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Reciprocal Regulation of Resistance-Nodulation-Division Efflux Systems and the Cpx Two-Component System in *Vibrio cholerae*

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The Cpx two-component regulatory system has been shown in *Escherichia coli* to alleviate stress caused by misfolded cell envelope proteins. The *Vibrio cholerae* Cpx system was previously found to respond to cues distinct from those in the *E. coli* system, suggesting that this system fulfills a different physiological role in the cholera pathogen. Here, we used microarrays to identify genes that were regulated by the *V. cholerae* Cpx system. Our observations suggest that the activation of the *V. cholerae* Cpx system does not induce expression of genes involved in the mitigation of stress generated by misfolded cell envelope proteins but promotes expression of genes involved in antimicrobial resistance. In particular, activation of the Cpx system induced expression of the genes encoding the VexAB and VexGH resistance-nodulation-division (RND) efflux systems and their cognate outer membrane pore protein TolC. The promoters for these loci contained putative CpxR consensus binding sites, and ectopic *cpxR* expression activated transcription from the promoters for the RND efflux systems. CpxR was not required for intrinsic antimicrobial resistance, but CpxR activation enhanced resistance to antimicrobial substrates of VexAB and VexGH. Mutations that inactivated VexAB or VexGH efflux activity resulted in the activation of the Cpx response, suggesting that *vexAB* and *vexGH* and the *cpxP-cpxRA* system are reciprocally regulated. We speculate that the reciprocal regulation of the *V. cholerae* RND efflux systems and the Cpx two-component system is mediated by the intracellular accumulation of an endogenously produced metabolic by-product that is normally extruded from the cell by the RND efflux systems.

Vibrio cholerae is a facultative human pathogen that causes cholera, a severe acute diarrheal disease that is estimated to afflict 3 to 5 million people annually (1). People acquire cholera by ingestion of *V. cholerae*-contaminated food or water (2). Once in the host environment, *V. cholerae* produces a variety of virulence factors that enable the pathogen to colonize the small intestine and to cause diarrhea. Two critical virulence factors coregulated by the virulence activator ToxR are the toxin-coregulated pilus (TCP), a type IV pilus that is essential for colonization, and cholera toxin (CT), an enterotoxin that causes the secretory diarrhea that is the hallmark of cholera (3–8). Like the expression of TCP, intestinal colonization is dependent upon *V. cholerae* overcoming host barriers in the human gastrointestinal tract. These barriers include antimicrobial compounds, such as bile salts, fatty acids, and components of the innate immune system. *V. cholerae* resistance to these factors is largely dependent upon the production of the resistance-nodulation-division (RND) family of efflux systems (9, 10).

RND efflux systems are tripartite transporters that are ubiquitous among Gram-negative bacteria. Each RND efflux system is made up of three components: an outer membrane porin homologous to *Escherichia coli tolC*, an integral cytoplasmic membrane pump protein belonging to the RND superfamily of transporters, and a periplasmic membrane fusion protein that links the outer membrane pore to the cytoplasmic membrane pump protein (11–15). These RND systems have garnered much attention with regard to xenobiotic resistance, as a number of RND systems have been shown to efflux numerous chemically unrelated antimicrobial compounds, including dyes, detergents, antibiotics, and antimicrobial peptides (16, 17). As such, many of these broad-spectrum RND efflux systems are intricately linked to the development of multiple-drug-resistant organisms. Although antibiotic resistance provides an easily scored phenotype for many efflux systems, phylogenetic analysis indicates that the RND efflux systems evolved independently of antibiotic selection (18, 19). Thus, the native role of the RND efflux systems in bacterial physiology remains unclear, but there is accumulating evidence to suggest that they influence bacterial physiology independently of their role in xenobiotic resistance. This is exemplified by reports implicating RND efflux systems in diverse phenotypes (reviewed in reference 20), such as biofilm formation (21, 22), iron acquisition (23), plant-bacterium interactions (24), lipid transport (25, 26), bacterial virulence (9, 10, 27), extrusion of toxic metal effectors (28), and removal of metabolic by-products from within the cell (29).

V. cholerae encodes six RND efflux systems that are required for antimicrobial resistance, virulence factor production, and intestinal colonization (9, 30, 31). Functional characterization of the RND systems revealed that four of the six systems (i.e., VexAB, VexCD, VexGH, and VexIJK) mediate resistance to antimicrobial compounds *in vitro* (9, 10, 31). The VexAB RND efflux system exhibits a high basal level of activity and provides *V. cholerae* with its intrinsic antimicrobial resistance, a function analogous to that

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of *E. coli*'s AcrAB. The VexAB system is a multiple-drug efflux system that mediates resistance to bile salts, nonionic detergents, and a variety of antibiotics (e.g., ampicillin, erythromycin, novobiocin, and polymyxin B). The three other RND efflux systems are functionally redundant with VexAB. VexCD is a bile-specific efflux system; VexGH mediates resistance to bile acids, nonionic detergents, ampicillin, and novobiocin; and VexIJK effluxes bile acids, nonionic detergents, and novobiocin. The two remaining RND efflux systems, VexEF and VexLM, do not appear to influence resistance to antimicrobials. All six of the RND efflux systems contribute to virulence (9, 30), and strains lacking the RND efflux systems are severely attenuated *in vivo* (9, 30). Inactivation of the RND efflux systems results in downregulation of the ToxR regulon, diminished CT and TCP production, and severe attenuation of growth in suckling mice (9, 32). The mechanism(s) by which the RND efflux systems influence virulence gene expression is unknown.

The Cpx two-component system is widely distributed among *Gammaproteobacteria*, including the *Enterobacteriaceae* and *Vibrionaceae*. In this regulatory system, CpxA functions as a membrane-associated sensor histidine kinase. Upon stimulation, CpxA autophosphorylates itself and then transfers the phosphate to a conserved aspartate residue on the cytoplasmic CpxR response regulator (reviewed in references 33 and 34). Phosphorylated CpxR (CpxR~P) then modulates the expression of its target genes by binding to a consensus binding sequence located in the genes' promoter regions. CpxR~P also regulates its own expression (i.e., the *cpxRA* operon) and the divergently transcribed *cpxP*. CpxP is a periplasmic protein associated with the *cpxRA* operon that appears to repress CpxR activation by interacting with CpxA and inhibiting its kinase activity and may also exhibit chaperone activity (35). *CpxA** mutants lead to constitutive activation of the Cpx system and have been useful in analyses of Cpx regulons. The *cpxA** mutation inactivates CpxA phosphatase activity, resulting in the accumulation of activated CpxR (i.e., CpxR~P) (36–38). The Cpx system has been most extensively studied in *E. coli*, where it has been shown to alleviate extracytoplasmic stress resulting from cell envelope perturbations that are generally associated with misfolded cell envelope proteins (39, 40). Consistent with this idea, the majority of stimuli that activate the Cpx system have been predicted to result in the production of misfolded or damaged cell envelope proteins (reviewed in references 33 and 34).

Although a number of Cpx-inducing stimuli have been described in *E. coli*, studies suggest that these stimuli are not conserved in *V. cholerae* (36). For example, the *E. coli* Cpx system is activated by increased osmolarity but not by increased salinity (36, 41). In contrast, the *V. cholerae* Cpx system functions in an opposite manner; it is not responsive to osmolarity but is activated by increased salinity (36). Additionally, the *E. coli* Cpx system is active under standard laboratory growth conditions, whereas the *V. cholerae* Cpx system is inactive. The differences in the physiological roles of the Cpx systems in *E. coli* and *V. cholerae* may be related to the distinct environmental niches that these organisms occupy and appear to be reflected in amino acid sequence variability in the signaling domain of CpxA (36, 42).

While the physiological roles of the *V. cholerae* and *E. coli* Cpx systems appear to differ, deletion of *tolC* activated the Cpx system in both organisms (36, 43). In *E. coli*, the activation of the Cpx system was linked to loss of TolC-dependent efflux (43). TolC functions as the outer membrane pore component of several *V. cholerae* transport systems, including RND family transporters (44). Thus, we speculated that the *tolC*-dependent activation of the *V. cholerae* Cpx system results from the loss of RND efflux activity. Here, we explored the linkage between RND efflux activity and the expression of the Cpx system. We show that CpxR functions as a positive regulator of the VexAB and VexGH RND efflux systems. Conversely, we found that mutation of *vexRAB* or *vexGH* resulted in the activation of the Cpx system, suggesting that the VexAB and VexGH RND efflux systems function in the regulation of the Cpx system. While the *V. cholerae* VexAB and VexGH RND efflux systems and the Cpx system were reciprocally regulated, the defect in virulence factor production in *V. cholerae* RND efflux mutants was independent of the Cpx system. Together, our findings revealed a genetic linkage between the *V. cholerae* Cpx system and RND-mediated efflux and suggested that the *V. cholerae* Cpx system is activated in response to the accumulation of RND efflux substrates.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used in this study are listed in Table 1. *Escherichia coli* EC100D*pir*$^+$ and SM10λ*pir* were used for cloning and plasmid mobilization, respectively. *E. coli* K-12 strains BW25113 and JW3883-1 (*cpxR*::Km) were used as hosts for the *E. coli vexRAB* and *vexGH* reporter assays (45). *V. cholerae* strains used in this study were derivatives of O1 El Tor strain N16961 (46). *V. cholerae* N16961 Δ*lacZ* Sm^r was used as the wild-type (WT) control strain for all experiments. Bacterial strains were grown at 37°C in LB broth or on LB agar. AKI broth was used for virulence-inducing conditions as described previously (10, 47). Bacterial stocks were maintained at −80°C in LB broth containing 25% glycerol. Growth medium was supplemented with carbenicillin (Cb) and streptomycin (Sm) at 100 μg/ml, kanamycin (Km) at 50 μg/ml, or chloramphenicol (Cml) at 1 μg/ml (for *V. cholerae*) or at 20 μg/ml (for *E. coli*) as required. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) was added to LB agar plates at 40 μg/ml unless otherwise indicated.

Plasmid construction. Plasmids and oligonucleotides used in this study are listed in Table 1. β-Galactosidase reporter constructs for *vexEF*, *vexGH*, *vexIJK*, and *vexLM* were constructed as follows. Briefly, the gene-specific PCR primer pairs (i.e., *P-VC0628-F-XhoI/P-VC0628-R-XbaI*, *P-VC0914-F-XhoI/P-VC0914-R-XbaI*, *P-VC1673-F-XhoI/P-VC1673-R-XbaI*, and *P-VCA0638-F-XhoI/P-VCA0638-R-XbaI*) were used to amplify the promoter region for each operon from the *V. cholerae* N16961 genome. The resulting PCR amplicons were then digested with XhoI and XbaI restriction endonucleases before being ligated into similarly digested pTL61T to generate pXB228 (*vexEF-lacZ*), pXB229 (*vexGH-lacZ*), pXB230 (*vexIJK-lacZ*), and pXB232 (*vexLM-lacZ*). The *breR-lacZ* reporter (pXB265) was similarly created using the PCR primer pair *P-VC1746-F-SmaI/P-VC1746-R-BamHI* and the restriction endonucleases indicated in the primer names. The DNA sequence of the reporter constructs were subsequently verified by DNA sequencing.

Mutant construction. Deletion of *V. cholerae cpxR* was performed as previously described (36). Briefly, pΔR was conjugated into *V. cholerae*, and cointegrants were selected for Sm/Km resistance. Several Sm/Km-resistant colonies were streaked onto LB agar (without salt) containing 5% sucrose to select for resolution of the integrated plasmid. Several resistant colonies were screened for Cb sensitivity to verify the loss of the plasmid and for Km resistance to verify the presence of the Km cassette inserted into the *cpxR* gene. PCR using the *cpxR-F* and *cpxR-R* primers was then used to confirm the presence of the *cpxR*::Km insert.

Introduction of the chromosomal *cpxP-lacZ* reporter into *V. cholerae* was performed as previously described (42). Briefly, the pJL1P'Z reporter construct was conjugated into *V. cholerae* strains, and cointegrants were selected for Sm/Cb resistance. Several Sm/Cb-resistant colonies were then streaked onto LB agar (without salt) containing 5% sucrose to select for

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

Strain, plasmid, or primer	Characteristic(s) or sequence[a]	Source
Strains		
E. coli		
EC100D*pir*	*supE44* Δ*lacU169* (ϕ80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1* (λ*pirR6K*)	Epicenter
SM10λ*pir*	*thi-1 thr leu tonA lacY supE recA*::RP4-2-Tc::Mu *km* (λ*pirR6K*)	Lab collection
BW25113	F$^-$ Δ(*araD-araB*)*567 lacZ4787*Δ::*rrnB3* LAM$^-$ *rph-1* Δ(*rhaD-rhaB*)*568 hsdR514*	45
JW3883-1	F$^-$ Δ(*araD-araB*)*567 lacZ4787*Δ::*rrnB3* LAM$^-$ *rph-1* Δ(*rhaD-rhaB*)*568 hsdR514 cpxR*::*km*	45
V. cholerae		
JB58	*V. cholerae* O1 El Tor strain N16961 Δ*lacZ* Sm^r	Lab collection
*cpxA** mutant	JB58::*cpxA**	36
Δ*cpxR* mutant	JB58 Δ*cpxR*	36
JB485	JB58 Δ*vexB* Δ*vexD* Δ*vexF* Δ*vexH* Δ*vexK* Δ*vexM*	9
DT1452	JB485 Δ*cpxR*	This study
MKW589	Δ*cpxR lacZ*::*cpxP-lacZ*$_{Ec}$	36
DT1458	JB58 *lacZ*::*cpxP-lacZ*$_{Ec}$	This study
DT1572	JB58 Δ*vexB lacZ*::*cpxP-lacZ*$_{Ec}$	This study
DT1574	JB58 Δ*vexD lacZ*::*cpxP-lacZ*$_{Ec}$	This study
DT1578	JB58 Δ*vexH lacZ*::*cpxP-lacZ*$_{Ec}$	This study
DT1687	JB58 Δ*vexK lacZ*::*cpxP-lacZ*$_{Ec}$	This study
DT1576	JB58 Δ*vexB* Δ*vexD lacZ*::*cpxP-lacZ*$_{Ec}$	This study
DT1580	JB58 Δ*vexB* Δ*vexH lacZ*::*cpxP-lacZ*$_{Ec}$	This study
DT1689	JB58 Δ*vexB* Δ*vexK lacZ*::*cpxP-lacZ*$_{Ec}$	This study
DT1582	JB58 Δ*vexD* Δ*vexH lacZ*::*cpxP-lacZ*$_{Ec}$	This study
DT1584	JB58 Δ*vexB* Δ*vexD* Δ*vexH lacZ*::*cpxP-lacZ*$_{Ec}$	This study
DT1691	JB58 Δ*vexB* Δ*vexD* Δ*vexK lacZ*::*cpxP-lacZ*$_{Ec}$	This study
DT1478	JB58 Δ*vexB* Δ*vexD* Δ*vexH* Δ*vexK lacZ*::*cpxP-lacZ*$_{Ec}$	This study
DT1480	JB58 Δ*vexB* Δ*vexD* Δ*vexF* Δ*vexH* Δ*vexK lacZ*::*cpxP-lacZ*$_{Ec}$	This study
DT1482	JB58 Δ*vexB* Δ*vexD* Δ*vexH* Δ*vexK* Δ*vexM lacZ*::*cpxP-lacZ*$_{Ec}$	This study
DT1462	JB58 Δ*vexB* Δ*vexF* Δ*vexH* Δ*vexK* Δ*vexM lacZ*::*cpxP-lacZ*$_{Ec}$	This study
DT1476	JB58 Δ*vexD* Δ*vexF* Δ*vexH* Δ*vexK* Δ*vexM lacZ*::*cpxP-lacZ*$_{Ec}$	This study
DT1586	DT1452 *lacZ*::*cpxP-lacZ*$_{Ec}$	This study
DT1460	JB485 *lacZ*::*cpxP-lacZ*$_{Ec}$	This study
Plasmids		
pTL61T	*lacZ* transcriptional reporter plasmid, Cb^r *oriRK2*	79
pXB228	pTL61T containing the *vexEF* promoter region	This study
pXB229	pTL61T containing the *vexGH* promoter region	This study
pXB230	pTL61T containing the *vexIJK* promoter region	This study
pXB231	pTL61T containing the *vexCD* promoter region	9
pXB232	pTL61T containing the *vexLM* promoter region	This study
pXB233	pTL61T containing the *vexRAB* promoter region	9
pXB265	pTL61T containing the *breR* promoter region	This study
pΔR	*cpxR*::Km allelic-exchange vector	36
pJL1P'Z	Allelic-exchange vector for placing *cpxP-lacZ* into the *V. cholerae* genome	36
pBAD33-*cpxR*	pBAD33 expressing *cpxR*	36
pBAD33	Arabinose-regulated expression plasmid, Cml^r, p15A origin of replication	80
PCR primers		
cpxR-F	GGTCAAGTGACGTATAGGGAGCG	
cpxR-R	GAGGTAGGGTCAATACCGCGAAC	
lacZ5	CTCTAGAAGCTTCTAGCTAGAGGG	
lacZ6	CCGCCACCTGACGTCTAAGAAACC	
P-166c-F-XhoI	TTCTCGAGGGGTCCGGAGACGTACT	
P-166c-R-XbaI	CGTCTAGAGGAGCTGTTTATCGCCG	
P-VC0628-F-XhoI	GGCTCGAGATATTTGATCGGCGGAGT	
P-VC0628-R-XbaI	GGCTCGAGATATTTGATCGGCGGAGT	
P-VC0914-F-XhoI	GCCTCGAGCACATCGCTCAAGTGCGC	
P-VC0914-R-XbaI	CGTCTAGATCTTTGGCCGATAGCACA	
P-VC1673-F-XhoI	GGCTCGAGACCGCAGCCTTGCTGGG	
P-VC1673-R-XbaI	AATCTAGACCCACCAGCAAAGTGGA	
P-VC1746-F-SmaI	AACCCGGGAATTCGGCTTTTTCTTTCCAAATCGGCAGTG	
P-VC1746-R-BamHI	AAGGATCCAATCAGCGCCAACCGTTTTTGCTCACTGAG	
P-VCA638-F-XhoI	GGCTCGAGGGGTTTGGTCGGCATCT	
P-VCA638-R-XbaI	CGTCTAGAGTGCGATACTCCAACTTA	

[a] Δ, with a deletion; *lacZ*$_{Ec}$, *lacZ* from *E. coli*.

resolution of the integrated plasmid. Sucrose-resistant colonies were then screened for Cb sensitivity to verify plasmid loss before the *cpxP-lacZ* insertion was confirmed by PCR using the *lacZ*5 and *lacZ*6 primers. Construction of the *V. cholerae* RND efflux mutant strains was previously reported (9, 30, 31, 48).

Transcriptional reporter assays. Strains were grown under the indicated growth conditions, and culture aliquots were taken in triplicate at various time points postinoculation to quantify β-galactosidase activity as previously described (49). All reporter experiments were performed independently at least three times. The *V. cholerae lacZ*::*cpxP-lacZ* reporter

strains were cultured under AKI conditions as follows. Overnight cultures were diluted 1:10,000 into 73 ml of AKI broth in a 30-mm-diameter glass cylinder. The cultures were then incubated statically at 37°C for 4 h, after which 10-ml aliquots were transferred into 125-ml Erlenmeyer flasks and grown at 37°C with shaking. Aliquots were then collected at the times indicated below for the β-galactosidase assay. The two-plasmid reporter system utilized overnight cultures of *V. cholerae* containing pBAD33-*cpxR* or pBAD33 (negative control) and a *lacZ* reporter (i.e., *vexRAB-lacZ* or *vexGH-lacZ*). The cultures were diluted 1:100 in LB broth with or without arabinose to achieve the final concentrations indicated (Fig. 4), and the cultures were incubated with shaking at 37°C. Aliquots were then collected at 4 h to measure gene expression using the β-galactosidase assay.

Microarray analysis. RNA was isolated for the *cpxA** microarrays from the WT and the *cpxA** mutant strain that were grown in LB broth at 37°C with shaking to an optical density at 600 nm (OD_{600}) of ~1.0. The subsequent steps of cDNA preparation and labeling, microarray hybridization, and data collection were carried out as previously described (50). The experiment was repeated independently three times, with two technical replicates for each individual experiment. The resulting data were processed as follows. The background subtracted spot intensities for each gene were subjected to global normalization before being averaged across all experimental samples. Genes exhibiting a change in expression of ≥2-fold with a coefficient of variation of ≤0.6 across all experiments were defined as being differentially regulated.

Identification of CpxR binding sites. Putative CpxR binding sites were identified in the promoter regions of the differentially regulated genes using Clone Manager Professional software (Sci-Ed Software). The search was limited to 300 bp preceding the ATG start codon for each gene and used the published CpxR consensus binding site (i.e., $GTAAN_6GTAA$), with an allowance for limited mismatches without gap insertion (51). To estimate the frequency of CpxR binding sites in the *V. cholerae* genome, we randomly selected 25 genes (15 from the large chromosome and 10 from the small chromosome) using the random-number generator function in Microsoft Excel. The promoters of these genes were then searched as described above for the presence of the CpxR consensus binding site.

Analysis of Cpx expression on agar plates. A chromosomal *cpxP-lacZ* reporter was used to examine the expression of the Cpx system in a panel of various RND efflux mutants. Overnight cultures of the reporter strains were diluted 100-fold into LB broth and grown at 37°C with shaking for 1 h. The cultures were then normalized to an OD_{600} of 0.1; equal volumes of the cultures were spun down, and the pellet was resuspended in an equal volume of phosphate-buffered saline (PBS). The resuspended cultures were then diluted 1,000-fold before 2 μl was inoculated onto LB agar plates containing 160 μg/ml X-gal. As a positive control, the same cultures were also inoculated onto LB agar plates containing 160 μg/ml X-gal and 500 μM $CuCl_2$. The inoculated plates were incubated overnight at 37°C before being photographed using a digital camera.

Antimicrobial susceptibility tests. Antimicrobial susceptibility tests were performed as previously reported (31) using the following antibiotic disks purchased from Becton, Dickinson (Franklin Lakes, NJ, USA): ampicillin (10 μg/ml), tetracycline (10 μg/ml), gentamicin (10 μg/ml), erythromycin (15 μg/ml), and polymyxin B (300 μg/ml). Test compounds that were not commercially available were prepared by spotting 20 μl of concentrated stock onto 6-mm blank disks from Becton, Dickinson (i.e., 1 M $CuCl_2$, 10% sarcosyl, and 10% deoxycholate). LB agar plates were inoculated with a lawn of the test strains using overnight cultures before the disks were aseptically placed on the surfaces of the agar plates. The plates were then inoculated overnight at 37°C before the zones of growth inhibition were measured in mm using a ruler. The Student *t* test was used to determine statistical significance for the zones of growth inhibition relative to that of the WT.

Plating efficiency. Overnight cultures of the tested *V. cholerae* strains were diluted 100-fold into LB broth or LB broth containing 2 M KCl before being incubated at 37°C with shaking for 1 h. The cultures were then normalized to an OD_{600} of 0.25, at which time equal aliquots of each strain were spun down in a microcentrifuge and the resulting cell pellets resuspended in PBS. Serial dilutions of the cultures were then inoculated onto LB agar and thiosulfate-citrate-bile sucrose (TCBS) agar plates using an Eddy Jet 2 spiral plater (IUL Instruments). The agar plates were then incubated overnight at 37°C before the viable cells were enumerated using a Flash & Go automatic colony counter (IUL Instruments).

Cholera toxin quantitation and TcpA Western blotting. CT production was determined by the GM_1 enzyme-linked immunosorbent assay (ELISA) as previously described (30) using purified CT (Sigma) as a standard. TcpA production was determined by Western immunoblotting as previously described (30) using polyclonal rabbit antisera against TcpA that was graciously provided by Jun Zhu (University of Pennsylvania). Immunoreactive proteins on the Western blots were visualized using the SuperSignal West Pico chemiluminescence detection kit (Pierce Biotechnology).

Microarray data accession number. The microarray data have been deposited in NCBI's Gene Expression Omnibus database and are available through accession number GSE55037.

RESULTS

Identification of CpxR-regulated genes. To gain a better understanding of the genetic basis of the *V. cholerae* Cpx response, we used microarrays to identify CpxR-regulated genes. This was done by defining the effect of the *cpxA** mutation on the *V. cholerae* transcriptome. The *cpxA** mutation inactivates the CpxA phosphatase activity, which results in accumulation of CpxR in its activated form (i.e., CpxR~P). Analysis of the microarray revealed that the levels of 25 transcripts were changed ≥2-fold in response to the constitutive activation of CpxR; 22 genes were upregulated, and three genes were downregulated (Table 2). We hypothesized that if CpxR directly regulated the expression of these genes, then their respective promoters would likely contain a CpxR consensus binding sequence. We therefore searched the putative promoter regions of the 25 genes for sequence similarity to the published CpxR consensus binding site ($GTAAN_6GTAA$) (51). Eighteen of the genes (72%) contained a putative CpxR consensus sequence in their respective promoters; all but one of the genes was upregulated by CpxR. An analysis of 25 randomly selected promoters identified two genes (8%) containing CpxR consensus binding sequences. Seven genes did not contain CpxR binding sites and were likely regulated in an indirect manner. The preferential location of CpxR consensus binding sites in the promoters of positivity-regulated genes suggests that CpxR functions primarily as a transcriptional activator in *V. cholerae*.

Transcripts for *cpxP* and *cpxR*, which are known to be regulated by CpxR, were in the list of upregulated genes, lending credence to our approach. CpxP is located next to *cpxR* and is expressed from a divergent promoter. A CpxR consensus binding sequence is located in the intergenic region separating these two genes, a location that is consistent with CpxR's known regulation of its own expression as well as the divergently transcribed *cpxP* gene (40, 52). In contrast to that in *E. coli*, the Cpx response in *V. cholerae* did not appear to activate genes involved in alleviating membrane stress (e.g., *dsbA*, *degP*, and *fkpA*) resulting from misfolded membrane proteins (51, 53, 54), a finding that may reflect functional differences between the Cpx responses in the *Vibrionaceae* and the *Enterobacteriaceae*.

Notably, 11 of the 25 CpxR-regulated genes were involved in maintaining the permeability barrier of the cell (Table 2). Five of the upregulated genes encoded the production of two broad-spectrum RND efflux systems: VexAB, VexGH, and their cognate

TABLE 2 CpxR-regulated genes

Gene category[a]	Open reading frame	Expression ratio (SD) relative to expression in the *cpxA** mutant	CpxR binding site?	Gene	Gene product
Cellular processes	vc2691	67.0 (16.8)	Yes[b]	*cpxP*	Periplasmic protein CpxP, putative
Cell envelope protein	vc1854	0.5 (0.1)	No	*ompT*	OmpT porin
Metabolism	vca0151	5.2 (2.3)	Yes		Oxidoreductase, putative
	vca0249	2.3 (0.3)	Yes		Cytochrome b_{561}, putative
	vca0538	10.4 (3.3)	Yes[c]		Cytochrome b_{561}
Regulatory function proteins	vc0166	3.2 (0.7)	Yes (2 sites)	*vexR*	Transcriptional regulator, TetR family
	vc2692	8.1 (1.7)	Yes[b]	*cpxR*	Transcriptional regulator CpxR
Transport and binding proteins	vc0042	0.5 (0.2)	Yes	*trkH*	Potassium uptake protein
	vc0164	2.4 (0.2)	Yes[d]	*vexB*	RND efflux pump VexB
	vc0165	2.2 (0.3)	Yes[d]	*vexA*	Membrane fusion protein VexA
	vc0913	14.8 (2.4)	Yes	*vexG*	Membrane fusion proteins VexG
	vc0914	12.4 (2.2)	Yes[e]	*vexH*	RND efflux pump VexH
	vc2436	2.1 (0.3)	Yes	*tolC*	Outer membrane pore protein TolC
	vca0576	4.2 (2.2)	Yes	*hutA*	Heme transport protein HutA
	vca0603	2.6 (1.4)	No		ABC transporter substrate-binding protein
	vca0782	4.3 (1.9)	Yes		ABC transporter, ATP-binding protein
Hypothetical and conserved hypothetical proteins	vc0191	2.0 (0.9)	Yes		
	vc0806	0.5 (0.1)	No		
	vc0915	2.4 (1.1)	No		
	vc0938	5.5 (1.3)	No		
	vca0126	2.0 (0.5)	No		
	vca0139	51.1 (25.4)	Yes (3 sites)		
	vca0162	3.0 (1.2)	No		
	vca0539	11.2 (2.0)	Yes[c]		
	vca0781	3.1 (1.2)	Yes[f]		

[a] Gene categories were derived from the work of Heidelberg et al. (46).
[b] The CpxR consensus binding site was located in the intergenic region between the divergently transcribed *cpxP* and *cpxR* genes.
[c] vca0538 and vca0539 are in an operon that contains three CpxR consensus binding sites located upstream of vca0538.
[d] vc0166, vc0165, and vc0164 are in an operon with two CpxR consensus binding site located upstream of vc0166.
[e] vc0913 and vc0914 are in an operon with a CpxR consensus binding site located upstream of vc0913.
[f] vca0782 and vca0781 are in an operon with a CpxR consensus binding site located upstream of vca0782.

outer membrane pore component, TolC. Furthermore, a putative CpxR consensus binding sequence was found in the promoter regions of *vexRAB*, *vexGH*, and *tolC*, suggesting that CpxR regulates their expression (Fig. 1). One of the downregulated genes was *ompT*, which encodes a ToxR-regulated porin that possesses a large-diameter pore. Repression of *ompT* is associated with decreased susceptibility to low-molecular-weight antimicrobial compounds, such as bile salts (55–57).

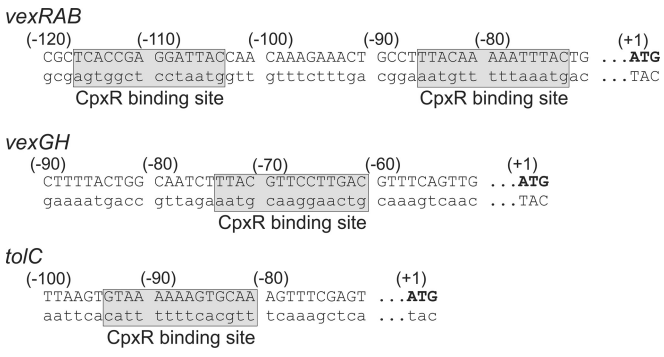

FIG 1 CpxR consensus binding sites in the *vexRAB*, *vexGH*, and *tolC* promoters. Putative CpxR consensus binding sequences in the promoter regions of the *vexRAB*, *vexGH*, and *tolC* genes are indicated by gray boxes. The start codon for each gene is marked by boldface. Numbering is relative to the start codon for each gene.

CpxR is a positive regulator of the *V. cholerae* RND efflux systems. The expression of the *vexRAB* and *vexGH* RND efflux systems increased in the *cpxA** mutant (Table 2). This finding, combined with the presence of CpxR consensus binding sequences in the *vexRAB* and *vexGH* promoters (Fig. 1), suggested that CpxR likely regulates the expression of these two RND efflux systems. To test this hypothesis and to validate the microarray results, we quantified *vexRAB* and *vexGH* expression in the WT, in the *cpxA** mutant (which constitutively activates CpxR), and in a *cpxR* deletion mutant during growth in LB broth and during growth under AKI conditions. These growth conditions were selected, as they represent conditions where the RND efflux systems have been shown to contribute to both antimicrobial resistance and virulence factor production (10). We measured reporter expression at two time points (2 and 4 h postinoculation for the LB cultures and 3.5 and 6.5 h postinoculation for the AKI cultures) to control for potential growth-dependent effects on expression.

With the activation of CpxR, in the *cpxA** background, *vexRAB* expression was significantly increased relative to that in the WT under AKI growth conditions. In contrast, the absence of CpxR in

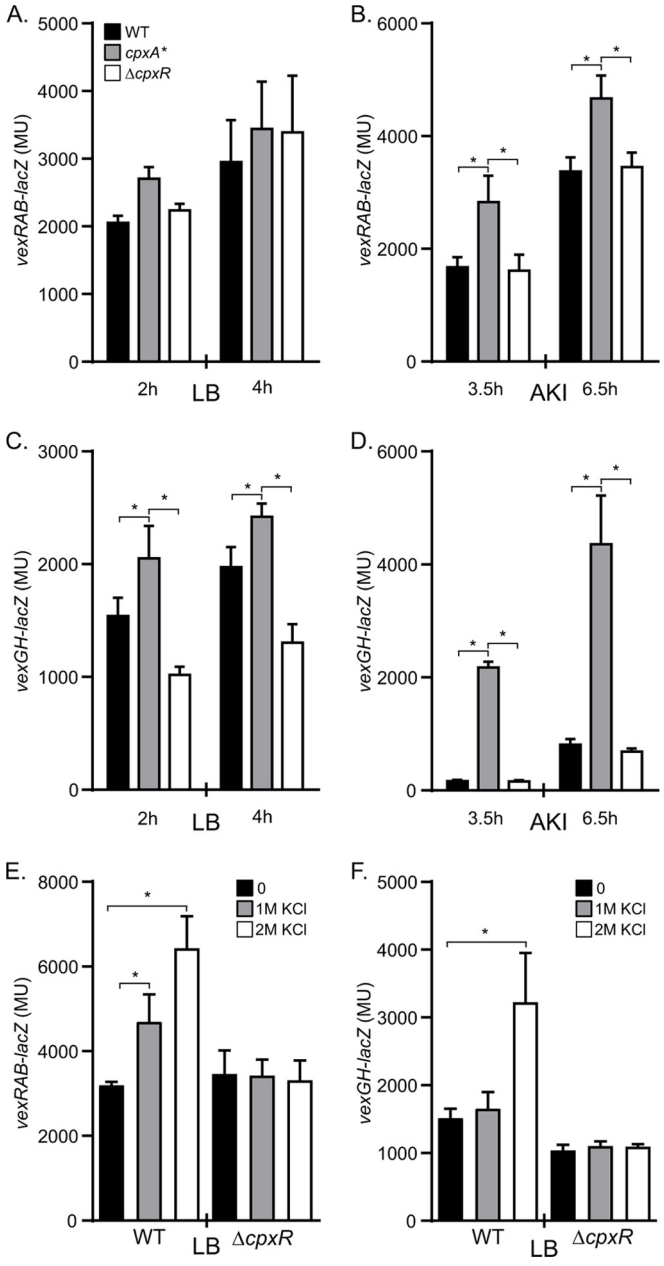

FIG 2 Expression of *vexRAB* and *vexGH* in *V. cholerae cpx* mutants. *V. cholerae* strains bearing either a *vexRAB-lacZ* (A, B, and E) or a *vexGH-lacZ* (C, D, and F) reporter were grown in LB broth (A and C), under AKI conditions (B and D), or in LB broth containing KCl at the indicated concentrations (E and F). Culture aliquots were taken at 2 h and 4 h (A and C), 3.5 and 6.5 h (B and D), or at 4 h (E and F) and assayed for β-galactosidase activity. The presented data are the means ± standard deviations (SD) of results from three independent experiments. Statistical significance was determined by analysis of variance (ANOVA) with the Tukey-Kramer multiple-comparison test (A, B, C, and D). (E and F) Values were compared to those with 0 KCl. *, $P < 0.001$.

the Δ*cpxR* background did not influence *vexRAB* expression under either condition (Fig. 2A and B). Thus, CpxR does not regulate the basal-level expression of *vexRAB* but can enhance *vexRAB* expression under conditions where the Cpx system is activated. The expression level of *vexGH* was increased during growth in LB broth and under AKI conditions in the *cpxA** background (Fig. 2C and D). However, the basal level of *vexGH* expression under AKI conditions appeared to be much lower than was observed in LB broth. Unlike with *vexRAB* deletion, *cpxR* deletion resulted in a significant reduction in *vexGH* expression during growth in LB broth (Fig. 2C) but not during growth under AKI conditions (Fig. 2D), implying that CpxR positively affects the basal-level expression of *vexGH* during growth in LB broth but not under AKI conditions. Collectively, these observations indicate that there are medium-dependent differences in the levels of activation of the Cpx system and that the Cpx system is likely inactive during growth under virulence gene-inducing conditions. These findings validate the microarray results and support the conclusion that CpxR is a positive regulator of the *vexRAB* and *vexGH* RND efflux systems.

Since *vexRAB* and *vexGH* appeared to be positively regulated by CpxR, we predicted that their expression should increase with activation of the Cpx system. We therefore quantified *vexRAB* and *vexGH* expression in the WT and a Δ*cpxR* mutant following growth in LB broth containing various concentrations of KCl, a known inducer of the *V. cholerae* Cpx system (36). The results showed a KCl concentration-dependent increase in *vexRAB* expression. Growth in 1 M KCl resulted in a 1.5-fold increase in *vexRAB-lacZ* expression, while growth in 2 M KCl caused a 2-fold increase in *vexRAB-lacZ* expression relative to that after growth in LB broth without KCl (Fig. 2E). This phenotype was found to be dependent on CpxR, as deletion of *cpxR* abolished the KCl-dependent induction of *vexRAB* (Fig. 2E). The expression of *vexGH* was also induced by KCl, as evidenced by a 2-fold increase in *vexGH-lacZ* expression during growth in 2 M KCl relative to that of the LB broth-grown control (Fig. 2F). The induction of *vexGH* by KCl was also dependent on CpxR, as deletion of *cpxR* abolished the KCl-dependent increase in *vexGH* expression (Fig. 2F). Together, these results confirm that the data obtained with the *cpxA** mutant reflects activated CpxR and provides additional evidence to support the conclusion that the *vexRAB* and *vexGH* RND efflux systems are positively regulated by the Cpx system in a CpxR-dependent manner.

Activation of the Cpx response enhances *V. cholerae* resistance to antimicrobial compounds. Since both the *vexRAB* and *vexGH* RND efflux systems were upregulated in the *cpxA** mutant (Fig. 2), we predicted that the *cpxA** mutant would exhibit enhanced resistance to antimicrobial compounds that are substrates for these two RND efflux systems (e.g., bile salts and other detergent-like molecules). To test this hypothesis, we calculated the plating efficiency of CpxR-activated *V. cholerae* on TCBS agar. Growth of *V. cholerae* on TCBS agar is dependent upon the expression of the RND efflux systems, which provide resistance to bile salts and other detergent-like compounds that are present in this medium (9, 30, 31, 58, 59). In these experiments, we compared the plating efficiencies of the *cpxA** and Δ*cpxR* strains to that of the WT. We also compared the plating efficiencies of the WT and the Δ*cpxR* mutant grown in LB broth with and without 2 M KCl. The results showed that there was a 4.4-fold increase in the recovery of the *cpxA** mutant relative to that of the WT, while the recovery of the Δ*cpxR* mutant was not significantly different from that of the WT (Fig. 3). Activation of the Cpx system by growth in 2 M KCl resulted in a 3-fold increase in the recovery of the KCl-grown cells relative to the recovery of cells grown in LB broth without KCl. Growth of the Δ*cpxR* mutant in 2 M KCl did not have a significant effect on its recovery, which confirmed that this

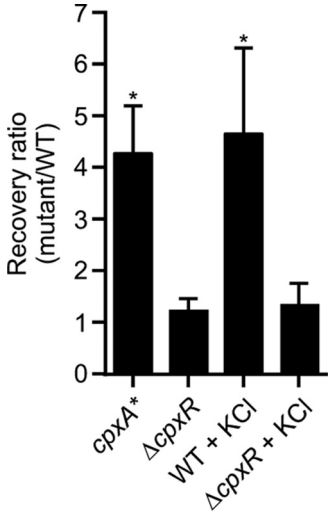

FIG 3 Effect of CpxR activation on the recovery of *Vibrio cholerae* on TCBS agar. The *V. cholerae* WT, *cpxA** mutant, and Δ*cpxR* mutant were cultured in LB broth or LB broth containing 2 M KCl before aliquots were diluted in PBS and plated in duplicate onto LB agar and TCBS agar plates for enumeration. The plates were incubated overnight at 37°C before the resulting colonies were counted. The recovery ratio for each mutant was calculated by the following equation: (the number of mutant colonies on TCBS/the number of mutant colonies on LB agar)/(the number of WT colonies on TCBS/the number of WT colonies on LB agar). The values are the means of results of three independent experiments ± SD. Statistical significance relative to WT values was determined by ANOVA with the Tukey-Kramer multiple-comparison test. *, $P < 0.001$.

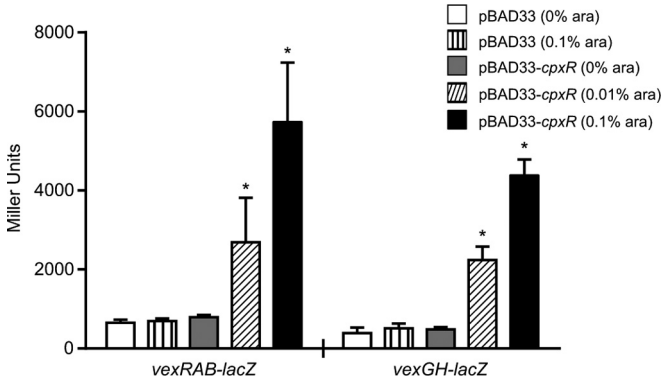

FIG 4 Ectopic expression of *cpxR* activates *vexRAB* and *vexGH* expression in *V. cholerae*. *V. cholerae* strains containing the indicated RND efflux system reporters and either pBAD33-*cpxR* or pBAD33 were grown in LB broth at 37°C in the presence or absence of arabinose (ara) as described in Materials and Methods. After 4 h of growth, triplicate aliquots were taken and assayed for β-galactosidase activity. The presented data are the means ± SD of results from three independent experiments. Statistical significance was determined by ANOVA with the Tukey-Kramer multiple-comparison test. *, $P < 0.001$.

phenotype was dependent on *cpxR* (Fig. 3). In conjunction with the previous data (Fig. 2), these results provide further evidence to support the idea that activation of the Cpx system induces *vexRAB* and *vexGH* expression in a CpxR-dependent manner, thereby enhancing antimicrobial resistance.

Next, the effect of the Cpx system on *V. cholerae* antimicrobial susceptibility was investigated by examining the resistance of the WT, *cpxA**, and Δ*cpxR* strains to antimicrobial agents by disk diffusion assays. The results showed that the *cpxA** mutant exhibited increased resistance to ampicillin but not to other tested antimicrobial compounds (see Table S1 in the supplemental material). Since VexAB and VexGH are the only two RND efflux systems that contribute to ampicillin resistance, this finding is consistent with the idea that both of these RND efflux systems are upregulated in the *cpxA** mutant. It is not surprising that differences in susceptibility were not observed for the other RND-dependent antimicrobial substrates, as the contributions of VexAB and VexGH to resistance to these substrates is masked due to redundancy among the six RND efflux systems (9, 30, 31). There was no significant difference in susceptibility to any of the compounds tested between the WT and the Δ*cpxR* mutant strain, suggesting that the tested xenobiotics did not function to activate the expression of the Cpx response. This was verified by plating *V. cholerae* containing a *cpxP-lacZ* reporter on LB agar containing subinhibitory amounts of these xenobiotics; no differences in expression of the reporter in the presence or absence of any of the compounds were noted (data not shown), confirming the supposition that the Cpx system was not activated in response to these antimicrobial compounds. Based on these observations, we conclude that the Cpx system is not required for *V. cholerae*'s intrinsic resistance to xenobiotic antimicrobial compounds, but its activation was able to enhance the pathogen's resistance to antimicrobials through increased expression of *vexRAB* and *vexGH*.

Ectopic expression of *cpxR* activates *vexRAB* and *vexGH* expression. The observation that the *vexRAB* and *vexGH* promoters contain CpxR consensus binding sequences (Fig. 1) and were upregulated in a *cpxA** background (Fig. 2) suggested that CpxR was a positive regulator of *vexRAB* and *vexGH*. To confirm this hypothesis, we expressed *cpxR* from the arabinose-inducible promoter in pBAD33 in *V. cholerae* harboring either the *vexRAB-lacZ* or the *vexGH-lacZ* reporter. This resulted in a dramatic arabinose-dose-dependent increase in both *vexRAB* and *vexGH* expression (Fig. 4). In contrast, *cpxR* expression did not affect the expression of any other RND efflux system (see Fig. S1 in the supplemental material), confirming that CpxR was specific for *vexRAB* and *vexGH*. These results confirmed our hypothesis that CpxR is a positive regulator of the *vexRAB* and *vexGH* RND efflux systems. Taken together, these observations are consistent with the hypothesis that CpxR functions as an activator at the *vexRAB* and *vexGH* promoters.

Mutation of *vexB* and *vexH* activate the Cpx system. Our collective findings revealed that the VexAB and VexGH RND efflux systems are components of the Cpx response. This suggested the possibility that active efflux by the RND efflux systems might function to suppress the Cpx response. To test this hypothesis, we used a chromosomal *cpxP-lacZ* reporter as an indicator of the activation state of the Cpx system; *cpxP* expression is regulated by CpxR (36). We compared levels of *cpxP* expression in the WT and JB485, a strain lacking all six RND efflux systems (9). JB485 produced dark-blue colonies on LB agar–X-gal plates, whereas the WT and the JB485 Δ*cpxR* mutant yielded white colonies (Fig. 5A). Control cultures grown on agar plates containing 500 μM $CuCl_2$ (a Cpx inducer) showed induction of *cpxP* in the WT but not in the *cpxR* mutants, validating the idea that the *cpxP-lacZ* construct faithfully reports the activation state of the Cpx system in each strain. Thus, the absence of RND efflux activity induces the Cpx system, a finding consistent with the idea that the RND efflux

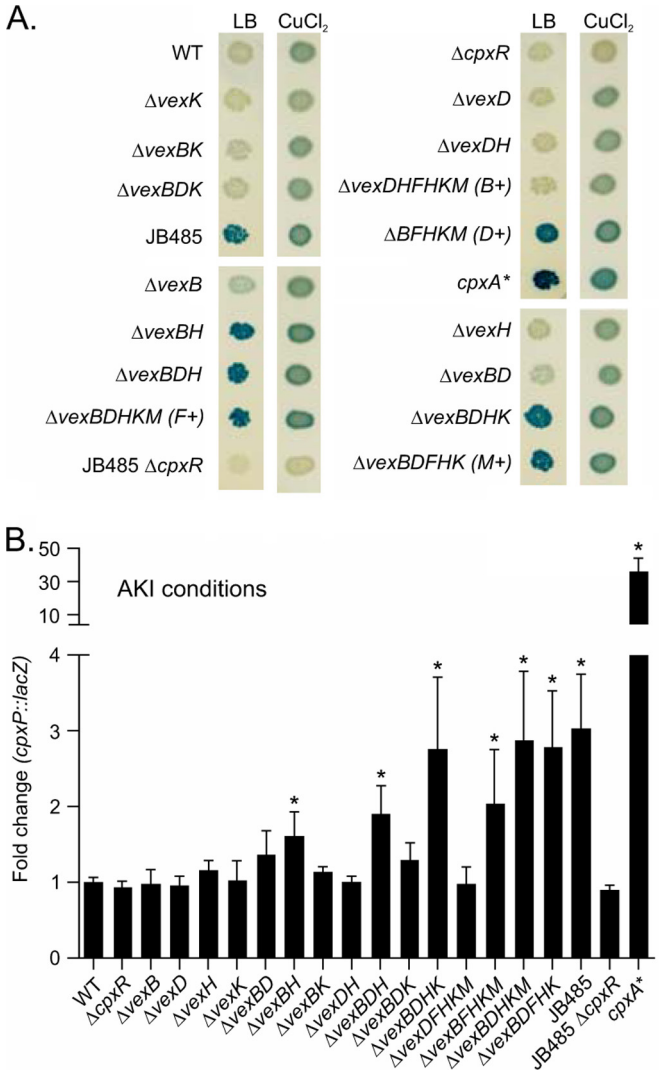

FIG 5 Induction of the Cpx system in *V. cholerae* RND efflux mutants. Expression of a chromosomal *cpxP-lacZ* reporter in *V. cholerae* strains with the indicated characteristics. (A) The strains were inoculated onto the surfaces of LB agar–X-gal plates with and without 500 μM $CuCl_2$ before the plates were incubated overnight at 37°C and photographed. (B) The same *V. cholerae cpxP-lacZ* fusion strains were cultured under AKI conditions for 5 h, after which culture aliquots were collected and their β-galactosidase activities assayed. The fold change was calculated as the β-galactosidase activity (measured in Miller units) in the mutant divided by that in the WT. The bars represent the means ± SD of results from three independent experiments. Statistical relevance to WT values was calculated using a one-way ANOVA with Dunnett's *post hoc* test. *, $P < 0.001$.

systems normally suppress the activity of the Cpx system in *V. cholerae*.

Previous studies have shown that four of the six RND systems (*vexB*, *vexD*, *vexH*, and *vexK*) contribute to the *in vitro* resistance of *V. cholerae* to antimicrobial compounds with both distinct and redundant roles (9, 30, 31). We therefore sought to determine which of the RND efflux systems contributes to Cpx suppression by examining *cpxP* expression in a panel of strains that included both single and multiple RND efflux mutants. For these experiments, the *cpxP-lacZ* reporter was introduced into the chromosome of each of the RND mutants. Expression of *cpxP* was then examined on LB agar–X-gal plates and LB agar–X-gal plates containing $CuCl_2$ (as a positive control). LB agar was used to screen for *cpxP* expression because previous studies have shown that the Cpx system was poorly expressed in LB broth (36). Among the four single RND efflux mutants (*vexB*, *vexD*, *vexH*, and *vexK* mutants), only the *vexB* mutant produced light-blue colonies on LB agar–X-gal (Fig. 5A); the *vexH* mutant produced colonies that appeared white to the naked eye but were discernible as faint blue under magnification, while the *vexD* and *vexK* mutant colonies were white. These observations suggest that *cpxP* expression is activated in the absence of either *vexB* or *vexH* (albeit to a very low level in the absence of *vexH*) but that the absence of either *vexD* or *vexK* does not induce *cpxP* expression. Furthermore, a *vexBDK* triple mutant produced colonies that were similar in color to the *vexB* single mutant, supporting the idea that *vexD* and *vexK* do not influence *cpxP* expression under these conditions. When the *vexB* and *vexH* deletions were combined, the resulting strain produced dark-blue colonies similar to those of JB485 (Fig. 5A), consistent with the hypothesis that these two RND efflux systems are functionally redundant for this phenotype. We hypothesized that if Cpx activation was a result of efflux activity provided by *vexGH* and/or *vexRAB*, then a strain lacking all five RND efflux systems except *vexRAB* should suppress expression of the Cpx system relative to that in JB485. Indeed, this was what we observed with the Δ*vexDFHKM* (*vexB*$^+$) strain, which produced faint-blue colonies that were similar in color to those of the Δ*vexH* mutant (Fig. 5A). We conclude that efflux activity provided by the VexAB and VexGH RND efflux systems maintains the Cpx system in a suppressed state during growth of *V. cholerae* on LB agar.

We also examined whether the RND efflux systems influence the expression of the Cpx system during growth under virulence-inducing conditions. For these experiments, each RND mutant was grown under AKI conditions before being assayed for *cpxP* expression. There was no difference in *cpxP* expression among any of the single-deletion mutants (Fig. 5B). However, when a *vexB* mutant was combined with a *vexH* deletion, we observed an approximately 2-fold increase in *cpxP* expression. The *vexBK* and *vexDH* mutations did not appear to affect *cpxP* expression. This suggested that during growth under AKI conditions, *vexB* and *vexH* function in a redundant manner to limit the activation of the Cpx system. There was an increase in *cpxP* expression in the *vexBDHK* mutant relative to that in the *vexBDH* mutant, suggesting that the VexIJK RND efflux system contributed to the suppression of the Cpx system. The expression of *cpxP* in the *vexBDHKM* (*vexF*$^+$) and *vexBDFHK* (*vexM*$^+$) mutants was similar to that in the *vexBDHK* mutant. Expression of *cpxP* in the *vexDFHKM* (*vexB*$^+$) mutant was similar to that in the WT, confirming the idea that the VexAB RND efflux system is sufficient to complement the absence of the five other RND efflux systems. The increase in *cpxP* expression in JB485 relative to that in the *vexBFHKM* (*vexD*$^+$) mutant suggests that the VexCD RND efflux system contributes to the suppression of the Cpx system during growth under AKI conditions. These findings are reminiscent of our previous observations regarding CT and TCP production in RND efflux-deficient *V. cholerae*, where all six RND systems were required for high-level CT and TCP production (30). The concordance of these observations raises the intriguing possibility that the factor responsible for activation of the Cpx system during AKI growth may also contribute, by a Cpx-independent mechanism, to the virulence attenuation observed in JB485 (9).

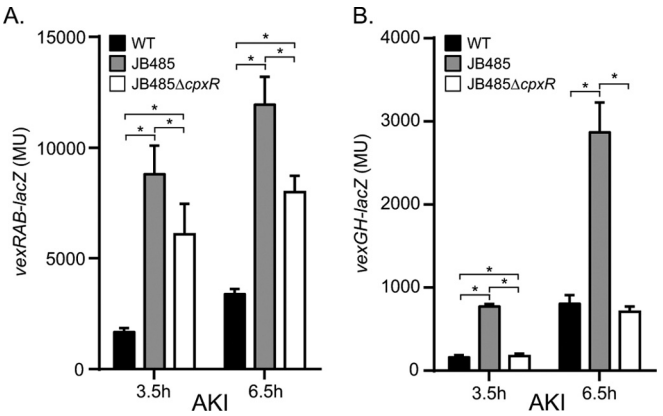

FIG 6 CpxR activates *vexRAB* and *vexGH* expression in the absence of RND efflux activity. *V. cholerae* strains containing a *vexRAB* (A) or *vexGH* (B) reporter plasmid were grown under AKI growth conditions. Aliquots were taken at 3.5 and 6.5 h and assayed for β-galactosidase activity. The presented data are the means ± SD of results from three independent experiments. Statistical significance was determined by ANOVA with the Tukey-Kramer multiple-comparison test. *, $P < 0.001$.

CpxR contributes to *vexRAB* and *vexGH* expression in RND efflux-negative *V. cholerae*. The above data suggested that the Cpx system was activated by loss of the RND efflux systems (Fig. 5). Based on this and the finding that CpxR activated *vexRAB* and *vexGH* expression (Fig. 2 and 4), we hypothesized that the expression of *vexRAB* and *vexGH* in JB485 would be upregulated in a CpxR-dependent manner. To test this, we quantified *vexRAB* and *vexGH* expression in the WT, JB485, and JB485 Δ*cpxR* strains during growth under AKI conditions. AKI conditions were selected, as they showed the most dramatic effect on the expression of *vexRAB* and *vexGH* (Fig. 2). The results showed a significant increase in the expression of both *vexRAB* and *vexGH* in strain JB485 relative to that in the WT at 3.5 and 6.5 h (Fig. 6A and B). The level of *vexRAB* expression in JB485 Δ*cpxR* was reduced relative to that in JB485 but was still greater than in the WT (Fig. 6A). This suggests that CpxR contributes to the induction of *vexRAB* in JB485 but that additional factors also contribute to *vexRAB* upregulation. In contrast to what occurred in the *vexRAB* mutant, the expression level of *vexGH* returned to WT levels in the JB485 Δ*cpxR* mutant, indicating that CpxR was responsible for the upregulation of *vexGH* in JB485 (Fig. 6B). Together, these results provide additional evidence supporting the conclusion that the loss of RND efflux activity results in CpxR activation and that CpxR functions as a positive regulator of *vexRAB* and *vexGH*.

The Cpx system does not affect CT or TCP production. The RND efflux systems were shown to be required for the production of both CT and TCP in *V. cholerae* (9). The mechanism by which the RND systems repressed CT and TCP production, while still unknown, was linked to repression of the ToxR regulon (9). The upregulation of the Cpx system in the RND null mutant suggested the possibility that CpxR functions to repress CT and TCP production. We investigated this hypothesis by quantifying CT and TCP production in the WT, *cpxA**, Δ*cpxR*, JB485, and JB485 Δ*cpxR* strains. If *cpxR* was responsible for attenuated CT and TCP production, then its deletion in the RND-negative background should result in increased CT and TCP production. Likewise, CT and TCP production should be decreased in the WT by the *cpxA** mutation due to constitutive activation of the Cpx system. Our results showed that there was no significant difference in CT or TCP production between the WT, *cpxA**, and Δ*cpxR* strains or between JB485 and JB485 Δ*cpxR* (Fig. S2). This indicated that the defect in virulence factor production in JB485 was not a result of the activation of the Cpx system. These results also suggested that the Cpx system does not function to regulate virulence factor production in *V. cholerae*, a result that is consistent with previous findings showing that the Cpx system was dispensable for *V. cholerae* colonization of the infant mouse small intestine (36).

DISCUSSION

Bacteria have evolved overlapping mechanisms to sense and respond to stress that can result from exposure to toxic molecules, from adhesion to abiotic surfaces, or from misfolded proteins. The Cpx two-component system represents one such system that in *E. coli* mitigates envelope stress resulting from protein misfolding (34). *V. cholerae* also encodes a Cpx two-component system that shares a conserved genetic organization with the *E. coli* Cpx system (36). However, there are differences in the amino acid sequences of the sensor domains of the *V. cholerae* and *E. coli* CpxA and CpxP proteins, and the *V. cholerae* Cpx system does not respond to stimuli that activate the *E. coli* Cpx system (36). Thus, the Cpx systems may play different roles in the physiology of these two related *Gammaproteobacteria*.

Our characterization of the *V. cholerae* CpxR transcriptome supports the idea that there are differences between the *V. cholerae* and *E. coli* Cpx regulons. One key difference that we found is that several *V. cholerae* genes involved in protein fate (e.g., *degP* and *dsbA*) either were not detected as being regulated or were found to be repressed by CpxR (Table 2). This finding is similar to findings for *Haemophilus ducreyi*, where *degP* was also noted to be absent from the Cpx regulon (60). Together, these observations suggest that the physiological role for Cpx systems may not be universally conserved among Gram-negative bacteria. Although *degP* and *dsbA* appear to be regulated independently of CpxR in *V. cholerae*, the activation of the Cpx system in *V. cholerae dsbC* and *dsbD* mutants (36), which likely results in misfolded cell envelope proteins, suggests that the Cpx system can respond to misfolded proteins. The mechanism by which this occurs remains to be determined but may involve an alternate sigma factor (sigma E) (*rpoE*), which has been shown to functionally overlap the Cpx system in responding to extracytoplasmic stress in *E. coli* (53, 61).

The list of *V. cholerae* CpxR-regulated genes included a number of genes that mediate uptake and efflux of low-molecular-weight antimicrobial compounds. This included the *ompT* porin (which was repressed) and the *vexRAB*, *vexGH*, and *tolC* genes (which were upregulated). The latter loci encode the production of two broad-spectrum RND efflux systems that contribute to *V. cholerae* resistance to multiple antimicrobial compounds and pathogenesis (9, 10, 31). Several lines of evidence suggest that the *vexRAB* and *vexGH* operons are regulated by CpxR: the promoters of both operons contain CpxR consensus binding sequences (Fig. 1), both operons were upregulated in the *cpxA** mutant (Fig. 2), ectopic *cpxR* expression activated their expression in *V. cholerae* (Fig. 4), and KCl (a Cpx system activator) induced their expression in the WT but not in a Δ*cpxR* mutant (Fig. 2). This conclusion is buttressed by the observations that the *cpxA** mutant exhibited increased resistance to ampicillin (an antibiotic substrate of the VexAB and VexGH RND systems) and exhibited a growth advantage on TCBS agar (see Table S1 in the supplemental material and

Fig. 3) and that induction of the Cpx system with 2 M KCl provided a growth advantage for the WT on TCBS but not for a Δ*cpxR* mutant (Fig. 3). While our data strongly support the conclusion that CpxR is an activator of these two RND efflux systems, CpxR was not required for *V. cholerae*'s intrinsic resistance to antimicrobial compounds (Fig. 3 and Table S1). This suggests that the Cpx system may contribute to xenobiotic resistance under Cpx-inducing conditions by further increasing the expression of *vexRAB* and *vexGH*; however, the Cpx system is not required for *V. cholerae*'s intrinsic resistance to xenobiotics.

Besides finding that the *V. cholerae* Cpx response promotes the expression of RND efflux systems, we found that inactivation of RND efflux stimulates the Cpx response. Mutation of *vexB* and/or *vexH* resulted in activation of the *V. cholerae* Cpx system. It is interesting to note that this phenotype was also observed with the deletion of RND efflux systems in *H. ducreyi* and *Klebsiella pneumoniae* (62, 63), which suggests that the genetic linkage between the Cpx system and the efflux systems is not unique to *V. cholerae*. The mechanism(s) by which the RND efflux systems modulate the activity of the Cpx system is not known. However, since RND efflux systems function in small-molecule export, we speculate that this phenotype is the result of the intracellular accumulation of an endogenously produced small molecule in the RND efflux mutants. Recent studies suggest that a natural function of RND efflux systems may be to remove metabolic waste from within the cell (64), which is consistent with this hypothesis. In *E. coli*, there is evidence to suggest that in the absence of RND-mediated efflux, metabolites accumulate in the cell and activate the expression of the MAR, Bae, and Cpx stress response systems (29, 43, 64–67). These regulatory systems then activate the expression of the *acrAB*, *acrD*, and *mdtABCD* RND efflux systems and other stress-mitigating genes. Although *V. cholerae* appears to lack the MAR and Bae systems, we propose that a similar mechanism occurs in *V. cholerae*. We speculate that an as-yet-unidentified cellular metabolite accumulates in the absence of RND efflux and activates the Cpx system. Our observation that different RND efflux pumps were required to suppress the Cpx system during growth on LB agar versus growth under AKI conditions suggests that the Cpx system may respond to the accumulation of multiple different endogenous molecules. Determining the identities of such effector molecules is a key challenge for future studies.

We previously proposed that a small molecule accumulated in *V. cholerae* in the absence of RND-mediated efflux and repressed CT and TCP production during growth under AKI conditions (30). This proposal was supported by the facts that *V. cholerae* virulence factor production was dependent upon all six RND efflux systems and that there was functional redundancy among the RND systems for this phenotype (9, 30, 32). The similarity of these results to the Cpx data, along with the inclusion of the ToxR-regulated *ompT* in the list of CpxR-responsive genes, suggests that attenuation of CT and TCP production in the RND-deficient strain may be due to the activation of the Cpx system. Although the Cpx system has been linked to virulence in other pathogens (68–73), our data indicate that the Cpx system does not affect *V. cholerae* virulence factor production. This was evidenced by the fact that *cpxR* deletion, constitutive activation of the Cpx system (i.e., *cpxA**), or chemical activation of the Cpx system (i.e., with $CuCl_2$) did not affect CT or TCP production. This conclusion is further supported by a previous study which showed that the Cpx system was dispensable for *V. cholerae* intestinal colonization (36).

While our findings strongly suggest that the Cpx system does not exert a significant influence over the expression of the ToxR-regulated virulence factors CT and TCP, we cannot exclude the possibility that the Cpx system plays a role in the regulation of other genes *in vivo*. Late in infection, *V. cholerae* is thought to encounter growth conditions conducive to the induction of the Cpx system, including high cell density, nutrient limitation, and the likely accumulation of metabolic waste/by-products (74, 75). The finding that both the *vexAB* and *vexGH* RND efflux systems are upregulated late during infection in humans and animals (74–77) is consistent with the hypothesis that the Cpx system is also induced late in infection. Thus, it is tempting to speculate that the Cpx system may contribute to late gene expression during infection. Late induced genes contribute to important phenotypes. This includes genes that contribute to *V. cholerae* survival in the environment and genes that contribute to the hyperinfectious phenotype associated with human-shed vibrios and is thought to be a key factor responsible for the epidemic spread of cholera (75, 78).

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