

Vibrio cholerae LeuO Links the ToxR Regulon to Expression of Lipid A Remodeling Genes

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Vibrio cholerae is an intestinal pathogen that causes the diarrheal disease cholera. Colonization of the intestine depends upon the expression of genes that allow *V. cholerae* to overcome host barriers, including low pH, bile acids, and the innate immune system. ToxR is a major contributor to this process. ToxR is a membrane-spanning transcription factor that coordinates gene expression in response to environmental cues. In previous work we showed that ToxR upregulated *leuO* expression in response to bile salts. LeuO is a LysR family transcription factor that contributes to acid tolerance, bile resistance, and biofilm formation in *V. cholerae*. Here, we investigated the function of ToxR and LeuO in cationic antimicrobial peptide (CAMP) resistance. We report that ToxR and LeuO contribute to CAMP resistance by regulating *carRS* transcription. CarRS is a two-component regulatory system that positively regulates *almEFG* expression. AlmEFG confers CAMP resistance by glycosylation of lipid A. We found that the expression of *carRS* and *almEFG* and the polymyxin B MIC increased in mutants lacking *toxRS* or *leuO*. Conversely, *leuO* overexpression decreased the polymyxin B MIC. Furthermore, we found that LeuO directly bound to the *carRS* promoter and that ToxR-dependent activation of *leuO* transcription regulated *carRS* transcription in response to bile salts. Our results suggest that LeuO functions downstream of ToxR to modulate *carRS* expression in response to environmental cues. This study extends the functional role of ToxR and LeuO in environmental adaptation to include cell surface remodeling and CAMP resistance.

Vibrio cholerae is a Gram-negative facultative human pathogen and the causative agent of the diarrheal disease cholera. *V. cholerae* is native to aquatic ecosystems, where it often associates with chitinous aquatic organisms (reviewed in reference 1). People acquire *V. cholerae* by ingestion of contaminated food or water. Following ingestion, *V. cholerae* colonizes the small intestine, where it produces virulence factors that result in a severe secretory diarrhea that is the hallmark of the disease cholera. The secretory diarrhea then contributes to *V. cholerae* transmission to new hosts and dissemination into the aquatic ecosystem.

The ability of *V. cholerae* to rapidly transition between the aquatic ecosystem and the host is essential for its success as a pathogen. Colonization of the human gastrointestinal tract is mediated by transcriptional responses that facilitate adaptation to dynamic environments in the gastrointestinal tract. Following ingestion, *V. cholerae* activates the expression of virulence factors that are essential for both colonization and disease development. This includes the production of the enterotoxin cholera toxin (CT) and an adhesin called the toxin coregulated pilus (TCP) (2, 3). The expression of many *V. cholerae* virulence genes is coordinately regulated by the ToxR regulon (4). The ToxR regulon is divided into two branches: the ToxT-dependent branch and the ToxT-independent branch. In the ToxT-dependent branch, ToxR and TcpP, two membrane-associated regulators, function as co-activators of *toxT* expression in response to environmental cues (5, 6). ToxT then directly activates the expression of the gene encoding a number of virulence factors, including those for CT and TCP production (7). In the *toxT*-independent branch, ToxR regulates the expression of the *ompU* and *ompT* porins (8, 9). Recent studies in our laboratory have shown that ToxR also regulates the expression of the LysR family transcription factor *leuO* independent of *toxT* (10, 11).

ToxR is a membrane-associated regulator that contains a periplasmic domain that is connected to a cytoplasmic DNA bind-

ing domain by a single transmembrane-spanning domain (6, 12, 13). The periplasmic domain is thought to function as a sensor which transduces extracellular cues to effect the activity of the cytoplasmic DNA binding domain. ToxR has been shown to directly regulate *ompU*, *ompT*, and *leuO* expression in response to bile salts (10, 14). In the presence of bile salts, ToxR activates *ompU* expression while repressing *ompT* expression. OmpU production appears to contribute to bile resistance due to the properties of its pore, which restricts the diffusion of negatively charged molecules (e.g., bile salts) across the outer membrane (15, 16). ToxR-dependent expression of *ompU* also contributes to cationic antimicrobial peptide (CAMP) resistance. OmpU serves as a sensor for the sigma E membrane stress response, and cells that lack *ompU* (i.e., *ompU* or *toxR* mutants) fail to activate the sigma E membrane stress response upon membrane perturbation, a phenotype that has been linked to increased CAMP susceptibility (17, 18). ToxR also activates *leuO* transcription in response to bile salts and cyclic dipeptides (10, 11). LeuO has been linked to multiple phenotypes in *V. cholerae*, including biofilm production, virulence repression, acid tolerance, and bile resistance (10, 11, 19, 20). Taken together, these results suggest that in addition to reg-

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ulating virulence factor production, ToxR also contributes to the regulation of numerous genes involved in environmental adaptation.

V. cholerae is exposed to a variety of antimicrobial compounds within the human gastrointestinal tract, including bile salts and products of the innate immune system, like CAMPs. *V. cholerae* growth in the presence of bile salts is dependent upon the expression of antimicrobial efflux systems combined with reduced cell permeability that is achieved by modulating porin production (21, 22). CAMP resistance is also mediated by multiple factors. CAMPs are short (~12 to 50 amino acids) amphipathic and hydrophobic peptides that typically exhibit a net positive charge (23). In Gram-negative bacteria, the initial interaction between CAMPs and the cell surface is thought to be driven by the electrostatic attraction between positively charged CAMPs and the negatively charged lipopolysaccharide (LPS). The binding of CAMPs to LPS results in outer membrane disruption and further CAMP uptake. Once across the outer membrane, CAMPs can disrupt the cytoplasmic membrane and/or inhibit critical cytoplasmic processes, resulting in cell death. *V. cholerae* resistance to CAMPs is mediated by active efflux via the VexAB-TolC RND-efflux system (22), expression of the extracytoplasmic stress response (17), and covalent modification of LPS (24–27). LPS remodeling has been shown to be critical for high-level CAMP resistance. In *V. cholerae* the production of hexa-acylated lipid A via the MsbB acyltransferase confers resistance to polymyxin B (PXB), a CAMP antibiotic (28). Hexacylated lipid A can be further modified with glycine and diglycine residues by AlmEFG to effect high-level PXB resistance (25–27). Glycylation of lipid A results in a net increase in the positive charge of lipid A which reduces the electrostatic interactions between CAMPs and lipid A, leading to CAMP resistance.

V. cholerae LPS glycylation is regulated by CarRS (also known as VprAB), a two-component regulatory system (24, 25). CarRS was first identified as a calcium-responsive negative regulator of biofilm production (29). Two subsequent studies showed that CarRS also regulated CAMP resistance by positively regulating *almEFG* expression (24, 25). Mutations in *carR* or *almEFG* resulted in an ~100-fold decrease in *V. cholerae* PXB resistance and attenuated intestinal colonization (24, 25), highlighting the importance of lipid A modification in *V. cholerae*. The molecular mechanisms controlling *carRS* expression are unknown. Previous studies showed that *carRS* expression was influenced by environmental cues, with *carRS* expression being activated by PXB and repressed by bile salts and calcium (25, 29).

In this study, we investigated the function of *V. cholerae* *toxR* and *leuO* in PXB resistance. We found that deletion of *toxR* or *leuO* resulted in increased PXB resistance. This phenotype was linked to *carRS* expression. Transcriptional reporter assays and electrophoretic mobility shift assays (EMSAs) revealed that LeuO functioned downstream of ToxR to directly repress *carRS* expression. This finding suggested that LeuO was a *carRS* repressor, a conclusion that was further supported by the results showing that *leuO* overexpression increased *V. cholerae* PXB susceptibility. We further found that both ToxR and LeuO were required for bile salt-dependent repression of *carRS* but did not influence *carRS* expression in response to PXB or calcium. Our collective results demonstrate that ToxR and LeuO effect cell surface remodeling and PXB resistance by repression of *carRS*. Our findings expand the function of ToxR and LeuO in environmental adaptation to

include regulation of CAMP resistance via the CarRS two-component system.

MATERIALS AND METHODS

Strains, media, and growth conditions. The bacterial strains used in this study are listed in Table 1. *Escherichia coli* strain SM10 λ pir was used for plasmid mobilization. *E. coli* strain EC100D+pir was used for the two-plasmid β -galactosidase reporter assays and for cloning experiments. *E. coli* strain ER2566 was used for purification of proteins for the EMSAs. The *V. cholerae* strains used in this study were derivatives of O1 El Tor strain N16961 (34). The *V. cholerae* N16961 Δ *lacZ* Sm^r strain 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. Bacterial stocks were maintained at –80°C in LB broth containing 25% glycerol. Culture medium was supplemented with carbenicillin (Cb) and streptomycin (Sm) at 100 μ g/ml, kanamycin (Km) at 50 μ g/ml, or chloramphenicol (Cm) at 25 μ g/ml as required. Arabinose was added to growth medium at the concentrations indicated in the figure legends to induce expression from the arabinose-regulated promoter in pBAD18Km and pBAD33.

Plasmid and mutant construction. Plasmids and oligonucleotides used in this study are listed in Table 1. The *carRS-lacZ* reporter, pVA289, was generated as follows. The P_{*carRS*}-pVA289-F and P_{*carRS*}-pVA289-R PCR primers were used to amplify the *carRS* promoter from the *V. cholerae* N16961 genome. The resulting PCR amplicon was digested with XhoI and BamHI restriction endonucleases before being ligated into similarly digested pTL61T to create pVA289. The *carRS-lacZ* reporter pXB340 was generated in an identical manner using the P-vc1320-F-XhoI and P-vc1320-R-XbaI PCR primers. The *almEFG-lacZ* reporter pXB342 was generated using P-vc1579-F-XhoI and P-vc1579-R-XbaI PCR primers. The *almE* (VC1579) deletion plasmid pWM91:: Δ *almE* was made by cross-over PCR as previously described (35, 36). Briefly, the *almE*-F1/*almE*-R2 and *almE*-F2/*almE*-R1 primer pairs were used in separate PCRs with N16961 chromosomal DNA. The resulting ~1-kb amplicons were purified and used as templates in a second PCR amplification with the flanking *almE*-F1/*almE*-R1 PCR primers. The resulting ~2-kb amplicon was then digested with SacI and SmaI restriction endonucleases before being ligated into similarly digested pWM91 to generate pWM91:: Δ *almE*. The *V. cholerae* *almE* deletion mutant was constructed by conjugating pWM91:: Δ *almE* into JB58 with selection for resistance to Sm and Cb. Several Sm- and Cb-resistant cointegrants were then plated onto LB no-salt agar containing 5% sucrose. Sucrose-resistant and Cb-sensitive colonies were then screened by PCR to confirm deletion of *almE* using the *almE*-F1/*almE*-R1 primers to identify strain XBV302. The *almE* deletion in XBV302 was subsequently verified by DNA sequencing.

β -Galactosidase assays. *V. cholerae* strains carrying the indicated *lacZ* reporter plasmids were cultured overnight in LB broth at 37°C with shaking. The cultures were then diluted 100-fold into fresh LB broth or LB containing 0.05% deoxycholate, 5 mM CaCl₂, or polymyxin B at the indicated concentrations and grown at 37°C with shaking. Culture aliquots were then collected in triplicate at the indicated time points to quantify β -galactosidase production as previously described (37). *E. coli* containing an expression plasmid (pBAD33, pVA126, or pXB302) plus the *carRS-lacZ* reporter plasmid pVA289 was cultured overnight in LB broth with shaking at 37°C. The overnight cultures were then diluted 100-fold into fresh LB broth containing antibiotics and 0.005%, 0.01%, or 0.05% arabinose, and the cultures were incubated at 37°C with shaking. Culture aliquots were collected in triplicate at mid-exponential phase (optical density at 600 nm [OD₆₀₀] of ~0.5) to quantify β -galactosidase activity. All of the reporter experiments were performed independently at least three times. Gene expression from the β -galactosidase reporters were calculated and displayed as Miller units (MU).

Purification of the maltose binding protein (MBP) and LeuO-MBP. Protein purification for the electrophoretic mobility shift assays (EMSAs) was done as previously described (19). Briefly, overnight cultures of *E. coli* ER2566 carrying pMAL-c2 or pMAL-c2::*leuO* (pVA175) were diluted

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

Strain, plasmid, or oligonucleotide	Relevant characteristic(s) or sequence (5' to 3')	Source or reference
Strains		
<i>Escherichia coli</i>		
EC100D+ <i>pir</i>	<i>supE44 ΔlacU169</i> (φ80 <i>lacZ</i> ΔM15) <i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i> (<i>λpirR6K</i>)	Epicenter
SM10 <i>λpir</i>	<i>thi-1 thr leu tonA lacY supE recA::RP4-2-Tc::Mu kmR</i> (<i>λpirR6K</i>)	Laboratory collection
ER2566	F ⁻ <i>glnV44(AS) galK2(Oc) rpsL704(strR) xylA5 mtl-1 argE3(Oc) thiE1 tfr-3 λ DE3 λ sBamHI ΔEcoRI-B int::(lacI::PlacUV5::T gene1) i21 Δnin5</i>	New England BioLabs
<i>Vibrio cholerae</i>		
JB3	<i>V. cholerae</i> O1 El Tor strain N16961, Sm ^r	Laboratory collection
JB58	<i>V. cholerae</i> O1 El Tor strain N16961, Sm ^r , Δ <i>lacZ</i>	Laboratory collection
XBV222	JB58 Δ <i>leuO</i>	11
DT733	JB58 Δ <i>toxRS</i>	11
JB461	JB3 Δ <i>toxRS</i>	30
XBV302	JB58 Δ <i>almE</i>	This study
XBV532	<i>V. cholerae</i> O1 El Tor strain N16961, Sm ^r , Δ <i>lacZ</i> Δ <i>ompU</i>	17
XBV534	XBV532 Δ <i>leuO</i>	This study
XBV537	XBV532 Δ <i>toxRS</i>	This study
Plasmids		
pTL61T	<i>lacZ</i> transcriptional reporter plasmid, Cb ^r	31
pXB266	pTL61T containing the <i>leuO</i> promoter region	11
pVA289	pTL61T containing the <i>carRS</i> promoter region	This study
pXB340	pTL61T containing the <i>carRS</i> promoter region	This study
pXB342	pTL61T containing the <i>almEFG</i> promoter region	This study
pBAD18Km	Arabinose-regulated expression plasmid, Km ^r	32
pXB269	pBAD18Km expressing VC2486 and <i>leuO</i>	11
pBAD33	Arabinose-regulated expression plasmid, Cm ^r	32
pVA126	pBAD33 expressing <i>leuO</i>	19
pXB302	pBAD33 expressing <i>toxRS</i>	10
pMAL-c2	IPTG-inducible expression vector for fusion of proteins to MBP and cytoplasmic expression, Cb ^r	New England BioLabs
pVA175	pMAL-c2 expressing <i>leuO</i>	19
pWM91	Suicide plasmid vector used for allelic exchange, Cb ^r	33
pWM91::Δ <i>almE</i>	pWM91::ΔVC1579	This study
Oligonucleotides		
P _{<i>carRS</i>} -pVA289-F	AAACTCGAGAACACGCGGCGAGGAATTGAGTCAG	
P _{<i>carRS</i>} -pVA289-R	CGGGGATCCGATAATGTAGAGACTGGGTGG	
P-vc1320-F-XhoI	ACCCTCGAGTAATCACTGAGAGTGTAGCC	
P-vc1320-R-XbaI	TTTTCTAGATGGGGACCTCGTATTTACGG	
P-vc1579-F-XhoI	AAACTCGAGATGTTGCGTCTATTGGCGCGCG	
P-vc1579-R-XbaI	AATTCTAGATCATGTCTTGATAGGTGT	
<i>almE</i> -F1	CCCCCGGGCCACCAAGATACAAACTA	
<i>almE</i> -F2	TACAATTCTGCGGCGAGTCAGACATA	
<i>almE</i> -R1	ATGAGCTCGCTGCATCATGTCCGGCTA	
<i>almE</i> -R2	TGTCTGACTCGCCGAGAATTGTATG	
<i>carRS</i> -F-EMSA	GCGGGAGTCGGCAGCGGGCGAGGAATTGAGTCAGAAGCC	
<i>carRS</i> -R-EMSA	GCGGGAGTCGGCAGCGGAGACTGGGTTGGTTAGACATGGGG	
5' BIO	5'-biotin-GCGGGAGTCGGCAGCG	

100-fold into fresh LB broth and incubated at 37°C with shaking until they reached an OD₆₀₀ of ~0.5 when isopropyl β-D-1-thiogalactopyranoside (IPTG) was added at 0.3 mM. The cultures were then incubated for an additional 2 h before the cells were harvested by centrifugation. The cell pellets were then resuspended in column buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA) plus 1 mM phenylmethylsulfonyl fluoride before being lysed with an M-11P Microfluidizer according to the manufacturer's instructions (Microfluidics). The resulting cell lysates were cleared of particulate matter by centrifugation at 9,900 rpm for 20 min at 4°C. The clarified supernatant was then diluted 1:6 in column buffer before being passed through a 0.8- by 7.0-cm chromatography column containing 1 ml

of amylose resin (New England BioLabs). Following washing with column buffer, bound proteins were eluted from the resin using elution buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 10 mM maltose). Protein concentrations were determined using the Coomassie plus (Bradford) assay kit according to the manufacturer's instructions (Thermo Scientific). Protein purification was assessed by SDS-PAGE with Coomassie brilliant blue R-250 staining.

EMSAs. EMSAs were performed as previously described (19). The DNA fragment containing the *carRS* promoter (the nucleotide sequence between -400 and +20 relative to the *carR* translational start site) was PCR amplified from the N16961 genome using *carRS*-F-EMSA and

carRS-R-EMSA oligonucleotide primers. The resulting PCR fragment was then gel purified, and 100 ng was used as a template for a second PCR using the biotinylated 5' BIO oligonucleotide primer purchased from IDT to produce the end-labeled DNA probe. The *vexR* DNA fragment was previously described and consisted of the nucleotide sequence between -129 and -46 relative to the *vexR* translational start site (38). The biotin-labeled probes (1.5 nM *carRS*, 3 nM *vexR*) were incubated with purified LeuO-MBP or MBP in amounts ranging from 0 to 25 μ M in binding buffer containing 10 mM Tris (pH 7.4), 150 mM KCl, 0.1 mM dithiothreitol (DTT), 0.1 mM EDTA (pH 8.0), and 200 μ g/ml sheared salmon sperm. The 10- μ l binding reaction mixtures were incubated at room temperature for 20 min before being subjected to electrophoresis on a non-denaturing 5% TBE-PAGE gel in 0.25 \times Tris-borate-EDTA (TBE) buffer at 200 V for 45 min. The gel was prerun at 100 V for 1 h in 0.25 \times TBE prior to sample addition. The DNA in the gel was then transferred to a nylon membrane in 0.5 \times TBE buffer at 380 mA for 1 h. The nylon membrane was then UV cross-linked at 120,000 μ J using a Stratelinker 1800 (Stratagene) before the biotin-labeled DNA fragments were detected using a chemiluminescent nucleic acid detection module (Thermo Scientific) and visualized using FluorChem E (Protein Simple).

Antimicrobial susceptibility assays. Antimicrobial susceptibility was assayed by the Etest strip method according to the manufacturer's instructions (bioMérieux) or the gradient agar plate methods as previously described (22). Strains tested by the Etest strip method were grown in LB broth at 37°C overnight. These cultures were diluted in LB to an OD₆₀₀ of 0.5 before being examined using Etest strips according to the manufacturer's directions. Mid-log-phase cultures were generated by inoculating overnight cultures at 1:100 into fresh LB broth, or LB supplemented with 0.05% deoxycholate, and growing the cultures at 37°C to an OD₆₀₀ of 0.5 before use. The MIC for each respective strain was recorded at the PXB concentration displaying a clear zone of inhibition following overnight incubation at 37°C. The gradient agar plate method was used to test susceptibility for strains bearing pBAD18Km or pBAD18Km-*leuO* (39, 40). Overnight cultures of the respective strains were inoculated at 1:100 into fresh LB supplemented with 0.1% arabinose and grown at 37°C to an OD₆₀₀ of 0.5 before being inoculated onto the PXB gradient agar plates supplemented with 0.1% arabinose. After overnight incubation, growth of each strain across the gradient was measured. The MIC for each strain was then calculated as the percentage of growth across the plate multiplied by the antimicrobial concentration used in the plate. The presented results are the means and standard deviations from three independent biological replicates. Statistical significance for the tested strains was determined using a one-sample Student's *t* test.

Polymyxin B killing assay. Overnight cultures of *V. cholerae* WT strain JB58, Δ *leuO* strain XBV222, and Δ *toxRS* strain JB461 were diluted 100-fold into fresh LB broth with or without 0.05% deoxycholate. The inoculated cultures were then incubated with aeration at 37°C until they reached an OD₆₀₀ of \sim 0.5. Culture aliquots were then collected by centrifugation and the cell pellets resuspended in phosphate-buffered saline (PBS) to an OD₆₀₀ of 0.1. Serial dilutions of each strain were then plated onto LB agar plates to enumerate the cell titer at time zero (CFU_{input}). The remaining cells were then collected by centrifugation and resuspended in LB broth containing 500 μ g/ml polymyxin B and incubated at 37°C with aeration for 60 min, when an aliquot was collected and washed in PBS before serial dilutions were plated onto LB agar to quantify the viable cells (CFU_{output}). The percent survival of each strain was then calculated by dividing the number of cells recovered following 60 min of exposure to polymyxin B by the number of input cells (i.e., time zero). The reported data represent the averages \pm standard deviations from four independent experiments.

RESULTS

ToxR and LeuO negatively regulate *carRS* expression. During the course of our studies on *V. cholerae* LeuO, we observed that *leuO* deletion resulted in increased PXB resistance. This suggested

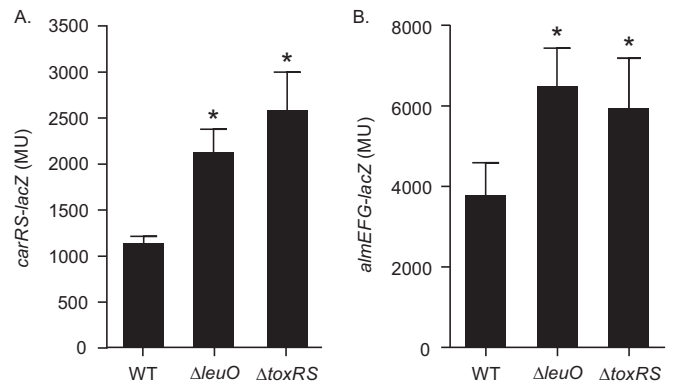


FIG 1 ToxR and LeuO regulate *carRS* and *almEFG* expression. WT strain JB58, Δ *leuO* strain XBV222, and Δ *toxRS* strain DT733 carrying the *carRS-lacZ* reporter pXB340 (A) or the *almEFG-lacZ* reporter pXB342 (B) were grown in LB broth to mid-log phase, at which time the expression of the reporter genes was quantified using a β -galactosidase assay. The presented data are the means \pm standard deviations from three independent experiments. Statistical significance was determined using one-way analysis of variance (ANOVA) with Dunnett's test, comparing each mean value to that of the WT. *, $P < 0.01$.

that *leuO* regulates *V. cholerae* CAMP resistance. Since the *carRS* two-component regulatory system was recently shown to be critical for PXB resistance (24, 25), we examined whether *leuO* or its upstream positive regulator, *toxR*, affected *carRS* transcription. We therefore introduced a *carRS-lacZ* reporter (pXB340) into WT strain JB58 (Δ *lacZ*) and its isogenic Δ *leuO* and Δ *toxRS* mutant strains. The resulting strains were then cultured to mid-logarithmic phase in LB broth when *carRS-lacZ* expression was quantified. The results showed an \sim 2-fold increase in *carRS* expression in both the Δ *leuO* and Δ *toxRS* mutants relative to the WT (Fig. 1A), suggesting that both ToxR and LeuO were negative regulators of *carRS*. The observation that *carRS* expression increased similarly in the Δ *toxRS* mutant and the Δ *leuO* mutant suggested that ToxR indirectly regulates *carRS* via *leuO*.

V. cholerae CarRS mediates PXB susceptibility by positively regulating the expression of the LPS remodeling genes *almEFG* (24). We hypothesized that if ToxR and LeuO were regulating PXB resistance via *carRS* repression, then deletion of *toxRS* or *leuO* should result in a corresponding increase in *almEFG* expression. To test this, we introduced an *almEFG-lacZ* reporter plasmid (pXB342) into the WT and the Δ *leuO* and Δ *toxRS* mutant strains and quantified *almEFG-lacZ* expression as described above. As expected, *almEFG* expression increased by \sim 2-fold in both the Δ *leuO* and Δ *toxRS* mutants relative to the WT (Fig. 1B), supporting our hypothesis. Taken together, these results suggested that ToxR and LeuO modulate PXB resistance via repression of *carRS*.

LeuO directly represses *carRS* transcription. The above-described reporter data indicated that both ToxR and LeuO negatively regulated *carRS* expression but did not address whether ToxR or LeuO acted directly at the *carRS* promoter. To examine this, we tested the effect of *toxRS* and *leuO* overexpression on *carRS-lacZ* expression in *E. coli*. We hypothesized that if either regulatory protein acted directly at the *carRS* promoter, then their overexpression in a heterologous host would repress expression of a *carRS-lacZ* reporter. We therefore cultured *E. coli* bearing pVA289 (*carRS-lacZ*) and either pBAD33 or pBAD33-*leuO* in the presence of various concentrations of arabinose and quantified *carRS-lacZ* expression. The results showed that the addition of

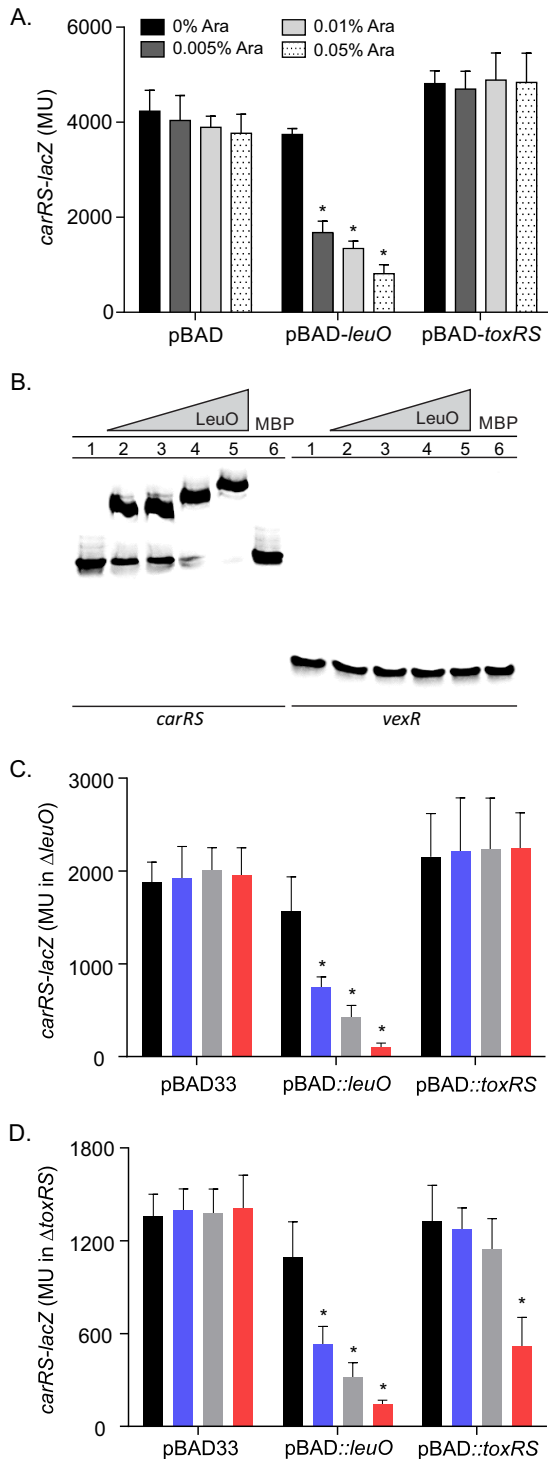


FIG 2 LeuO acts directly on the *carRS* promoter. (A) *E. coli* carrying either pBAD33, the pBAD33-*leuO* plasmid pVA126, or the pBAD33-*toxRS* plasmid pXB302 plus the *carRS-lacZ* reporter plasmid pVA289 was grown in LB broth containing the indicated amount of arabinose (Ara) to mid-logarithmic phase and assayed for β -galactosidase activity as described in Materials and Methods. The presented data are averages \pm standard deviations from three independent experiments. Statistical analysis was conducted using two-way ANOVA with Tukey's posttest. *, $P < 0.0001$. (B) Electrophoretic mobility shift assay showing the binding of LeuO to the *carRS* promoter. Biotin-labeled DNA fragments containing *carRS* (1.5 nM) or *vexR* (3 nM) promoter were incubated with LeuO-MBP at 0 μ M (lane 1), 2.5 μ M (lane 2), 5 μ M (lane 3), 10 μ M

arabinose did not affect *carRS-lacZ* expression in the negative-control cultures bearing pBAD33 (Fig. 2A). The addition of arabinose to the cultures bearing pBAD33-*toxRS* also did not affect *carRS-lacZ* expression, suggesting that ToxR represses *carRS* expression indirectly. In contrast, there was an arabinose dose-dependent reduction in *carRS-lacZ* expression in the cultures containing pBAD33-*leuO*, suggesting that LeuO directly represses *carRS* transcription.

The above-described results suggested that LeuO acts directly at the *carRS* promoter; however, they did not exclude the possibility that LeuO affected *carRS* expression via an intermediate protein that was conserved in *E. coli* and *V. cholerae*. To determine if LeuO was capable of acting directly on the *carRS* promoter, we performed an EMSA with MBP-LeuO and the *carRS* promoter as previously described (19). The results showed that incubation of MBP-LeuO with a DNA probe spanning the *carRS* promoter resulted in a mobility shift in the *carRS* probe starting at 2.5 μ M MBP-LeuO (Fig. 2B, lane 2) and was further shifted with increasing concentrations of MBP-LeuO (lanes 4 and 5). It is unclear whether the additional shifting of the probe resulted from MBP-LeuO multimerization, as has been reported with LeuO in *Salmonella enterica* serovar Typhi (41). Incubation of the *carRS* probe with MBP alone did not result in a mobility shift (lane 6), indicating that LeuO-MBP binding to the *carRS* promoter probe was due to LeuO and not due to nonspecific binding by the MBP. To further test the specificity of LeuO binding at the *carRS* promoter, we repeated these experiments using a DNA probe derived from the *V. cholerae vexR* promoter. The results showed that neither MBP-LeuO (lanes 2 to 5) nor MBP (lane 6) affected the mobility of the *vexR* DNA probe. This result is consistent with the conclusion that MBP-LeuO binding to the *carRS* promoter was not due to nonspecific interactions between MBP-LeuO and DNA. Based on these results, we concluded that LeuO likely functioned downstream of ToxR to directly repress *carRS* transcription.

Our reporter data suggested that ToxR indirectly regulated *carRS* via LeuO (Fig. 2A). ToxR also directly regulates *toxT* expression in *V. cholerae*, but this phenotype was not replicated in *E. coli* due to the lack of a *toxT* coregulator (i.e., TcpPH) (42). Based on this fact, we could not rule out the possibility that ToxR directly regulated *carRS* in *V. cholerae*. To address this, we examined whether ToxR could activate *carRS* expression in *V. cholerae* mutants lacking *leuO* or *toxRS*. We introduced pXB340 (*carRS-lacZ*) and either pBAD33, pBAD33-*leuO*, or pBAD33-*toxRS* into XBV222 (Δ *leuO* mutant) and JB461 (Δ *toxRS* mutant). The resulting strains were then cultured to mid-log phase in the presence of increasing concentrations of arabinose when *carRS-lacZ* expression was quantified. The results showed that the addition of arabinose did not have any effect on *carRS* expression in the empty vector control in either mutant background (Fig. 2C and D). Overexpression of *leuO* resulted in an arabinose concentration-dependent decrease in *carRS* expression in both mutants (Fig. 2C

(lane 4), or 25 μ M (lane 5) or with MBP at 25 μ M (lane 6) prior to electrophoresis. (C) *V. cholerae* Δ *leuO* strain carrying either pBAD33, pBAD33-*leuO*, or pBAD33-*toxRS* plus the *carRS-lacZ* reporter plasmid pVA289 was grown in LB broth containing 0% (black), 0.005% (purple), 0.05% (grey), or 0.01% (red) arabinose to mid-logarithmic phase and assayed for β -galactosidase activity as described in Materials and Methods. (D) Same as panel C, except the *V. cholerae* Δ *toxRS* strain was used. Statistical analysis was conducted using two-way ANOVA with Tukey's posttest. *, $P < 0.005$.

TABLE 2 Polymyxin B susceptibility of *V. cholerae* strains

Strain	Polymyxin B MIC ($\mu\text{g/ml}$) ^a	
	Log phase	Stationary phase
WT	128	32
$\Delta\textit{leuO}$	384	64
$\Delta\textit{toxRS}$	384	2
$\Delta\textit{ompU}$	128	3
$\Delta\textit{ompU} \Delta\textit{toxRS}$	384	3
$\Delta\textit{ompU} \Delta\textit{leuO}$	384	3

^a The MICs for the indicated strains were determined using the Etest with LB broth cultures.

and D), providing additional support for the conclusion that LeuO directly represses *carRS* expression. Significantly, expression of *leuO* from pBAD33-*leuO* still repressed *carRS* in the *toxRS*-negative background, indicating that LeuO functions independently of ToxR. In contrast, overexpression of *toxRS* repressed *carRS* expression in the $\Delta\textit{toxRS}$ (*leuO*⁺) background but not in the $\Delta\textit{leuO}$ mutant background. The observation that ToxR only represses *carRS* transcription in the presence of *leuO* is consistent with the conclusion that ToxR functions indirectly at the *carRS* promoter. This conclusion is further supported by the fact that the *carRS* promoter lacks a consensus ToxR binding site that is found in the promoter region of many ToxR-regulated genes, including *leuO* (10, 43).

LeuO regulates polymyxin B resistance in *V. cholerae*. It has been reported that CarRS contributes to PXB resistance by positively regulating the expression of the *almEFG* lipid A glycosylation system (24, 25). Since our data described above indicated that LeuO was a *carRS* repressor (Fig. 1 and 2), we hypothesized that a *leuO* mutant should exhibit increased resistance to PXB as a result of *carRS* derepression. To test this, we determined the PXB MIC for mid-log-phase cultures of the WT and an isogenic $\Delta\textit{leuO}$ mutant using Etests according to the manufacturer's instructions (bioMérieux). The findings revealed that *leuO* deletion resulted in a 3-fold increase in the PXB MIC relative to that of the WT (Table 2), confirming that *leuO* negatively regulated PXB resistance. Since ToxR positively regulates *leuO* expression, we also examined a $\Delta\textit{toxRS}$ mutant. The $\Delta\textit{toxRS}$ mutant displayed a MIC that was identical to that of the $\Delta\textit{leuO}$ mutant. Given that ToxR positively regulates *leuO*, this finding is consistent with the conclusion that ToxR indirectly represses *carRS* via *leuO*.

Production of the OmpU porin has been reported to confer a survival advantage to *V. cholerae* upon PXB exposure in killing assays due to its function in the activation of the sigma E extracytoplasmic stress response (17, 18). To determine whether *toxRS* and *leuO* affected the PXB MIC via the sigma E membrane stress response, we examined the effect of *leuO* and *toxRS* mutations on the PXB MIC in an *ompU*-negative strain which is deficient for sigma E activation. The PXB MIC results showed that mid-log-phase cultures of the respective *ompU* mutants phenocopied their respective *ompU*-positive parental strains (Table 2). The fact that *ompU* deletion did not affect PXB sensitivity when combined with deletions of *leuO* or *toxRS* indicates that *leuO* functioned independently of the sigma E membrane stress response to regulate *V. cholerae* PXB resistance. The fact that the PXB MIC increased in the $\Delta\textit{ompU} \Delta\textit{toxRS}$ mutant, which cannot activate sigma E, suggests that ToxR has pleiotropic effects on PXB resistance, being

TABLE 3 Polymyxin B susceptibility of *V. cholerae* strains overexpressing *leuO* at mid-log phase

Strain	MIC ^a ($\mu\text{g/ml}$) (SD)
WT (pBAD18Km)	118 (13.4)
WT (pBAD18Km- <i>leuO</i>)	40 ^b (15.9)
$\Delta\textit{leuO}$ (pBAD18Km)	294 (45.6)
$\Delta\textit{leuO}$ (pBAD18Km- <i>leuO</i>)	56 ^c (5.6)

^a The MIC was determined using PXB gradient agar plates with mid-log-phase LB broth cultures.

^b *P* value of ≤ 0.01 relative to WT pBAD18Km.

^c *P* value of ≤ 0.01 relative to $\Delta\textit{leuO}$ pBAD18Km.

required for both *leuO* expression and the induction of the sigma E membrane stress response via OmpU (11, 17).

We performed complementation experiments to confirm that *leuO* repressed PXB resistance in *V. cholerae*. In these experiments, we ectopically expressed *leuO* from the arabinose-regulated promoter in pBAD18Km in WT and $\Delta\textit{leuO}$ strains before determining the PXB MIC of the resulting cultures on PXB gradient agar plates. The results showed that *leuO* expression decreased the PXB MIC in both the WT and the $\Delta\textit{leuO}$ mutant (Table 3). Overexpression of *leuO* resulted in an ~ 3 -fold decrease in the PXB MIC in the WT and an ~ 5 -fold decrease in the PXB MIC in the $\Delta\textit{leuO}$ mutant. The fact that *leuO* overexpression decreased the PXB MIC in both the WT and the $\Delta\textit{leuO}$ mutant was consistent with the hypothesis that *leuO* regulates PXB resistance via *carRS* repression.

Growth-phase-dependent regulation of PXB susceptibility. During the course of our studies, we observed that *V. cholerae* susceptibility to PXB varied according to the growth phase. To confirm this observation, we repeated the PXB MIC studies using *V. cholerae* stationary-phase cultures. The results revealed a significant decrease in the PXB MIC in stationary-phase cells (Table 2). This was evidenced by 4-fold and 6-fold decreases in the PXB MIC for the WT and the $\Delta\textit{leuO}$ mutant, respectively. The MIC for the $\Delta\textit{leuO}$ mutant was still elevated relative to that of the WT, suggesting that *leuO* still contributed to PXB resistance at stationary phase (Table 2). Deletion of *toxRS* resulted in a 16-fold decrease in the PXB MIC relative to that of WT stationary-phase cells. This contrasts with mid-log-phase cells, where *toxRS* deletion increased the PXB MIC. To determine if the *toxRS* phenotype was related to its role in the induction of the sigma E stress response via *ompU* (17), we assessed an isogenic $\Delta\textit{ompU}$ mutant at stationary phase. The *ompU* mutant exhibited a PXB MIC that was similar to that of the $\Delta\textit{toxRS}$ mutant. Further deletion of *toxRS* or *leuO* in the $\Delta\textit{ompU}$ background did not affect the PXB MIC in stationary-phase cells. The observation that the $\Delta\textit{ompU}$ and $\Delta\textit{toxRS}$ mutants exhibited similar decreases in their PXB MICs in stationary-phase cells suggests that the OmpU-dependent activation of the sigma E extracytoplasmic stress response is important for PXB resistance at stationary phase. The fact that deletion of *leuO* in the $\Delta\textit{ompU}$ background did not affect the PXB MIC suggests that the contribution of OmpU, and likely the sigma E membrane stress response, is epistatic to *leuO* for PXB resistance at stationary phase. These results provide additional support for the conclusion that ToxR has pleiotropic effects on PXB resistance due to its requirement for the expression of both *leuO* and *ompU*.

LeuO represses *carRS* expression in response to bile salts. Previous reports indicated that *leuO* and *carRS* were inversely regulated in response to bile salts, with *leuO* being upregulated and

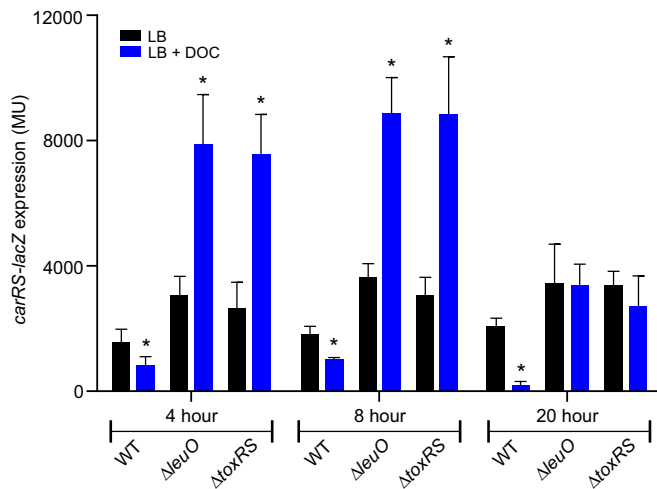


FIG 3 Expression of *carRS* is repressed by deoxycholate in a LeuO-dependent manner. *V. cholerae* WT strain JB58, $\Delta leuO$ mutant strain XBV222, and $\Delta toxRS$ mutant strain DT733 carrying pXB340 (*carRS-lacZ*) were cultured in LB broth in the presence or absence of 0.05% deoxycholate (DOC) to mid-log phase (4 h), late-log phase (8 h), and stationary phase (20 h), and then β -galactosidase activity was quantified as described in Materials and Methods. The presented data are means \pm standard deviations from three independent experiments. Statistical analysis was conducted using a paired *t* test. *, $P < 0.05$ relative to the control cultured in LB broth.

carRS being repressed (10, 25). This suggested the possibility that LeuO and, thus, ToxR are responsible for *carRS* repression by bile salts. To test this, we grew the WT, $\Delta leuO$, and $\Delta toxRS$ strains containing the *carRS-lacZ* reporter (pXB340) in LB broth with or without 0.05% deoxycholate. We then quantified *carRS-lacZ* expression at mid-log phase (4 h), late log phase (8 h), and stationary phase (20 h). We observed increased *carRS* expression in the *leuO* and *toxRS* mutants relative to the WT during growth in LB broth at all three time points (Fig. 3). The addition of deoxycholate repressed *carRS* expression in the WT at all three time points, which is consistent with previous reports (25). The deoxycholate-dependent repression of *carRS* was abrogated in both the *toxRS* and *leuO* mutants, a finding that is consistent with the notion that ToxR indirectly represses *carRS* expression via LeuO (10). Surprisingly, in the absence of *leuO* or *toxRS*, deoxycholate stimulated *carRS* expression in mid- and late-log-phase cultures by >2 -fold relative to cells grown in LB broth alone (Fig. 3). This bile salt-dependent enhancement was lost in the stationary-phase cells. This suggests that deoxycholate can activate *carRS* expression by an unknown mechanism in the absence of *leuO* and *toxRS*. Although the mechanism by which this occurs is unknown, this regulatory signal could be important under environmental conditions where ToxR is diminished (44).

Our data suggested a model where bile salts activated the ToxR-dependent expression of *leuO*. LeuO then repressed *carRS*, which resulted in reduced *almEFG* expression, decreased LPS glycosylation, and increased PXB susceptibility. To test this model, we determined the PXB MIC for WT, $\Delta leuO$, $\Delta toxRS$, $\Delta ompU$, $\Delta ompU \Delta leuO$, and $\Delta ompU \Delta toxRS$ strains following growth to mid-log phase in LB broth containing 0.05% deoxycholate (Table 4). The results showed that growth of the WT in deoxycholate decreased its PXB MIC by 4-fold relative to growth in LB broth (Tables 2 and 4). In contrast, deoxycholate did not affect the PXB

MIC for the $\Delta leuO$ and $\Delta toxRS$ mutants relative to their growth in LB broth alone. This indicated that the decreased PXB MIC observed in WT cells grown in deoxycholate was dependent on *toxR*-dependent expression of *leuO* in response to deoxycholate and not due to nonspecific effects of growth in deoxycholate. The PXB MIC for the $\Delta ompU$ mutant was slightly lower than that for the WT, which was consistent with *ompU* contributing to PXB resistance. The MIC for the $\Delta ompU \Delta leuO$ mutant was unchanged relative to that of cells grown in the absence of deoxycholate. However, growth in deoxycholate reduced the PXB MIC for the $\Delta ompU \Delta toxRS$ mutant (1.5-fold) relative to the same strain grown in the absence of deoxycholate. Since bile salts enhance ToxR activity at target promoters (10), the latter result suggests that ToxR contributes to PXB resistance by both *leuO*-dependent and *leuO*-independent mechanisms.

The above-described results suggested the possibility that the inverse regulation of *leuO* and *carRS* by deoxycholate affects *V. cholerae* survival upon challenge with CAMPs. To investigate this, we performed PXB survival assays with the WT and $\Delta leuO$ and $\Delta toxRS$ mutants. An isogenic *almE* mutant was included as a negative control. The strains were independently cultured to mid-log phase in LB broth or LB broth plus 0.05% deoxycholate. The cells were then challenged by exposure to a high concentration of PXB (500 μ g/ml) for 60 min, and then survival was assessed by plating for viable cells. The results showed a decrease in survival of the WT following growth in deoxycholate relative to cells grown in the absence of deoxycholate (Fig. 4). There was an ~ 2 -log drop in survival of the $\Delta leuO$ and $\Delta toxRS$ mutants following growth in LB broth. Conversely, growth of the $\Delta leuO$ and $\Delta toxRS$ mutants in deoxycholate imparted a >2 -log increase in survival relative to cells grown in LB alone. These results appear to contradict the MIC results where growth of the $\Delta leuO$ and $\Delta toxRS$ mutants in deoxycholate did not affect the PXB MIC. This discrepancy likely reflects the different experimental approaches used in each study. Killing assays assess the immediate physiological state of the cell and are not dependent on cell replication during PXB challenge. In contrast, MIC determination requires cell replication to visualize the results, which allows the expression of adaptive responses that dilute the physiological effects that were imparted to the cells during growth in the presence of deoxycholate. Finally, the *almE* mutant was not recovered under the test conditions, confirming previous reports that mutations into this locus resulted in a PXB hypersensitive phenotype (27).

The results of the killing assays further supported the notion that *leuO* expression adversely affects PXB resistance. The fact that the $\Delta leuO$ and $\Delta toxRS$ mutants showed a >2 -log survival increase following growth in deoxycholate indicates the presence of an

TABLE 4 Sensitivity of *V. cholerae* strains to polymyxin B following exposure to deoxycholate

Strain	PXB MIC ^a (μ g/ml)
WT	16
$\Delta leuO$	384
$\Delta toxRS$	384
$\Delta ompU$	12
$\Delta ompU \Delta toxRS$	256
$\Delta ompU \Delta leuO$	384

^a The MICs for the indicated strains were determined using the Etest following growth to mid-log phase in LB broth containing 0.05% deoxycholate.

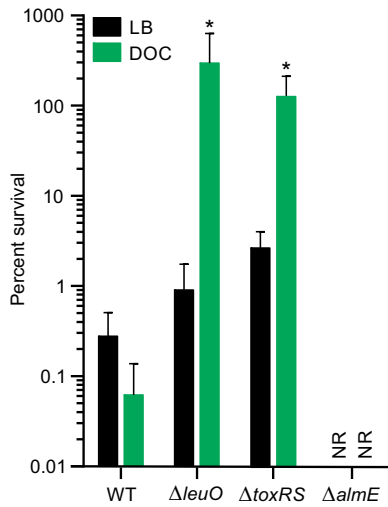


FIG 4 *V. cholerae* survival following PXB challenge. *V. cholerae* WT strain JB58, isogenic $\Delta leuO$ strain XB222, $\Delta almE$ strain XB302, and $\Delta toxRS$ strain JB461 were grown in LB broth or LB broth plus 0.05% deoxycholate (DOC) to mid-logarithmic phase before being incubated in LB broth containing a lethal concentration of polymyxin B. Aliquots were collected at 60 min after exposure to polymyxin B and plated onto LB agar to quantify the viable cells as described in Materials and Methods. The percent survival for each strain is reported relative to the input cell count. The data presented are averages \pm standard deviations from four independent experiments. Statistical analysis was conducted using two-way ANOVA. *, *P* value of <0.05 relative to the input. NR, none recovered.

uncharacterized bile-dependent regulatory circuit that positively regulates CAMP resistance in the absence of *leuO*. This suggests that LeuO functions to antagonize this regulatory circuit in the presence of bile. This also suggests that the increased survival of the *toxRS* and *leuO* mutants following growth in deoxycholate is due to the deoxycholate-dependent upregulation of *carRS*.

LeuO does not regulate *carRS* expression in response to PXB or Ca^{2+} . In addition to being regulated by bile salts, *carRS* expression was also reported to be regulated in response to antimicrobial peptides and calcium (Ca^{2+}) where antimicrobial peptides activated *carRS* expression (25) and extracellular Ca^{2+} repressed *carRS* expression (29). Since *leuO* regulated *carRS* in response to bile salts, we investigated whether LeuO also regulated *carRS* expression in response to these stimuli. We examined whether Ca^{2+} or PXB affected *leuO* expression. To do this, we cultured WT containing a *leuO-lacZ* reporter (pXB266) in the presence and absence of 5 mM $CaCl_2$ or subinhibitory amounts of PXB (1/32 and 1/16 of its MIC) and quantified *leuO* expression. The results showed that PXB did not affect *leuO* expression at either concentration (Fig. 5), suggesting that *leuO* is not involved in regulating *carRS* in response to PXB. In contrast, growth of *V. cholerae* in the presence of 5 mM $CaCl_2$ resulted in an ~25% decrease in *leuO* expression. This indicates that *leuO* expression is regulated in response to Ca^{2+} in *V. cholerae*. However, as Ca^{2+} represses both *leuO* and *carRS* transcription, this result indicated that Ca^{2+} -dependent repression of *carRS* occurs by a mechanism that is independent of *leuO*. Taken together, these results support the conclusion that *leuO* expression is modulated by calcium and suggest that LeuO is not involved in the PXB- or Ca^{2+} -dependent regulation of *carRS* expression in *V. cholerae*.

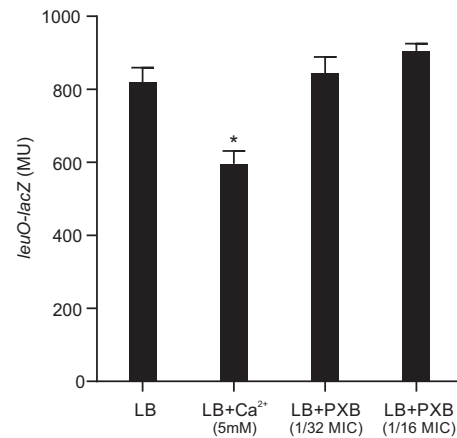


FIG 5 Expression of *leuO* is regulated by calcium but not polymyxin B. *V. cholerae* WT strain JB58 carrying the *leuO-lacZ* reporter plasmid pXB266 was cultured in LB broth in the presence or absence of 5 mM $CaCl_2$ or the indicated concentrations of PXB to mid-logarithmic-growth phase before being assayed for β -galactosidase activity. The presented data are means \pm SD from three independent experiments. Statistical analysis was conducted using a paired *t* test. *, *P* < 0.05 .

DISCUSSION

V. cholerae is a natural inhabitant of aquatic ecosystems. Its entry into the human host represents a dramatic environmental shift where the cells are confronted with a number of barriers to colonization, including exposure to bile salts and products of the innate immune system. ToxR plays a critical role in the transition to the human host and overcoming these barriers (10, 11, 19, 45–47). In addition to regulating the production of essential virulence factors, ToxR also functions to modulate the composition of the outer membrane to fine-tune its barrier properties. In response to bile salts ToxR activates *ompU* expression while repressing *ompT* expression; the preferential expression of *ompU* enhances *V. cholerae* resistance to the toxic effects of bile salts (21). ToxR-dependent expression of *ompU* has also been linked to CAMP resistance due to the requirement for OmpU in the induction of the sigma E membrane stress response (17). In this study, we have shown that the function of ToxR extends beyond porin regulation to include the CarRS-AlmEFG lipid A glycylation system (Fig. 6). We showed that ToxR indirectly regulated *carRS* and *almEFG* expression, and PXB resistance, via *leuO*. This was evident by the fact that deletion of *toxRS* or *leuO* increased *V. cholerae* resistance to PXB, while *leuO* overex-

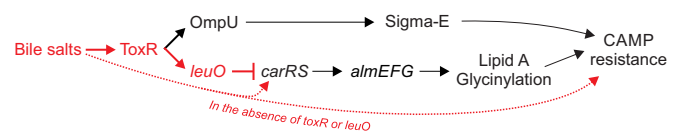


FIG 6 Model for the function of ToxR and LeuO in antimicrobial peptide resistance. ToxR-dependent transcription of *leuO* is activated in response to bile salts. LeuO then directly represses *carRS* by binding to its promoter. This results in decreased expression of *almEFG*, which reduces lipid A glycylation and results in increased susceptibility to CAMPs. ToxR also activates expression of the major outer membrane porin *ompU* in response to bile salts. OmpU serves as a membrane stress sensor for activation of the sigma E membrane stress response, which contributes to the mitigation of the deleterious effects of CAMPs. Lastly, in the absence of ToxR and/or LeuO, bile salts activate *carRS* transcription and increase CAMP resistance by an unknown mechanism. Pathways shown in red were described in this work.

pression decreased *carRS* and *almEFG* expression and increased PXB susceptibility. The bile salt deoxycholate stimulated the expression of this negative regulatory pathway in WT cells, but surprisingly it activated *carRS* expression and increased PXB resistance in cells lacking *toxRS* or *leuO*, which suggests that LeuO functions as a *carRS* antagonist in response to bile salts.

The linkage between ToxR, deoxycholate, and *carRS* expression suggests the intriguing possibility that ToxR regulates outer membrane remodeling in response to spatial cues in the host. Following ingestion, *V. cholerae* is initially localized to the intestinal lumen but then migrates to and colonizes the intestinal epithelium crypts. The intestinal crypts are separated from the lumen by a mucous layer which is thought to function as a diffusion barrier that retains CAMPs produced by enterocytes while limiting exposure of the intestinal epithelium to luminal bile salts (48). This would likely result in a bile salt and CAMP concentration gradient across the mucosa with CAMPs being concentrated near the epithelial surface and bile salts being most concentrated toward the lumen. In this scenario, it is conceivable that *V. cholerae* localized to the bile-rich, but CAMP-barren, lumen would down-regulate *carRS* expression via ToxR-dependent activation of *leuO*. However, as *V. cholerae* traverses the mucus layer to colonize the crypts, it is likely to be exposed to decreasing levels of bile salts and increasing levels of CAMPs. Reduced bile salts exposure in the mucous layer would result in reduced *leuO* expression, derepression of *carRS*, and increased lipid A glycosylation by AlmEFG, which would mitigate the effects of the increased CAMP exposure near the epithelial surface. While this model is speculative, evidence does suggest that *leuO*, *carRS*, and *almEFG* are all expressed *in vivo* (11, 24, 25); however, additional work will be required to determine if ToxR differentially regulates its target genes between the lumen and epithelial surface.

ToxR indirectly regulated *carRS* transcription via LeuO in response to deoxycholate. Although ToxR is a major virulence regulator, *toxR* is also part of the ancestral chromosome, and its regulon extends beyond horizontally acquired virulence genes. This conclusion is supported by microarray studies which implicated ToxR in the regulation of >150 genes in *V. cholerae*, including genes involved in cellular transport, energy metabolism, motility, iron uptake, and outer membrane proteins (30). This conclusion is further supported by a recently published ToxR chromatin immunoprecipitation sequencing (ChIP-seq) study showing that ToxR can function as an H-NS antagonist (49). We previously showed that ToxR directly activated *leuO* expression in response to environmental cues by a process that was dependent upon the presence of the periplasmic domain of ToxR (10, 11). This suggested that the periplasmic domain of ToxR functioned as an environmental sensor to activate *leuO* expression in response to extracellular stimuli (e.g., bile salts). LeuO itself has been linked to diverse phenotypes in *V. cholerae*, including biofilm formation, virulence, bile resistance, and acid tolerance (10, 11, 19, 20). Since many of these phenotypes have also been associated with ToxR, these results suggest that ToxR regulates many of its gene targets indirectly. This conclusion is supported by the aforementioned ChIP-seq studies indicating that ToxR directly regulated just 39 genes (49). The gene encoding LeuO was among the 39 genes identified in the ChIP-seq data set, confirming our previous reports that ToxR directly regulated *leuO* transcription (10, 11). Collectively these findings suggest that the ToxT-independent branch of the ToxR regulon is a hierarchical regulatory system that

likely plays a more extensive role in *V. cholerae* biology than previously appreciated.

The PXB MIC declined as *V. cholerae* entered stationary phase. The reason for this decline was unclear, but a similar phenomenon was also observed in *V. cholerae* O1 El Tor strain A1552 with both granulysin-derived peptides and PXB (50), which indicated that this was not a strain-specific phenotype. We noted that deletion of *leuO* or *toxRS* increased the PXB MIC in mid-log-phase cells but not in stationary-phase cells. This suggests that the effects of *carRS* derepression on PXB resistance in these mutants was abrogated at stationary phase. We also found that *ompU* mutation decreased the PXB MIC in stationary-phase cells and that additional deletion of *leuO* or *toxRS* in the *ompU*-negative strain did not further affect the PXB MIC. This contrasts with mid-log-phase cultures where *ompU* deletion did not affect the MIC and where $\Delta ompU \Delta leuO$ and $\Delta ompU \Delta toxRS$ double mutants exhibited increased PXB MICs. These results indicate that unknown factors affect PXB resistance in stationary-phase cells. These results also suggest that OmpU production is critical for PXB resistance at stationary phase and that the *ompU*-dependent resistance trait was dominant in *carRS* derepression. Although we do not know the reasons for the *ompU*-dependent defect, we speculate that decreased PXB resistance in stationary-phase cells reflects the inability of *ompU* mutants to express the sigma E membrane stress response (17). The sigma E stress response has been shown to be activated predominantly at stationary phase when the cells experience deleterious effects caused by nutrient limitation, pH alterations, oxidative stress, and the accumulation of toxic metabolites (51). It is therefore possible that the deleterious environmental conditions that are present in stationary-phase cultures, and absent from mid-log-phase cultures, enhance the bactericidal activity of PXB on stationary-phase cells, resulting in a decrease in the PXB MIC.

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