *E. coli* two-plasmid reporter experiments were performed as follows. *E. coli* bearing an expression plasmid (pBAD33, pXB302, or pDT1391) and a *lacZ* reporter plasmid (pXB266, pVA258, or pVA261) was cultured overnight in LB with shaking at 37°C. The overnight cultures were then diluted 1:100 into fresh LB with or without 0.08% arabinose, and the cultures were incubated at 37°C with shaking. Culture

aliquots were collected in triplicate at mid-exponential phase (OD_{600}, ~0.5) to quantify β-galactosidase activity, as previously described (34). All of the reporter experiments were performed independently at least three times. Expression from the *lacZ* reporter was calculated and is given in Miller units (MU).

The bioluminescence assays were performed as follows. *V. cholerae* strain JB58 containing pJB906 (*leuO-lux*) was cultured overnight in LB at 37°C with shaking. The overnight cultures were then diluted 1:100 into fresh LB and incubated at 37°C with shaking for 2 h. Aliquots (100 μl) of the culture were then diluted into 100 μl of LB plus the indicated substrates (i.e., dimethyl sulfoxide [DMSO], deoxycholate [DOC], or cFP) and distributed into triplicate wells of a white 96-well microtiter plate with a clear bottom (Corning). In these experiments, DOC was used at 0.0125%, cFP at 1 mM, and DMSO at 0.1%. The plates were then incubated at 37°C, and luminescence and the OD_{600} were measured at the time points indicated in Fig. 1 using a BioTek Synergy HT plate reader. The relative light units (RLU) for each sample were calculated by dividing the luminescence by the OD_{600}. The reported results are the averages and standard deviations (SD) of the results from three independent experiments.

Bile killing assay. Overnight cultures of the indicated strains were diluted 1:100 in LB with or without 0.1% arabinose (to induce expression from pBAD18) and incubated at 37°C with shaking for 4 h. Culture aliquots were then collected and washed once with phosphate-buffered saline (PBS) before being diluted in PBS to an OD_{600} of 0.5. Serial dilutions of each strain were then plated onto LB agar plates to determine the cell titer at time zero (CFU_{input}). Aliquots containing ~10^6 CFU of each strain were then added to PBS containing 10% bile (*toxR* mutant strains) or 20% bile (*toxR*-positive strains); the bile concentrations were reduced for the *toxR* mutant strains due to their increased bile sensitivity. The cultures were then incubated statically at room temperature for 60 min, at which point the aliquots were collected, washed in PBS, and plated onto LB agar to quantify the viable cells (CFU_{output}). The recovery ratio of each strain was then calculated as CFU_{output}/CFU_{input}. The fold change in recovery was then determined by dividing the recovery ratio for the mutant strains by the recovery ratio for the WT. The fold change in recovery for the *leuO*-overexpressing strain was determined by dividing the recovery ratio for the Δ*toxRS*::pBAD18-*leuO* mutant by the recovery ratio for the Δ*toxRS*::pBAD18 mutant. The presented data are the means and standard deviations (SD) of the results from three independent experiments. Statistical significance was determined using Dunnett's multiple-comparison test relative to a recovery ratio of 1.0.

RNA extraction and quantitative real-time PCR. RNA was extracted from *V. cholerae* cultures grown in modified T medium at 37°C with shaking to an OD_{600} of ~0.3, at which point a mixture of the amino acids asparagine, arginine, glutamic acid, and serine (NRES) (50 mM) or bile (0.2%) was added to the cultures. The cultures were then incubated for an additional 15 min at 37°C with shaking before RNA was extracted using TRIzol, according to the manufacturer's instructions (Ambion). The resulting RNA was quantified using a NanoDrop spectrophotometer (Thermo) before being used to make cDNA with the Maxima first-strand cDNA synthesis kit (Life Technologies), according to the manufacturer's instructions. The resulting cDNA was then used with gene-specific primers (Table 1) and the SYBR green PCR master mix (Thermo) to quantify gene expression using a StepOnePlus real-time PCR system (Applied Biosystems). The relative gene expression levels were then calculated using the $2^{(-\Delta\Delta CT)}$ method (where CT is the threshold cycle) (35) using the A subunit of DNA gyrase (*gyrA*) as an internal control. The presented results are the means ± standard deviations of the results from three independent biological replicates, with each biological replicate being generated from at least two technical replicates.

Membrane isolation. *V. cholerae* strain DT733 (Δ*toxRS*) containing pXB289 (pBAD18::*toxRS*), pXB286 (pBAD18::*toxR*$^{\Delta Ppd}S$), or pBAD18 was cultured in LB with shaking at 37°C to an OD_{600} of ~1.0, at which point expression from the arabinose promoter was induced by the addi-

tion of arabinose to 0.2%. The cultures were then incubated with shaking at 37°C for an additional hour, and then the cells were harvested by centrifugation. The cell pellet was resuspended in 10 mM Tris-HCl (pH 8.0) in 0.75 M sucrose. Spheroplasts were then induced by the addition of 150 μg/ml lysozyme, followed by the addition of 2 volumes of buffer A (10 mM Tris-HCl [pH 8.0], 10 mM EDTA) (36, 37). The cells were then lysed by passage through a model M-11P Microfluidizer (Microfluidics). Particulate matter was removed from the cell lysate by centrifugation at $8,000 \times g$ and 4°C for 10 min. The membrane-containing supernatant was then subjected to centrifugation in an SW-28 rotor (Beckman Coulter) at 24,000 rpm and 4°C for 2 h to pellet the membrane fraction. The membrane pellet was then suspended in 20% sucrose in buffer B (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) and applied to a two-step 60% and 70% sucrose gradient. The sucrose gradients were then subjected to centrifugation in an SW-28 rotor at 23,000 rpm and 4°C for 18 h. The inner and outer membrane fractions were then decanted from the sucrose gradients and diluted with 2 volumes of cold buffer B before being centrifuged in a Ti55 rotor (Beckman Coulter) at 47,000 rpm and 4°C for 1 h. The resulting inner membrane pellets were resuspended in cold buffer B and frozen at −20°C until used. Protein concentrations were determined using the Pierce Coomassie Plus protein assay, according to the manufacturer's directions.

EMSA. DNA probes for gel shift assays were generated by PCR using the primers listed in Table 1. The PCR primers for the electrophoretic mobility shift assays (EMSAs) were engineered to include a 5′ tail (5′-GC GGGAGTCGGCAGCG-3′), which facilitated biotinylation of the probes by a second PCR using the 5′BIO PCR primer, which hybridized to the 5′ tail. The 5′BIO PCR primer was purchased from the manufacturer (IDT) with a 5′ biotin label. The biotinylated probes were gel purified and quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific) before being used in the EMSAs. The DNA binding reactions were performed in a final volume of 10 μl of binding buffer (10 mM Tris-HCl [pH 7.4], 5 mM NaCl, 50 mM KCl, 1 mM EDTA, 50 μg/ml bovine serum albumin [BSA], 1.5 nM biotinylated probe, and 10 μg/ml sheared salmon sperm DNA) containing the indicated amounts of the *V. cholerae* inner membranes. The binding reaction mixtures were incubated at 30°C for 30 min before being subjected to electrophoresis at 150 V for 1 h on a nondenaturing 5% polyacrylamide Tris buffer-EDTA (TBE) gel that had been prerun with 5% thioglycolic acid for 1 h at 150 V in 1× TBE buffer. The resolved gels were electroblotted to positively charged nylon membranes in 0.5× TBE buffer at 380 mA for 1 h, before the nylon membrane was UV cross-linked at 120,000 μJ using a Stratalinker 1800 cross-linker (Stratagene). The biotinylated probes were then detected using the Pierce chemiluminescent nucleic acid detection module (Thermo Scientific) and visualized using a FluorChem E imaging system (ProteinSimple).

RESULTS

Bile salts induce expression of *leuO*. In response to bile salts, ToxR has been shown to activate *ompU* and *ctxAB* expression while repressing *ompT* expression (16, 38). Given that ToxR activates the expression of at least some of its target genes in response to bile salts, we hypothesized that ToxR may also regulate *leuO* expression in response to bile salts. To test this hypothesis, we introduced a *leuO-lacZ* reporter plasmid, pXB266, into WT strain JB58. The resultant strain was then cultured in the presence of bile or the bile salt deoxycholate to the mid-logarithmic growth phase, when *leuO* expression was quantified by the β-galactosidase assay. The results showed an ~5-fold increase in *leuO* expression in the presence of bile and an ~6-fold increase in *leuO* expression in the presence of deoxycholate compared to that in the LB control (Fig. 1A). These results confirmed the hypothesis that *leuO* expression was upregulated in the presence of bile or deoxycholate.

Bile salts and other components of bile exhibit detergent-like properties that can affect the permeability barrier of the outer
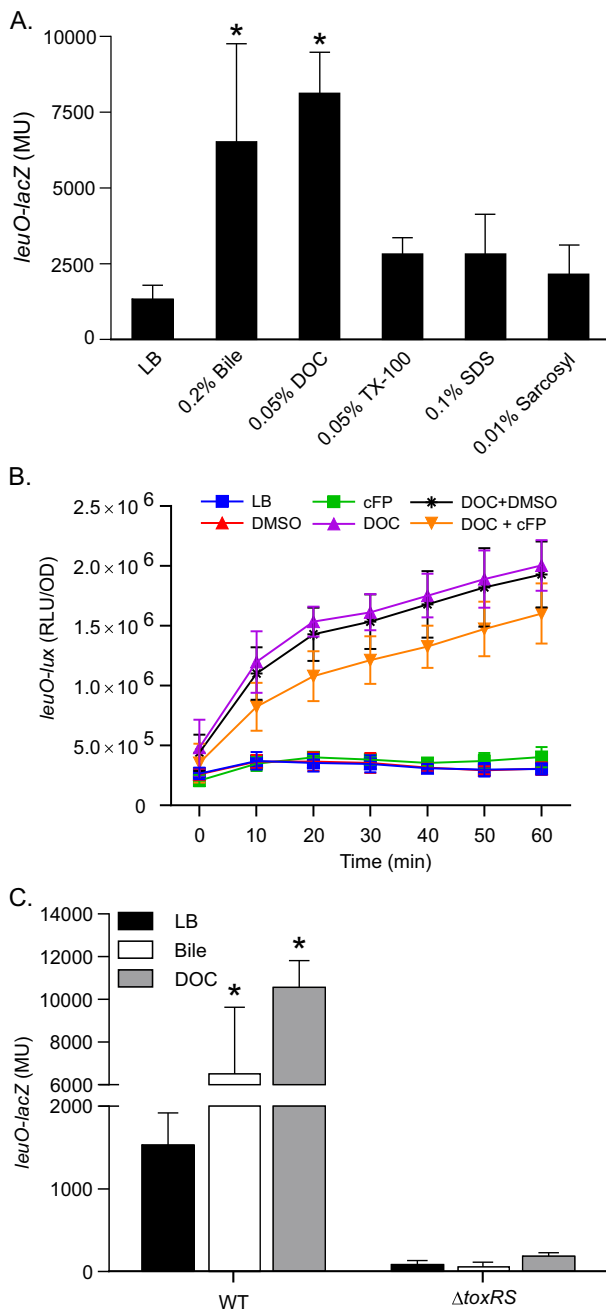
FIG 1 Induction of *leuO* transcription by bile and bile salts requires ToxR. (A) WT strain JB58 carrying the *leuO-lacZ* reporter plasmid pXB266 was grown in LB or LB supplemented with bile or the indicated detergents. Culture aliquots were collected at mid-logarithmic phase and assayed for *leuO-lacZ* expression by the β-galactosidase assay. (B) Expression of *leuO-lux* in the WT strain JB58::pJB906 (*leuO-lux*) during growth in LB containing the indicated compounds. DOC was used at 0.0125%, cFP at 1 mM, and DMSO at 0.1%. (C) WT strain JB58 and Δ*toxRS* strain DT733 carrying pXB266 (*leuO-lacZ*) were grown in LB or LB plus 0.2% bile or 0.1% deoxycholate. Culture aliquots were collected at mid-logarithmic phase and assayed for *leuO-lacZ* expression by the β-galactosidase assay. The presented data are the means ± SD of the results from three independent experiments. Statistical significance in panel A was determined using one-way analysis of variance (ANOVA) with Dunnett's posttest, comparing each mean with that for the LB control; *, $P < 0.01$. Statistical significance in panel B was determined using two-way ANOVA with Tukey's posttest; *, $P < 0.0001$. DOC, deoxycholate; TX-100, Triton X-100; SDS, sodium dodecyl sulfate; MU, Miller units; DMSO, dimethyl sulfoxide; RLU, relative light units.

membrane and compromise the integrity of the cytoplasmic membrane (10). This alluded to the possibility that *leuO* was upregulated as a result of the deleterious effects from the detergent properties of bile/deoxycholate on the cell envelope. If this were true, we hypothesized that exposure of *V. cholerae* to other classes of membrane-active detergents should also result in *leuO* upregulation. We therefore tested the effects of two anionic detergents (SDS and sarcosyl) and one nonionic detergent (Triton X-100) on *leuO-lacZ* expression. The results showed that exposure of JB58(pXB266) to these three detergents did not significantly affect *leuO* expression (Fig. 1A). Taken together, these results suggested that *leuO* induction was specific for bile and bile salts and was probably not a result of the detergent-like properties of bile or deoxycholate.

The finding that bile salts induced *leuO* expression prompted us to investigate if cFP and bile salts functioned synergistically to regulate *leuO* expression. To test this, we quantified *leuO* expression in the WT strain JB58 containing pJB906 (*leuO-lux*) during growth in LB containing DOC, cFP, or DOC and cFP. As expected, the addition of DOC to the medium activated *leuO* expression (Fig. 1B). However, the addition of cFP to the medium did not significantly increase *leuO* expression compared to that in the DMSO control. cFP did activate *leuO* expression at high (nonphysiological) concentrations of cFP (data not shown). As cFP was shown to activate *leuO* expression under AKI growth conditions, this result suggests that cFP activity is dependent on the growth conditions (23). The addition of both DOC and cFP to the growth medium resulted in a small but reproducible decrease in *leuO* expression compared to that in cells grown in medium containing DOC and DMSO. Although the presence of DOC and cFP appeared to decrease *leuO* expression, the differences were not statistically significant. Based on these results, we concluded that cFP and DOC do not work synergistically to increase *leuO* expression during growth under standard laboratory conditions. It remains to be determined how cFP and DOC affect *leuO* expression *in vivo*.

Upregulation of *leuO* by bile is dependent on ToxR. ToxR regulates OmpU and OmpT production in response to bile salts. This suggested the possibility that ToxR is responsible for the bile-dependent upregulation of *leuO* in response to bile and bile salts. To test this, we compared levels of *leuO-lacZ* expression in JB58(pXB266) and an isogenic Δ*toxRS* mutant, DT733(pXB266), which had been cultured in the presence and absence of bile or deoxycholate, as described above. The results showed that *leuO* expression in JB58 was increased upon exposure to deoxycholate and bile relative to growth in LB alone (Fig. 1C), as shown above. In contrast, *leuO* expression in the Δ*toxRS* mutant grown in LB decreased by ~15-fold relative to that in JB58. This indicates that ToxR is a positive regulator of *leuO*, confirming previous findings (23). The deletion of *toxRS* also abolished the bile- and deoxycholate-dependent upregulation of *leuO* (Fig. 1C). Together, these data indicated that ToxR was required for basal *leuO* expression and the increased *leuO* expression in response to bile and bile salts. Further, given that none of the other tested detergents affected *leuO* expression, we speculate that the ToxR-dependent upregulation of *leuO* was specific for bile salts and was not due to other components of bile or to a general membrane stress response.

ToxR acts directly on the *leuO* promoter. Previous studies showed that ToxR binds to direct repeat elements that represent a ToxR consensus binding sequence in the *toxT*, *ompU*, *ompT*, and
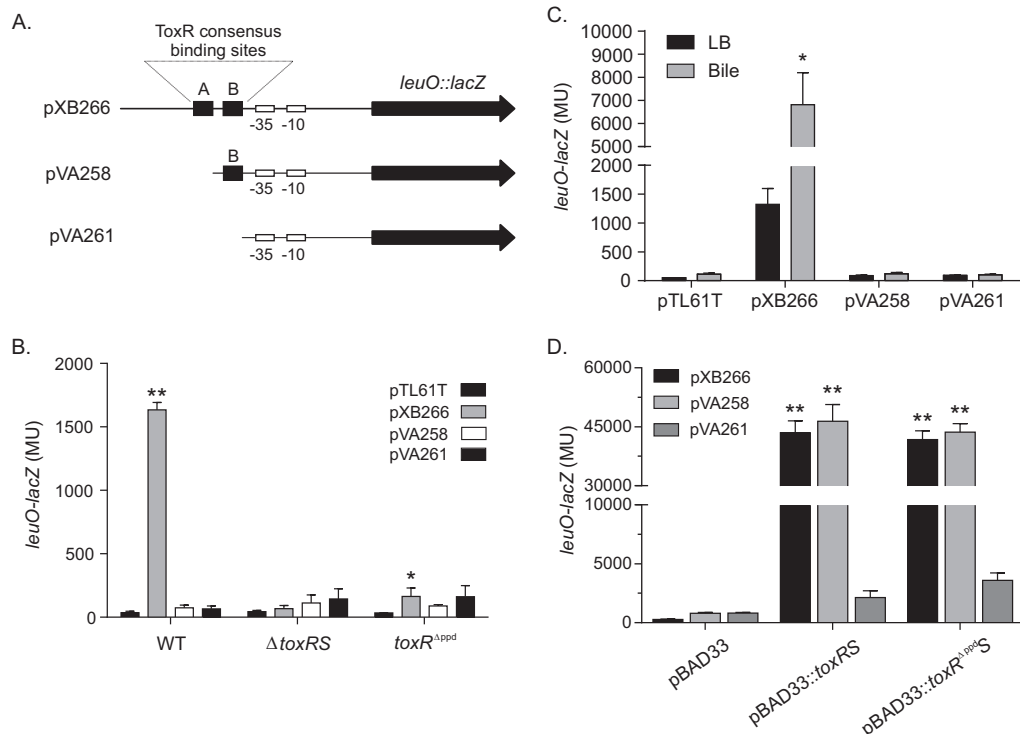
FIG 2 ToxR consensus binding sequences are required for ToxR activation of *leuO* transcription. (A) Schematic diagram of the *leuO* promoter in the indicated *leuO-lacZ* reporter plasmids. The locations of the two putative ToxR consensus binding sites are indicated by the letters A (distal) and B (proximal). The putative −10 and −35 promoter elements are indicated. pXB266 contains the native *leuO* promoter, which contains both ToxR consensus binding sites, pVA258 contains only the proximal ToxR binding site, and pVA261 lacks both ToxR binding sites but maintains the −35 and −10 basal promoter elements. (B) WT strain JB58, Δ*toxRS* strain DT733, and *toxR*^{ΔPpd} strain SS4 containing the indicated *leuO-lacZ* reporter plasmids were grown in LB to mid-logarithmic phase, at which point aliquots were collected and assayed for *leuO-lacZ* using the β-galactosidase assay. (C) WT strain JB58 containing the indicated *leuO-lacZ* reporter plasmids was grown in LB or LB plus 0.2% bile to mid-logarithmic phase, at which point aliquots were collected and assayed for *leuO-lacZ* expression using a β-galactosidase assay. (D) *E. coli* strains containing the indicated *toxRS* expression plasmid pBAD33::*toxRS*, pBAD33::*toxR*^{Δppd}*S*, or pBAD33 and one of the *leuO-lacZ* reporter plasmids, pXB266, pVA258, or pVA261, were grown in LB containing 0.08% arabinose to mid-logarithmic phase, at which point aliquots were collected and assayed for *leuO-lacZ* expression using the β-galactosidase assay. Statistical significance in panel B was determined using two-way ANOVA with Dunnett's posttest, comparing the mean result for each plasmid to that for the control, pTL61T, in the designated strain. Statistical significance in panel C was determined using two-way ANOVA with Tukey's posttest, comparing the result for the overexpression plasmid to that of the pBAD33 control plasmid with the designated second plasmid. *, $P < 0.05$; **, $P < 0.0001$.

ctxAB promoters to regulate their transcription (4, 18, 19, 39, 40). The gene encoding LeuO (VC2485) is located downstream from VC2486 in an apparent two-gene operon. Sequence analysis of the *leuO* promoter revealed the presence of two putative ToxR consensus binding sequences, suggesting that ToxR may directly regulate *leuO* expression (23). The distal ToxR binding site (i.e., site A in Fig. 2A) is located from −126 to −112 relative to the start codon for VC2486, while the proximal ToxR binding site (i.e., Fig. 2A, site B) is located on the complementary strand from −104 to −90 relative to the start codon for VC2486. To determine if both ToxR binding sites were required for *leuO* expression, derivatives of the *leuO* promoter lacking one or both ToxR consensus sequences were transcriptionally fused to the *lacZ* gene in pTL61T. All together, we generated three *leuO-lacZ* reporters: pXB266 (WT *leuO* promoter), pVA258 (deletion of the distal ToxR binding site), and pVA261 (no ToxR binding sites) (Fig. 2A).

We introduced these three *leuO-lacZ* reporter plasmids into WT strain JB58 and the Δ*toxRS* mutant strain DT733. The resulting strains were then grown to mid-logarithmic phase, when *leuO-lacZ* expression was quantified using β-galactosidase assays (Fig. 2B). Consistently with the above data, the results of these

tests showed high levels of β-galactosidase production in JB58 containing the native *leuO* promoter (i.e., pXB266) and very little β-galactosidase production in the Δ*toxRS* mutant containing the same promoter reporter. In contrast, very little β-galactosidase was produced in JB58 or the Δ*toxRS* mutant containing the *leuO* reporters lacking one (pVA258) or both (pVA261) ToxR consensus binding sites. These results confirmed that ToxR was required for *leuO* expression and suggested that both sites A and B were required for basal *leuO* expression in *V. cholerae*.

We next tested to see if the addition of bile could bypass the requirement for both ToxR binding sites in the *leuO* promoter to induce *leuO* expression. We therefore cultured WT strain JB58 bearing the three *leuO-lacZ* reporter plasmids (Fig. 2A) in the presence and absence of bile and quantified *leuO* expression. The results showed that only the full-length *leuO* promoter (i.e., pXB266) supported the activation of *leuO* expression in the presence of bile (Fig. 2C). Very little β-galactosidase was produced in JB58 containing the *leuO* reporters lacking one (pVA258) or both (pVA261) ToxR consensus binding sites in the presence or absence of bile. These results indicated that both ToxR binding sites are required for the bile-dependent induction of *leuO* expression.

The presence of the ToxR binding sequences in the *leuO* promoter suggested that ToxR acts directly on the *leuO* promoter. If this is true, we hypothesized that ToxR expression in a heterologous host would result in activation of the *leuO* promoter. To test this, we expressed *toxRS* from the pBAD33 arabinose-inducible promoter in an *E. coli* host that contained each of the three *leuO-lacZ* reporters described above (i.e., pXB266, pVA258, and pVA261). We cultured the recombinant strains to mid-logarithmic phase in the presence of arabinose (to induce *toxRS* expression) before quantifying *leuO-lacZ* expression using β-galactosidase assays. The results showed that the expression of *toxRS* from pBAD33::*toxRS* resulted in high and equal levels of expression from the native *leuO* promoter (pXB266) and the promoter lacking the distal ToxR binding site (pVA258) (Fig. 2D). β-Galactosidase production was greatly diminished in the strain bearing the *leuO* promoter that lacked both ToxR consensus sequences (pVA261). The ~18-fold increase in *leuO-lacZ* expression in the *E. coli*(pBAD33::*toxRS*) cultures bearing pXB266 and pVA258 relative to the strain bearing pVA261 suggests that ToxR directly binds to the ToxR consensus binding sites in the *leuO* promoter to facilitate activation of *leuO* transcription in *E. coli*. These results also imply that in *E. coli*, in contrast to *V. cholerae*, ToxR can bind to the proximal ToxR consensus site in pVA258 to activate transcription.

Activation of *leuO* requires the ToxR periplasmic domain. The periplasmic domain of ToxR was shown to be important for the cFP-dependent activation of *leuO* expression (23) but dispensable for basal *ompU* expression and virulence factor production (2, 23). We therefore examined whether the ToxR periplasmic domain contributed to *leuO* expression during growth in LB. To test this, we generated a *V. cholerae* mutant (SS4) that produced a *toxR* allele in which we deleted the carboxy-terminal periplasmic domain (i.e., *toxR*$^{\Delta PPd}$). This mutant allele is localized to the membrane and was previously shown to be functional (2, 23). We then introduced pXB266, pVA258, and pVA261 into the *toxR*$^{\Delta PPd}$ mutant SS4. The strains were then cultured as described above, and *leuO-lacZ* expression was quantified. The results showed that deletion of the ToxR periplasmic domain largely abolished *leuO* expression from all three *leuO-lacZ* reporters (Fig. 2B). This indicated that the ToxR periplasmic domain was important for *leuO* expression.

We also tested whether the ToxR periplasmic domain was necessary for the activation of *leuO* expression in *E. coli*. *E. coli* strains containing each of the *leuO-lacZ* reporter plasmids were transformed with plasmid pBAD33::*toxR*$^{\Delta PPd}$*S* (pXB286), and the resulting strains were cultured as described above before being assayed for *leuO-lacZ* expression. The results of these experiments mirrored the results obtained with *E. coli* expressing the WT *toxRS* allele. Overexpression of *toxR*$^{\Delta PPd}$*S* from pXB286 activated the WT *leuO* promoter in pXB266 and the *leuO* promoter lacking the distal ToxR binding site in pVA258 to similar levels (Fig. 2D). Further, the magnitude of activation was similar to that observed with the strain expressing the WT *toxRS* allele. This suggested that the *toxR*$^{\Delta PPd}$ allele produces a functional protein that is able to bind to the ToxR consensus sequences in the *leuO* promoter and to activate *leuO* transcription. This suggests that the inability of ToxR$^{\Delta PPd}$ to activate *leuO* expression in *V. cholerae* was not due to its inability to bind to DNA but rather due to other factors that are dependent on the presence of the ToxR periplasmic domain.

ToxR can directly bind the *leuO* promoter. To further sup-

port the conclusion that ToxR bound directly to the *leuO* promoter, gel electrophoretic mobility shift assays were performed with the *leuO* promoter. The EMSAs were performed with crude membrane fractions that were isolated from *V. cholerae* strain DT733 (Δ*toxRS*) containing pBAD18 or pBAD18 expressing *toxRS* or *toxR*$^{\Delta PPd}$*S*. As has been found with other gel shift assays using ToxR membrane fractions, the bound DNA probes do not enter the gel and are left in the wells of the gel; thus, the binding of ToxR to the labeled probe should be assessed by the disappearance of free probe and not by the presence of a shifted band (3). The results showed that the ToxRS-positive membrane fractions bound directly to the *leuO* promoter, which resulted in a decrease in the abundance of free *leuO* probe (Fig. 3A, lane 2). The decrease in free *leuO* probe was largely abolished in the presence of ToxRS-negative membrane fractions (lane 7). Taken together, these results indicated that ToxR can directly bind to the *leuO* promoter.

To determine whether ToxR binding to the *leuO* promoter was specific, we performed binding competition assays by including a 10-fold excess of unlabeled competitor DNA in the binding reaction mixtures. If ToxR bound specifically to the *leuO* promoter fragment, adding excess unlabeled *leuO* promoter would prevent a shift in the free probe. When excess *leuO* DNA was added in the assay, there was a reduced shift in the labeled *leuO* probe (Fig. 3A, lane 3). The *leuO* promoter was also competed with a known ToxR-specific promoter, the *ompU* promoter, which competed for *leuO* binding and prevented the shift in the free probe (lane 4). The addition of a nonspecific competitor encompassing the *vexR* promoter, which is not regulated by ToxR, did not alter the level of free *leuO* probe (lane 5), indicating that the observed shift was specific for the *leuO* promoter. These same binding conditions were also used for ToxRS-negative membranes (Fig. 3A, lanes 6 to 10). The results showed that although there was some decrease in free probe between the no-protein control and the ToxRS-negative membrane control, the decrease was much less than that observed with ToxRS-positive membranes and was unaffected by the addition of any of the unlabeled competitor probes. These results confirmed the specificity of the ToxR-positive membranes for the *leuO* promoter.

Gel shift assays were also performed to determine if deletion of the ToxR periplasmic domain altered ToxR binding to the *leuO* promoter. These experiments were performed as described above with membrane fractions from DT733 containing pBAD18 expressing *toxR*$^{\Delta PPd}$*S*. The results showed that the ToxR$^{\Delta PPd}$*S* membranes had shifts comparable to those of the ToxRS membranes. The ToxR$^{\Delta PPd}$*S* membrane fractions shifted the *leuO* promoter (Fig. 3B, lane 2). The *leuO* shift was competed by the addition of a 10-fold excess of unlabeled *leuO* or *ompU* promoter probes but not by the addition of the nonspecific *vexR* promoter probe (lanes 3 to 5). These same binding conditions were also used for ToxR$^{\Delta PPd}$*S*-negative membranes (Fig. 3B, lanes 6 to 10). The results showed a decrease in free probe between the no-protein control and the ToxR$^{\Delta PPd}$*S*-negative membrane control, but the decrease was much less than that observed with ToxR$^{\Delta PPd}$*S*-positive membranes and was unaffected by the addition of any of the unlabeled competitor probes; this confirmed the specificity of the ToxR-positive membranes for the *leuO* promoter. From these results, we concluded that the ToxR periplasmic domain was not required for ToxR binding to the *leuO* promoter.

ToxR periplasmic domain is important for responding to bile. The role of the ToxR periplasmic domain in environmental
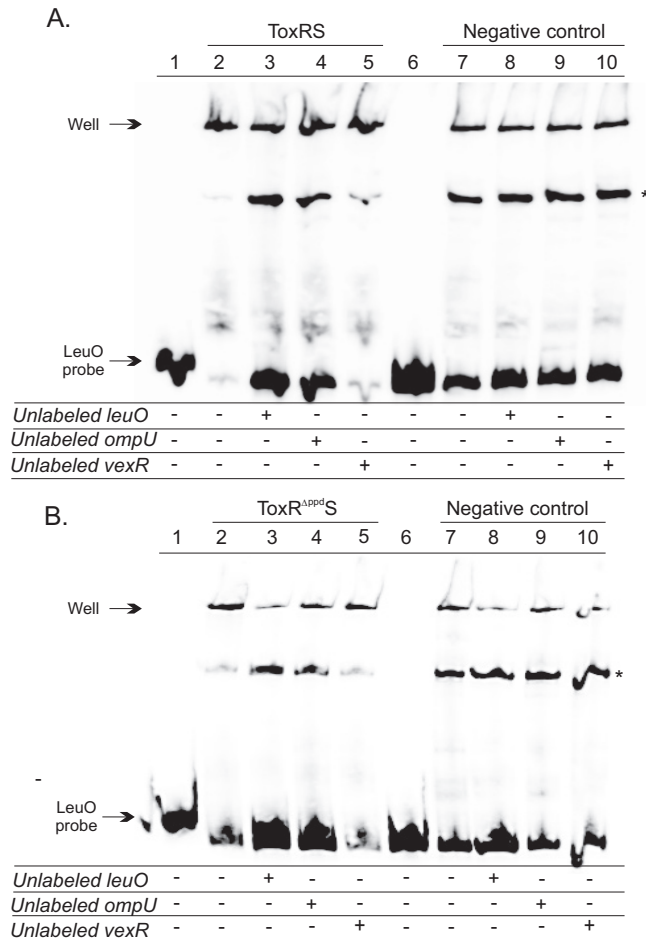
FIG 3 ToxR and ToxR$^{\Delta ppd}$ interact with the *leuO* promoter. A biotinylated DNA fragment encompassing the *leuO* promoter was incubated with *V. cholerae* cytoplasmic membrane fractions isolated from strain DT733 ($\Delta toxRS$), which overproduces ToxRS or ToxR$^{\Delta ppd}$S from pBAD18, described in Materials and Methods. The negative-control membranes are ToxRS negative and were isolated from DT733 containing the empty vector control (i.e., pBAD18). The binding reaction mixtures contained either no membranes (lanes 1 and 6) or equal amounts of the indicated membranes (lanes 2 to 5 and 7 to 10). Unlabeled competitor DNA probes encompassing the *leuO*, *ompU*, or *vexR* promoter were added as indicated to the binding reaction mixture at a 10-fold excess relative to the level of the biotinylated *leuO* DNA probe. Equal aliquots of binding reaction mixtures were then electrophoresed on nondenaturing 5% acrylamide gels, and the position of the *leuO* probe was visualized by chemiluminescence detection, as described in Materials and Methods. (A) ToxR binding to the *leuO* promoter. The ToxR binding mixture contained 3 mg/ml ToxRS-positive membranes, while the control binding reaction mixtures contained an equivalent concentration of ToxRS-negative membranes. (B) ToxR$^{\Delta ppd}$ binding to the *leuO* promoter. The ToxR$^{\Delta ppd}$ binding mixture contained 1 mg/ml ToxR$^{\Delta ppd}$S-positive membranes, while the control binding reaction mixtures contained an equivalent concentration of ToxRS-negative membranes. The asterisks denote an unknown nonspecific mobility shift.

sensing is poorly understood. ToxR inversely regulates *ompU* and *ompT* expression. OmpT is expressed during growth in minimal medium, while OmpU is expressed during growth in rich medium. The addition of bile salts or the amino acids asparagine, arginine, glutamic acid, and serine (NRES) to minimal medium results in ToxR-dependent porin switching that mimics growth in rich medium (i.e., expression of *ompU* and repression of *ompT*) [41]. The mechanism by which ToxR activates *ompU* expression
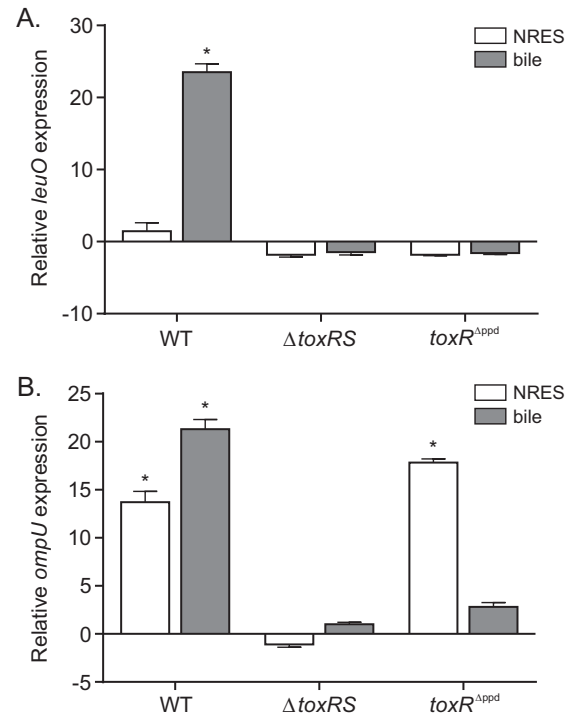

FIG 4 The ToxR periplasmic domain is required for *leuO* and *ompU* upregulation in response to bile. qRT-PCR was used to determine the effect of the amino acids NRES and bile on *leuO* (A) and *ompU* (B) expression during the growth of JB58 (WT), DT733 ($\Delta toxRS$), and SS4 (*toxR*$^{\Delta ppd}$) in modified T medium. The strains were cultured to an OD$_{600}$ of ~0.3 before being transferred to medium supplemented with either 50 mM NRES or 0.2% bile. The cultures were then grown for an additional 15 min before aliquots were collected for RNA isolation, as described in Materials and Methods. The presented data are the means ± SD of the results from three independent experiments. Statistical significance was determined using a one-sample *t* test comparing the sample mean to a hypothetical value of ±1. *, $P < 0.01$.

in minimal medium differs for bile salts and NRES [20]. The addition of NRES to minimal medium results in *toxR* upregulation, which is sufficient to increase *ompU* expression. In contrast, bile salts activate *ompU* expression via a process that does not result in *toxR* upregulation and may involve transcriptional activation [20].

We took advantage of the *ompT-ompU* switching system described above to test the contribution of the ToxR periplasmic domain on *leuO* and *ompU* expression in response to bile. We cultured WT strain JB58, the $\Delta toxRS$ strain DT733, and the *toxR*$^{\Delta ppd}$ strain SS4 in T minimal medium or T medium containing bile or NRES and quantified *leuO* and *ompU* expression by quantitative real-time PCR (qRT-PCR). The results showed that *leuO* expression was induced ~24-fold in response to bile and was unaffected by the addition of NRES to the WT strain (Fig. 4A). The addition of NRES or bile to the medium did not affect *leuO* expression in the *toxRS* or *toxR*$^{\Delta ppd}$ mutant. In contrast to *leuO*, *ompU* expression increased in response to both NRES (13-fold) and bile (22-fold) relative to that in the control cultures (Fig. 4B). The expression of *ompU* was abolished under all conditions in the $\Delta toxRS$ mutant. The expression of *ompU* in the *toxR*$^{\Delta ppd}$ mutant following exposure to NRES increased to a level that was similar to that observed in the WT, further confirming that the *toxR*$^{\Delta ppd}$ allele produced a functional protein in *V. cholerae*. The expression

TABLE 2 Bile killing assays

Strain	Fold change in recovery (mean [SD]) for:	
	10% bile	20% bile
$\Delta leuO$ mutant or WT	1	−4.5 (2.1)[a]
$\Delta toxRS$ mutant or WT	−11.3 (3.7)[a]	NR[b]
$toxR^{\Delta PPd}$ mutant or WT	−7.0 (1.4)[a]	NR
$\Delta toxRS$ pBAD18::*leuO* or $\Delta toxRS$ pBAD18 mutant	5.2 (1.4)[a]	NR

[a] $P < 0.01$.
[b] NR, not recovered.

of *ompU* in the $toxR^{\Delta PPd}$ mutant following exposure to bile resulted in a much lower level of *ompU* induction than that observed in the WT. Exposure to bile resulted in an ∼21-fold increase in *ompU* expression in the WT but only an ∼3-fold increase in the $toxR^{\Delta PPd}$ mutant (Fig. 4B). Taken together, these results provide additional evidence that the $ToxR^{\Delta PPd}$ protein is functional and that the periplasmic domain of ToxR is critical for the induction of *ompU* and *leuO* in response to bile. This suggests the possibility that bile or bile salts affect ToxR activity by a process that requires the periplasmic domain.

LeuO contributes to *V. cholerae* bile resistance. Since ToxR functions in bile resistance (7, 16), we hypothesized that LeuO may also contribute to *V. cholerae* bile resistance. To test this, we performed bile killing assays. In these experiments, we quantified the survival of WT *V. cholerae* (JB58) and isogenic *leuO*, *toxRS*, and $toxR^{\Delta PPd}$ deletion mutants upon exposure to a lethal concentration of bile for 1 h, as described in Materials and Methods. The results of these experiments showed an ∼4-fold decrease in the recovery of the *leuO* mutant strain XBV222 relative to the recovery of WT strain JB58 when exposed to 20% bile (Table 2). This suggested that the presence of *leuO* provided a survival advantage to *V. cholerae* in the presence of bile. The $\Delta toxRS$ mutant strain DT733 and the $toxR^{\Delta PPd}$ mutant SS4 were not recovered when exposed to 20% bile. This finding is consistent with the role of ToxR in bile resistance and is likely attributable to the combined dysregulation of *ompU*, *ompT*, and *leuO* expression in the $\Delta toxRS$ mutant. We therefore performed the killing assays with the *toxR* mutant strains using 10% bile. When exposed to 10% bile, the $\Delta toxRS$ mutant strain exhibited an ∼11-fold decrease in recovery relative to that of the WT, suggesting that the $\Delta toxRS$ mutant was more sensitive to bile than the $\Delta leuO$ mutant. The $toxR^{\Delta PPd}$ mutant exhibited an ∼7-fold decrease in recovery compared to that of the WT. This indicated that the $toxR^{\Delta PPd}$ mutant exhibited greater susceptibility to bile than did a $\Delta leuO$ mutant. As $ToxR^{\Delta PPd}$ has been shown to be sufficient for *ompU* expression under standard laboratory conditions, these results suggest the possibility that the periplasmic domain is required for the expression of other factors, in addition to *leuO*, that might contribute to bile resistance.

The above data suggest that LeuO contributed to *V. cholerae* survival in the presence of bile. If this is true, the overexpression of *leuO* in a $\Delta toxRS$ mutant should provide a survival advantage upon exposure to a lethal concentration of bile. We tested this by introducing pBAD18-*leuO* or pBAD18 into the $\Delta toxRS$ mutant DT733. We chose a $\Delta toxRS$ mutant for these experiments to negate *leuO* and *ompU* expression. We cultured the *toxRS* deletion strain bearing the pBAD18-*leuO* plasmid pVA94 or the empty

vector control (pBAD18) to log phase in the presence of 0.1% arabinose to induce *leuO* expression. Aliquots of the induced cultures were then exposed to a lethal concentration of bile before being processed as described above. The results showed that *leuO* overexpression resulted in an ∼5-fold increase in cell recovery relative to the recovery of the empty vector control (Table 2). This indicated that LeuO contributed to *V. cholerae* survival in the presence of bile by a mechanism that is likely independent of *ompU*. Taken together, the results of these experiments confirm the importance of ToxR in bile resistance and support the conclusion that ToxR activation of *leuO* expression contributes to bile resistance.

LeuO does not contribute to bile resistance through regulation of the RND efflux pumps. Our laboratory has previously shown that the *V. cholerae* resistance-nodulation-division (RND) family efflux systems are major contributors to *V. cholerae* bile resistance (11–15). We therefore tested whether the contribution of *leuO* to bile resistance was mediated by upregulation of any of the RND efflux systems. We quantified the expression of all six of the RND efflux pumps in the WT strain JB58 and the $\Delta leuO$ mutant strain XBV222 in response to bile using *lacZ* promoter reporter fusions. The strains were cultured in the absence or presence of bile to mid-logarithmic growth phase, at which point the expression of the individual RND efflux systems was quantified. The results showed that in the absence of bile, there were no significant differences between the expression of any of the RND efflux pumps in the $\Delta leuO$ mutant and that in the WT strain (Fig. 5). This indicated that LeuO did not contribute to the basal expression of any of the efflux pumps. When the same reporter strains were cultured in the presence of bile, as expected, *vexRAB* and *vexCD* expression levels were found to be significantly induced. However, there were no significant differences found in any of the RND efflux pump expression levels between the WT and *leuO* deletion strains in response to bile (Fig. 5). This indicated that LeuO does not regulate the expression of the RND efflux systems and is likely not working through the RND efflux pumps to contribute to bile resistance.

DISCUSSION

The ability of *V. cholerae* to respond to its environment is essential for its success as an enteric pathogen. This is critical upon entrance into the human host, where *V. cholerae* must express genes that are indispensable for colonization of the small intestine. Colonization and growth in the small intestine require the expression of virulence genes plus the expression of genes that combat antimicrobial agents present in the intestine. ToxR plays a critical role in this regard by regulating the expression of genes that are required for host adaptation. ToxR is thought to transduce *in vivo* signals to affect the expression of its target genes, but the mechanism by which this occurs is poorly understood.

In this study, we observed the upregulation of *leuO* expression in response to bile and the bile salt deoxycholate by a process that was dependent on ToxR (Fig. 1). Bile salts have detergent-like properties that make them bactericidal. The expression of *leuO* was not altered by other membrane-active detergents (e.g., SDS, Triton X-100, or sarcosyl), indicating that *leuO* induction was not the result of the general membrane stress response but instead was directly in response to bile and bile salts. The upregulation of *leuO* in response to bile suggested the possibility that *leuO* functions in bile resistance. Support for this conclusion was provided by the
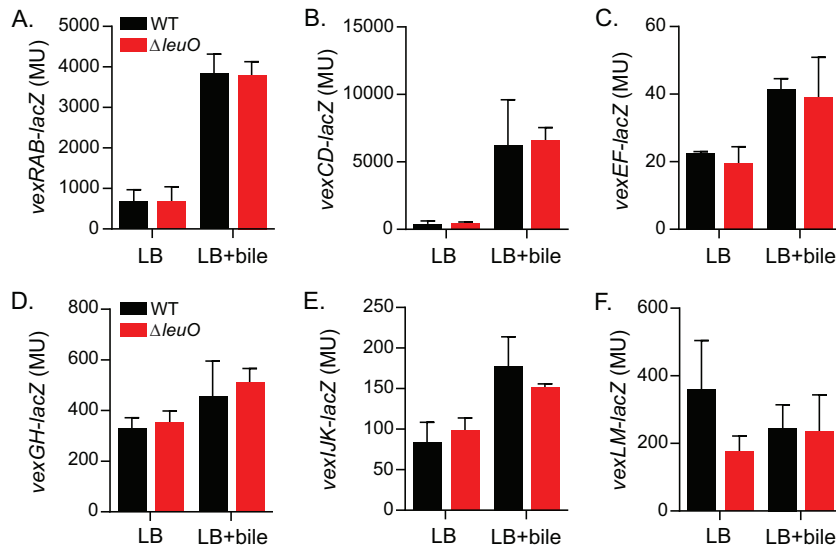
FIG 5 Effect of *leuO* on the expression of the *V. cholerae* RND efflux systems. *V. cholerae* WT strain JB58 and Δ*leuO* mutant strain XBV222 carrying *lacZ* promoter fusion reporter plasmids for *vexRAB-lacZ* (pXB233) (A), *vexCD-lacZ* (pXB231) (B), *vexEF-lacZ* (pXB228) (C), *vexGH-lacZ* (pXB229) (D), *vexIJK-lacZ* (pXB230) (E), or *vexLM-lacZ* (pXB232) (F). All strains were cultured in LB in the presence or absence of 0.2% bile before being assayed for β-galactosidase activity, as described in Materials and Methods. The presented data are the means ± SD of the results from three independent experiments.

observation that *leuO* deletion resulted in increased bile suscepti- bility, while *leuO* overexpression resulted in increased bile resis- tance (Table 2). In light of recent studies using an infant mouse model showing that *leuO* is expressed in the intestine (23), we speculate that the findings observed here may extend to the host. These results were also similar to what has been reported for OmpU, a porin that is associated with bile resistance in *V. cholerae* and whose expression is also activated by ToxR in response to bile and deoxycholate (16).

While the contribution of *leuO* to bile resistance is clear, the mechanism by which *leuO* impacts bile resistance was not re- solved. The Δ*leuO* mutant strain exhibited a bile susceptibility phenotype that was intermediate relative to that of the Δ*toxRS* mutant (Table 2). This suggested that LeuO likely affected bile resistance by a mechanism that was distinct from *ompU* or *ompT* regulation. This conclusion was confirmed by the finding that *leuO* overexpression provided a survival advantage in a *toxRS*- negative strain exposed to bile (Table 2). Bile resistance results from the synergistic affects of reduced outer membrane permea- bility and active efflux (14). In *V. cholerae*, the RND efflux systems are major contributors to bile resistance, suggesting a potential mechanism by which LeuO might affect bile resistance. However, our results showed that there was no difference in the levels of expression of any of the RND efflux systems in a *leuO* mutant grown in the presence and absence of bile (Fig. 5), suggesting that *leuO* affects bile resistance by a mechanism that is independent of the RND efflux systems. There are a number of other potential mechanisms by which LeuO might impact bile resistance, includ- ing the expression of other transport systems, the production of other porins, alterations in cell physiology, and alterations to the cell envelope.

The expression of *leuO* was previously found to be dependent on ToxR (23). Sequence analysis of the *leuO* promoter revealed the presence of two putative ToxR consensus binding sites, both of which were required for basal-level *leuO* expression in *V. cholerae*

(Fig. 2B). This finding was reminiscent of what was observed for ToxR activity at the *ompU* promoter, in which the most distal ToxR binding site was needed for full *ompU* activation in *V. chol- erae* (18). In contrast, ToxR activated expression from the *leuO* promoter lacking the distal ToxR binding site (i.e., pVA258) in *E. coli* (Fig. 2D). The lack of expression from the same mutant pro- moter in *V. cholerae* suggested that other factors affect *leuO* ex- pression in *V. cholerae*. We do not know what these factors are, but there are a number of potential explanations for this result. It is possible that other DNA binding proteins interact with the *leuO* promoter and impede ToxR binding at the proximal site. It is also possible that ToxR binds sequentially to the two ToxR binding sites in *V. cholerae* and has to bind to the distal site first, which then facilitates binding at the proximal site. This tandem fashion of binding is similar to that of other DNA binding domains of OmpR family proteins, which generally interact as dimers with direct repeat DNA sequences (40). This idea is also supported by coop- erative binding studies comparing ToxR oligomerization and the regulation of target promoters containing multiple operator ele- ments in *E. coli* and *V. cholerae* (42).

The periplasmic domain of ToxR has been implicated in the response to environmental signals, but how ToxR regulates its target genes in response to these signals is poorly understood. The finding that the ToxR periplasmic domain was required for the upregulation of both *leuO* and *ompU* in response to bile (Fig. 4) suggests the possibility that the periplasmic domain acts as a bile sensor, which can affect the activity of the cytoplasmic DNA bind- ing domain at its target promoters. The mechanism by which the periplasmic domain senses bile is unclear. There are a number of potential mechanisms by which bile might affect ToxR activity. Bile might facilitate ToxR interaction with ToxS. ToxS has been shown to contribute to ToxR stability and to enhance its activity at target genes (4–6). Alternatively, bile might affect conformational changes in the ToxR periplasmic domain that affect DNA binding. Bile might also affect disulfide bond formation in the two cysteine

residues located in the ToxR periplasmic domain. There is evidence that disulfide bond formation in the periplasmic domain of ToxR and ToxR-like proteins affects their activities. For example, disulfide bond formation in ToxR has been shown to contribute to *ompU* regulation in response to some growth conditions (43). ToxR and TcpP have also been shown to form homodimers and heterodimers (44, 45), and the bile salt taurocholate has been shown to induce intermolecular disulfide bond formation in the periplasmic domain of TcpP (46). Similarly, *E. coli* CadC has been shown to form disulfide bonds in response to pH, which result in the activation of *cadBA* transcription (47). Additional work will be required to differentiate between these potential mechanisms.

Previous studies showed that cFP activation of *leuO* resulted in the downregulation of the ToxR regulon (23). The data presented here show that *leuO* expression is also activated by bile and bile salts. While the fatty acid components of bile have been linked to downregulation of the ToxR regulon (48), bile salts have been shown to either be neutral or enhance virulence gene expression (38, 46). Thus, the role of LeuO in virulence gene regulation is a paradox. Although there are a number of potential explanations for the differential effects of LeuO on virulence, we suspect that bile salts and CDPs differentially affect the expression (or activities) of other proteins that contribute to virulence gene expression. For example, bile salts and cFP may differentially affect histone-like nucleoid-structuring protein (H-NS) or C-reactive protein (CRP), both of which have been shown to suppress the ToxR regulon and thus might contribute to the observed phenotype (49, 50). Studies to resolve the role of LeuO in virulence gene regulation are ongoing in our laboratory.

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