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Vibrio cholerae OmpR Represses the ToxR Regulon in Response to Membrane Intercalating Agents That Are Prevalent in the Human Gastrointestinal Tract

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ABSTRACT   Multidrug efflux systems belonging to the resistance-nodulation-division (RND) superfamily are ubiquitous in Gram-negative bacteria. RND efflux systems are often associated with multiple antimicrobial resistance and also contribute to the expression of diverse bacterial phenotypes including virulence, as documented in the intestinal pathogen *Vibrio cholerae*, the causative agent of the severe diarrheal disease cholera. Transcriptomic studies with RND efflux-negative *V. cholerae* suggested that RND-mediated efflux was required for homeostasis, as loss of RND efflux resulted in the activation of transcriptional regulators, including multiple environmental sensing systems. In this report, we investigated six RND efflux-responsive regulatory genes for contributions to *V. cholerae* virulence factor production. Our data showed that the *V. cholerae* gene VC2714, encoding a homolog of *Escherichia coli* OmpR, was a virulence repressor. The expression of *ompR* was elevated in an RND-null mutant, and *ompR* deletion partially restored virulence factor production in the RND-negative background. Virulence inhibitory activity in the RND-negative background resulted from OmpR repression of the key ToxR regulon virulence activator *aphB*, and *ompR* overexpression in wild-type cells also repressed virulence through *aphB*. We further show that *ompR* expression was not altered by changes in osmolarity but instead was induced by membrane-intercalating agents that are prevalent in the host gastrointestinal tract and which are substrates of the *V. cholerae* RND efflux systems. Our collective results indicate that *V. cholerae ompR* is an *aphB* repressor and regulates the expression of the ToxR virulence regulon in response to novel environmental cues.

KEYWORDS   cholera, OmpR, two-component regulatory systems, virulence regulation

The Gram-negative bacterium *Vibrio cholerae* is the causative agent of the life-threatening diarrheal disease cholera. *V. cholerae* is an aquatic organism that infects humans following the consumption of *V. cholerae*-contaminated food or water. After ingestion, *V. cholerae* colonizes the epithelium of the small intestine to cause disease by a process that is dependent upon virulence factor production. The two most important *V. cholerae* virulence factors are the toxin-coregulated pilus (TCP), which mediates intestinal colonization, and cholera toxin (CT), an enterotoxin that is responsible for the secretory diarrhea that is the hallmark of the disease cholera. CT and TCP production is under the control of a hierarchical regulatory system known as the ToxR regulon (1, 2). Activation of the ToxR regulon begins with the expression of two cytoplasmic transcriptional regulators, *aphA* and *aphB* (3, 4). AphA and AphB function synergistically to activate *tcpP* expression. TcpP then binds along with ToxR to the *toxT* promoter to activate *toxT* expression. ToxT directly activates the expression of the genes that encode CT and TCP production (2).

The expression of adaptive responses is important for the success of *V. cholerae* as

a pathogen. This includes tight regulation of the ToxR regulon, which is known to limit virulence factor production to the host gastrointestinal tract. Thus, the ToxR regulon has evolved to respond to specific environmental signals within the host (5). Other genes which are important for survival and persistence in aquatic ecosystems must be repressed during host entry for successful colonization (6–8). Late in infection, in preparation for host exit, *V. cholerae* downregulates virulence genes while activating genes required for dissemination and transmission (9–12). Although the genetic mechanisms involved in ToxR regulon activation have been extensively studied, less is known about how environmental signals influence ToxR regulon expression *in vivo*.

V. cholerae is exposed to disparate environments within both the aquatic ecosystem and the human gastrointestinal tract. *V. cholerae* survival and growth in these niches require rapid adaptation to environmental conditions. *V. cholerae* enters humans from aquatic ecosystems that are typically aerobic and alkaline. The bacterium must then pass through the gastric acid barrier of the stomach before entering the duodenum and migrating to the epithelial surface, where it colonizes the crypts of the small intestine. Successful transition between these dissimilar environments requires that *V. cholerae* modulates its transcriptional responses so that specific genes are only expressed during colonization of appropriate niches. In *V. cholerae*, like most bacteria, this is achieved by environmentally responsive regulatory systems that monitor the extracellular environment by use of a range of membrane-bound sensors such as ToxR and two-component signal transduction systems (TCSs) (13).

TCSs are widespread phospho-relay systems that modulate gene expression in response to environmental cues. They consist of a membrane-bound histidine kinase sensor protein coupled with a cytosolic response regulator. In the presence of appropriate stimuli, the sensor autophosphorylates a conserved histidine residue before transferring the phosphate to a conserved aspartate residue on the response regulator, activating the response regulator. Activated response regulators function to modulate adaptive responses by effecting the expression of target genes. Response regulators are typically transcription factors but can also function by other mechanisms (14). The adaptive responses mediated by TCSs are broad and include virulence, motility, metabolism, and stress responses.

One of the better characterized TCSs is the EnvZ-OmpR system that is ubiquitous in Gram-negative bacteria (15). EnvZ is the membrane-associated sensor kinase, and OmpR the response regulator that functions as a transcription factor. EnvZ-OmpR was first discovered in *Escherichia coli* and was shown to regulate the expression of its two major outer membrane porin proteins (OMP), *ompC* and *ompF*, in response to environmental osmolarity (16–18). The function of OmpR as an osmoregulator has been extended to a number of other bacterial genera (19–21). OmpR has also been linked to other phenotypes in Gram-negative bacteria, including virulence (19, 20, 22–26) and acidic tolerance (21, 27–31). The *V. cholerae* OmpR homolog, open reading frame (ORF) VC2714, has been little studied, and its role in *V. cholerae* biology is unknown.

The RND efflux systems are ubiquitous tripartite transporters in Gram-negative bacteria that play critical roles in antimicrobial resistance. Many RND efflux systems exhibit broad substrate specificity and have the capacity to efflux multiple substrates that are both structurally and functionally unrelated (32, 33). The RND systems play critical roles in antimicrobial resistance by exporting toxic compounds from the cytosol and periplasm into the extracellular environment. Although RND efflux pumps have been widely studied for their role in multiple antibiotic resistance, they also impact many other physiological phenotypes in bacteria (34). This was recently documented in *V. cholerae*, where the RND systems were shown to be required for cell homeostasis (35, 36). The absence of RND efflux in *V. cholerae* resulted in downregulation of the ToxR regulon and altered expression of genes involved in metabolic and environmental adaptation (37, 38), including several TCSs. The results of these studies suggested that RND-mediated efflux modulated homeostasis by effluxing cell metabolites, which served as concentration-dependent environmental cues to initiate transcriptional responses via periplasmic sensing systems. This observation suggested the possibility

that these TCSs may have contributed to the virulence attenuation observed in RND efflux-impaired *V. cholerae*.

In this work, we investigated six regulatory genes that were induced in the absence of RND-mediated efflux for their contribution to virulence factor production in *V. cholerae*. This revealed that VC2714, encoding a homolog of *E. coli* OmpR, functioned as a virulence repressor in *V. cholerae*. We documented that VC2714 repressed the expression of the key virulence regulator *aphB*. We further showed that *ompR* expression was regulated in response to detergent-like compounds, which are prevalent in the host gastrointestinal tract and are substrates of the RND transporters. Our collective results suggest that the *V. cholerae* EnvZ-OmpR TCS has evolved to regulate virulence in response to novel environmental stimuli.

RESULTS

***V. cholerae* OmpR represses virulence factor production.** The loss of RND-mediated efflux resulted in downregulation of the ToxR regulon and diminished CT and TCP production (37), suggesting that there are one or more factors linking efflux to virulence factor production. Transcriptional profiling of an RND-negative (ΔvexB ΔvexD ΔvexF ΔvexH ΔvexK ΔvexM) *V. cholerae* mutant during growth under AKI conditions (i.e., virulence-inducing conditions) showed that the expression of a number of regulatory genes, including several TCSs, was increased in the absence of RND efflux (38). We hypothesized that one or more of these regulatory genes may have contributed to RND efflux-dependent virulence repression. To test this, we expressed six regulators (i.e., VC0486, VC1320 and VC1319, VC1081, VC1638, VC1825, VC1320, and VC2714) from the arabinose-regulated promoter in pBAD33 in wild-type (WT) *V. cholerae* during growth under AKI conditions in the presence of 0.05% arabinose and quantified CT production (39). VC0486 encodes an uncharacterized DeoR family regulator. VC1320 (*carR*) and VC1319 (*carS*) encode the CarRS TCS that is involved in regulating lipopolysaccharide (LPS) remodeling and *vps* production (40–42); *carR* (pTB15) and *carRS* (pTB3) were independently expressed. VC1081 encodes an uncharacterized response regulator. VC1638 was recently shown to regulate the expression of *VCA0732* in response to polymyxin B (43). VC1825 is an AraC family regulator that regulates a phosphotransferase system (PTS) transporter (44). VC2714 encodes an uncharacterized response regulator. The results showed that only pTB11, expressing VC2714, repressed CT production (Fig. 1A). VC2714 encodes a homolog of the *E. coli* osmotic stress regulator OmpR with 92.1% amino acid sequence similarity and hereafter will be referred to as *ompR*.

To further verify that *V. cholerae* OmpR was a virulence repressor, we repeated the above experiment in WT *V. cholerae* harboring plasmid pTB11 during growth under AKI conditions in the presence of increasing arabinose concentrations and quantified CT and TcpA production. The results showed an arabinose-dependent inhibition of both CT and TcpA production (Fig. 1B). Based on these results, we conclude that OmpR functions as a virulence repressor in *V. cholerae*.

OmpR contributes to virulence repression in RND efflux-deficient *V. cholerae*. To verify that *ompR* was upregulated in RND-deficient *V. cholerae* as previously indicated in a transcriptomics data set (38), we introduced the *ompR-lacZ* transcriptional reporter plasmid pKD9 into WT and isogenic RND efflux-negative (ΔRND) *V. cholerae* strains and quantified *ompR* expression in both strains following growth in LB broth, in minimal T-medium, and under AKI conditions. The results showed significantly increased *ompR* expression in ΔRND relative to WT during growth under AKI conditions but no significant difference in LB broth or minimal T-medium (Fig. 2A). These findings confirm the previous study and suggest that the RND efflux-dependent induction of *ompR* is dependent on growth under AKI growth conditions.

We next tested if *ompR* contributed to the virulence repression observed in the RND-negative strain. To address this, we created *ompR* deletion strains in WT and RND-negative *V. cholerae* and quantified CT and TcpA production in WT, ΔRND, and their respective isogenic ΔompR mutants. Confirming previous studies (37), the RND-

A.

B.
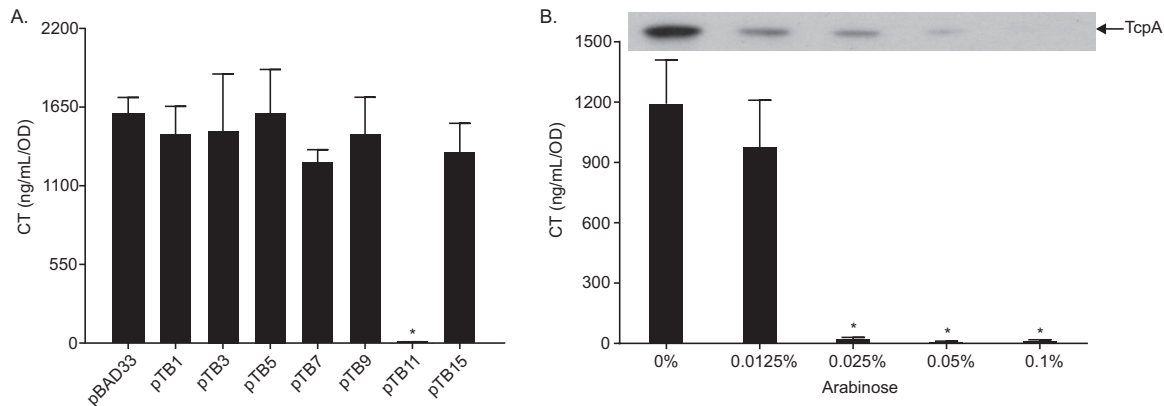
FIG 1 Overexpression of *ompR* represses virulence factor production. (A) WT *V. cholerae* harboring pBAD33 or the indicated expression plasmids was cultured under AKI conditions with 0.05% arabinose for 24 h when culture supernatants were used for CT quantitation by GM1 enzyme-linked immunosorbent assay (GM1-ELISA). The pBAD33-based expression plasmids carry the following genes: pTB1, VC0486; pTB3, VC1320 and VC1319; pTB5, VC1081; pTB7, VC1638; pTB9, VC1825; pTB11, VC2714; and pTB15, VC1320. The data represent the mean ± standard deviation (SD) of three independent experiments. *, $P < 0.0001$ relative to pBAD33 determined by Dunnett's multiple-comparison test. (B) WT *V. cholerae* harboring pTB11 (pBAD33-*ompR*) was cultured under AKI conditions with indicated arabinose concentrations for 24 h when culture supernatants were used for CT quantitation by GM1 ELISA and the cell pellets were used for TcpA immunoblotting. CT data represent the mean ± SD of a minimum of three independent experiments. *, $P < 0.0001$ relative to 0%, determined by Dunnett's multiple-comparison test. The TcpA immunoblot is representative of a minimum of three independent experiments.

negative strain produced significantly reduced amounts of CT and TcpA relative to WT (Fig. 2B). Consistent with the finding that OmpR is a *V. cholerae* virulence repressor, deletion of *ompR* in WT resulted in a slight but not statistically significant increase in both CT and TcpA production (Fig. 2B). Deletion of *ompR* in the ΔRND background partially restored CT and TcpA production in the ΔRNDΔ*ompR* strain relative to the parental ΔRND strain, but the magnitude of the increase did not reach WT levels

A.

B.
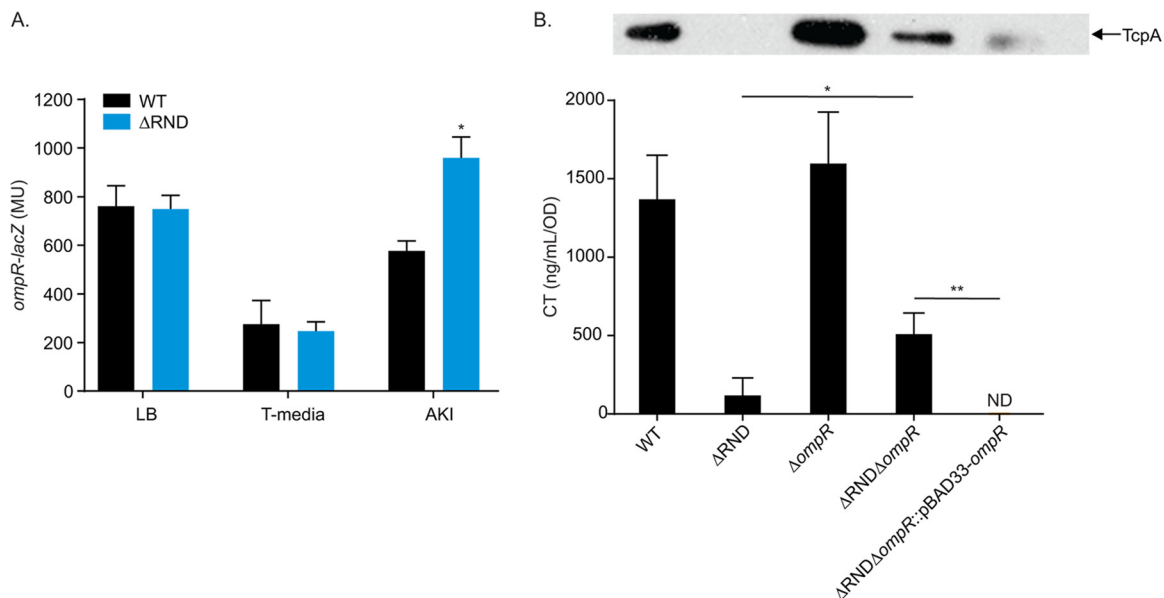
FIG 2 OmpR represses virulence factor production in RND efflux-negative *V. cholerae*. (A) WT and ΔRND *V. cholerae* strains harboring an *ompR-lacZ* reporter plasmid were cultured under the indicated conditions for 5 h when β-galactosidase activity was quantified. Data represent the mean ± SD of three independent experiments performed in triplicate. *, $P < 0.0001$ relative to WT determined by a *t* test. (B) The indicated *V. cholerae* strains were cultured under AKI conditions with the ΔRNDΔ*ompR*::pBAD33-*ompR* strain cultured in the presence of 0.05% arabinose. At 24 h, culture samples were collected, and CT and TcpA production was assessed by GM1 ELISA and TcpA immunoblotting, respectively. The CT data represent the mean ± SD of a minimum of three independent experiments. ND, nondetectable levels of CT. *, $P < 0.05$; **, $P < 0.01$ relative to the parental strain, determined by Tukey's multiple-comparison test. TcpA immunoblot is representative of a minimum of three independent experiments.
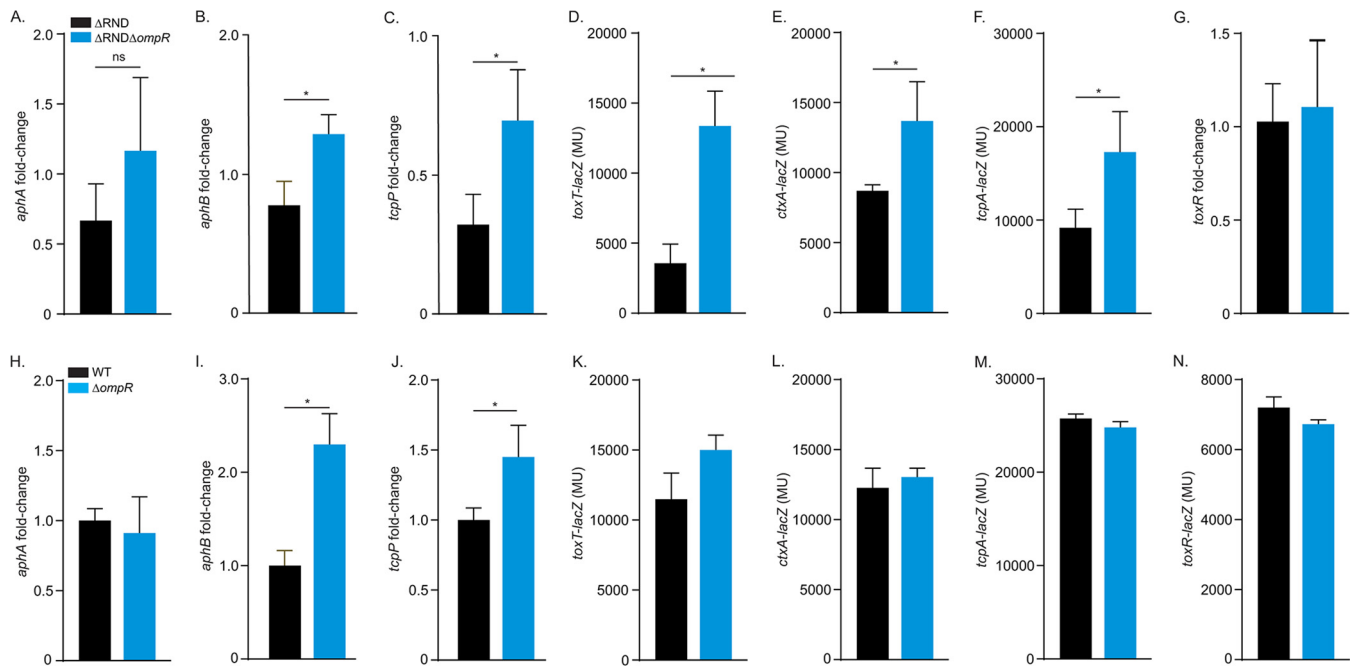
FIG 3 OmpR represses the ToxR regulon. *V. cholerae* strains were cultured under AKI conditions when gene expression was assessed using *lacZ* promoter reporters (panels D to F and K to N) or reverse transcription-quantitative PCR (qRT-PCR) (panels A to C and G to J) as described in Materials and Methods. (A to G) Reporter gene expression in ΔRND and ΔRNDΔompR *V. cholerae* strains. (H to N) Reporter gene expression in WT and ΔompR *V. cholerae* strains. The results presented in panels A to C and H to J were generated at 3.5 h postinoculation; results from the remaining assays were generated at 5 h postinoculation. Data represent mean and SD of at least three independent experiments performed in triplicate. *, $P < 0.05$ relative to parental strain determined by a *t* test.

(Fig. 2B). To determine if deletion of *ompR* was sufficient for the increased virulence factor production found between the ΔRNDΔompR and ΔRND strains, *ompR* was complemented in the ΔRNDΔompR strain by use of plasmid pTB11. Complementation of *ompR* in the ΔRNDΔompR strain resulted in repression of both CT and TcpA production, indicating that deletion of *ompR* was in fact responsible for increased virulence factor production in the ΔRNDΔompR strain. Together, these data suggest that *ompR* contributes to virulence attenuation in the RND-negative background but that other factors are also involved in virulence repression.

***V. cholerae* OmpR represses *aphB* expression.** The above results suggested that OmpR was a virulence repressor, but the mechanism by which it attenuated virulence factor production was unclear. As CT and TCP production is positively regulated by the ToxR regulon, we hypothesized that OmpR repressed components of the ToxR regulon. If this was true, then *ompR* deletion in ΔRND should increase the expression of the affected ToxR regulon genes relative to the parental strain ΔRND. We therefore compared ToxR regulon gene expression in ΔRND and its isogenic ΔompR mutant during growth under AKI conditions. The results showed that *ompR* deletion in the RND-negative strain ΔRND did not significantly affect *aphA* expression (Fig. 3A) but did result in increased expression of *aphB* and the ToxR regulon genes downstream from *aphB* (i.e., *tcpP*, *toxT*, *ctxA*, and *tcpA*) (Fig. 3B through F). ΔRND and ΔRNDΔompR had comparable levels of *toxR* expression, indicating that virulence repression by OmpR was not due to reduced *toxR* expression (Fig. 3G). As *aphB* is one of the most upstream regulators in the ToxR regulon, these results suggested that OmpR attenuated virulence factor production by repressing *aphB* in ΔRND.

To test if OmpR affected ToxR regulon expression in efflux-sufficient cells, we repeated the above experiments in the WT during growth under AKI conditions. The results showed that *ompR* deletion in the WT did not affect *aphA* expression but resulted in increased expression of *aphB* and its downstream target *tcpP* (Fig. 3I and J) but not the other ToxR regulon genes (Fig. 3H, J, K, L, M, and N). This is consistent with the observation that deletion of *ompR* did not significantly affect CT or TcpA production
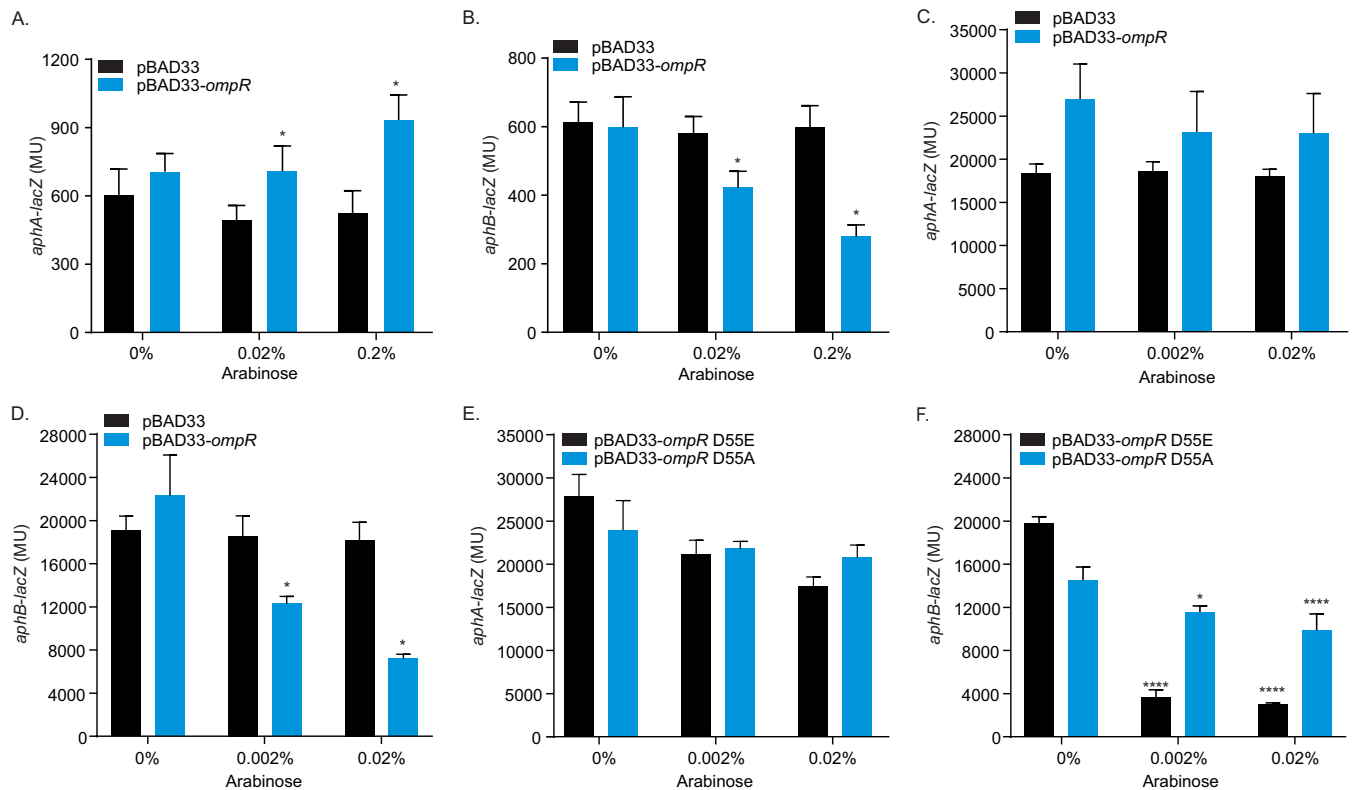
FIG 4 *V. cholerae* OmpR represses *aphB* expression. (A and B) WT *V. cholerae* harboring either pBAD33 or pBAD33-*ompR* (pTB11) with either *aphA-lacZ* (pXB202) or *aphB-lacZ* (pXB203) reporter plasmids was cultured under AKI conditions for 5 h with the indicated arabinose concentrations when β-galactosidase activity was quantified. Data represent the mean ± SD of three independent experiments performed in triplicate. (C and D) *E. coli* strain EC100 harboring either pBAD33 or pBAD33-*ompR* with either *aphA-lacZ* or *aphB-lacZ* reporter plasmids was cultured in LB broth for 5 h with the indicated arabinose concentrations when β-galactosidase activity was quantified. Data represent the mean ± SD of three independent experiments performed in triplicate. (E and F) *E. coli* strain EC100 harboring indicated pBAD33-*ompR* aspartate 55 mutant constructs (i.e., D55E or D55A) with either *aphA-lacZ* or *aphB-lacZ* reporter plasmids was cultured in LB broth with the indicated arabinose concentrations for 5 h when β-galactosidase activity was quantified. Data represent the mean ± SD of three independent experiments performed in triplicate. *, $P < 0.01$; ****, $P < 0.001$ relative to 0% arabinose determined by Sidak's multiple-comparison test.

in the WT (Fig. 2B). Collectively, these results support the conclusion that OmpR is an *aphB* repressor and that OmpR regulation of *aphB* is relevant in WT cells during growth under AKI conditions.

Ectopic *ompR* expression represses *aphB* transcription in *E. coli*. To further confirm that OmpR can repress *aphB*, we tested if ectopic *ompR* expression altered *aphB* expression in *V. cholerae* and the heterologous host *E. coli*. In the first set of experiments, we expressed *ompR* from pTB11 in WT *V. cholerae* bearing *lacZ* transcriptional reporters for *aphA* and *aphB* during growth under AKI conditions in the presence of varying arabinose concentrations to induce *ompR* expression. The results showed a small arabinose dose-dependent increase in *aphA* expression (Fig. 4A); the biological significance of this finding is unclear. By contrast, we observed an arabinose dose-dependent decrease in *aphB* expression (Fig. 4B), confirming that OmpR is an *aphB* repressor. Although OmpR may have a weak ability to induce *aphA* expression, its ability to repress *aphB* appears to be dominant, as the net consequence of *ompR* regulation of *aphA* and *aphB* is repression of *tcpP* (Fig. 3C and J).

In the second set of experiments, we expressed *V. cholerae ompR* from pTB11 in *E. coli* bearing *aphA-lacZ* or *aphB-lacZ* transcriptional reporters to address whether OmpR acted directly at the respective promoters. The *E. coli* strains were cultured to mid-log phase in the presence of varying arabinose concentrations when *aphA-lacZ* or *aphB-lacZ* expression was quantified. The results showed that arabinose addition had little effect on *aphA* expression (Fig. 4C). By contrast, there was an arabinose dose-dependent decrease in *aphB* expression (Fig. 4D), consistent with OmpR being an *aphB*

repressor. These results suggest that OmpR may act directly at the *aphB* promoter; however, we cannot exclude the possibility that OmpR could be acting through an intermediate that is present in both *E. coli* and *V. cholerae.*

As OmpR has been shown to function as an active transcription factor in both the unphosphorylated and phosphorylated forms, we investigated if the phosphorylation of *V. cholerae* OmpR plays a role in its repression of *aphB*. To this end, OmpR point mutants mimicking constitutive phosphorylation (D55E) and constitutive dephosphorylation (D55A) of the conserved aspartate were cloned into pTB11. The mutant *ompR* constructs were then expressed in *E. coli*, and *aphA* and *aphB* expression was quantified as before. Consistent with the above findings, expression of the phosphomimic *ompR* mutants did not significantly affect *aphA* expression (Fig. 4E). By contrast, expression of the *ompR*^{D55E} allele strongly repressed *aphB* expression, whereas expression of the *ompR*^{D55A} allele only marginally repressed *aphB*, and to a far lesser extent than the *ompR*^{D55E} allele (Fig. 4F). Taken together, these results indicate that both phosphoforms of *V. cholerae* OmpR have the capacity to repress *aphB* transcription, but the phosphorylated form (i.e., *ompR*^{D55E}) is predominately responsible for *aphB* repression. Collectively, these results support the conclusion that OmpR negatively regulates the ToxR regulon via directly repressing *aphB* transcription, and they suggest that the phosphorylated form of OmpR is responsible for this repression.

V. cholerae *ompR* is induced by bile salts and detergents. While the above data showed that OmpR functions as a virulence repressor through repression of *aphB*, we wished to address the environmental factors that modulate *ompR* expression in *V. cholerae*. OmpR has been extensively studied in *Enterobacteriaceae*, where it has been shown to function as an osmoregulator that mediates adaptive responses to osmotic stress (18, 22, 45). We therefore tested if *V. cholerae ompR* responded to changes in medium osmolarity by quantifying *ompR-lacZ* expression during growth under AKI conditions in standard AKI broth (86 mM NaCl), AKI broth with low NaCl (21.5 mM), and AKI with excess NaCl (250 mM). As shown in Fig. 5A, the NaCl concentration did not significantly affect *ompR* expression, suggesting that *ompR* was not regulated in response to osmolarity. Consistent with this, growth analysis showed that *ompR* was dispensable for growth in high-osmolarity broth up to 500 mM NaCl in both WT and ΔRND backgrounds (Fig. 5B and C). From these results, we conclude that *V. cholerae* OmpR is not regulated in response to medium osmolarity and therefore likely responds to different environmental stimuli than what is observed in *Enterobacteriaceae*.

The finding that *ompR* was induced in the absence of RND-mediated efflux (Fig. 2A) suggested that small molecules that accumulate intracellularly in the absence of RND efflux may play a role in *ompR* expression. Previous studies showed that a major function of the *V. cholerae* RND efflux systems was in resistance to hydrophobic and detergent-like molecules, including bile salts, fatty acids, and detergents (37, 46). We therefore tested if bile salts or detergents affected *ompR* expression as described above. The results showed that the addition of deoxycholate, bile salts, oxgall, and SDS to the growth media increased *ompR* expression (Fig. 5D). We also tested another small molecule, indole. Indole is a *V. cholerae* metabolite that is an RND efflux substrate and virulence repressor (46, 47). The data showed that indole did not affect *ompR* expression, suggesting that altered *ompR* expression was specific for compounds with detergent-like properties. As detergents are associated with envelope stress due to their membrane-intercalating properties, we hypothesized that *ompR* may be induced in response to envelope stress. To test this, we quantified *ompR* expression following the induction of membrane stress by ethanol treatment (48). The results of these experiments showed that there was an ethanol dose-dependent increase in *ompR* expression (Fig. 5E). Taken together, these findings suggest that *V. cholerae ompR* is likely regulated in response to membrane perturbations resulting from exposure to membrane-intercalating agents.

C18-depleted AKI medium nullifies *ompR* induction in an RND-negative _V. cholerae_ strain. Based on the results above, we hypothesized that hydrophobic and/or
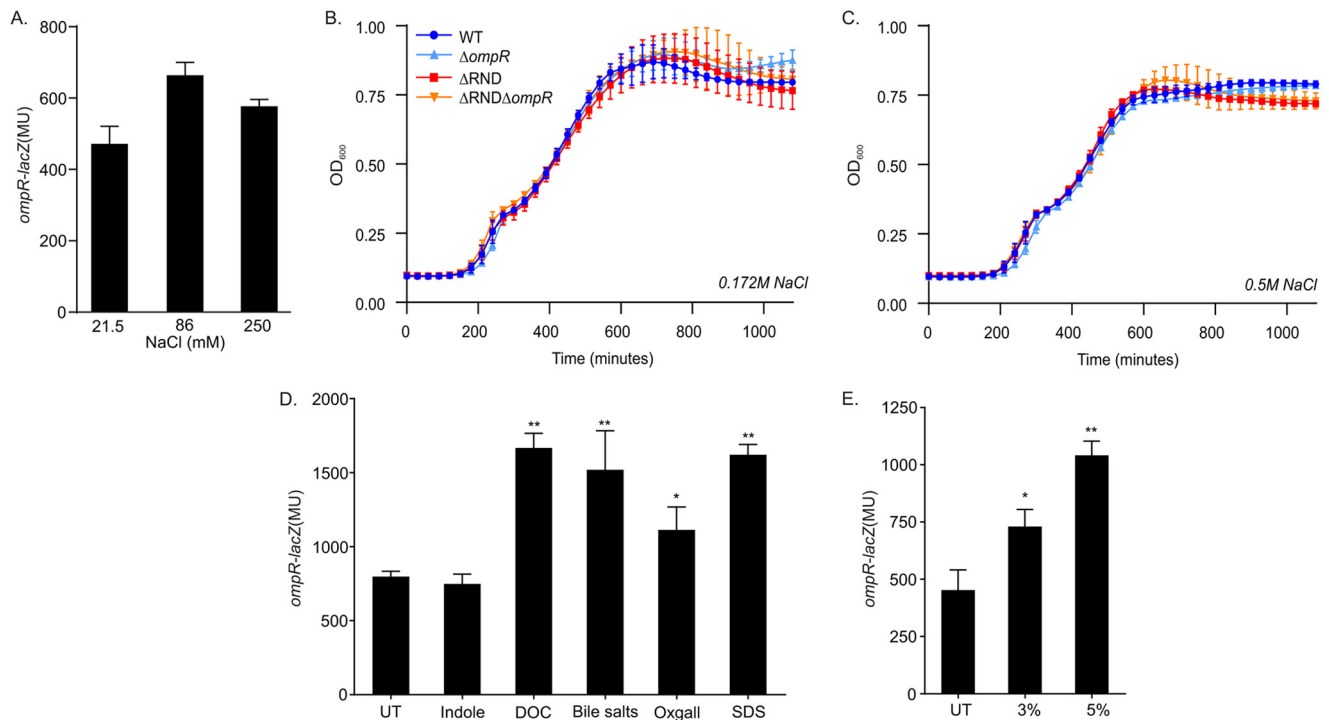
FIG 5 *V. cholerae ompR* does not respond to osmolarity but is induced by membrane-intercalating agents. (A) WT *V. cholerae* harboring an *ompR-lacZ* reporter plasmid (pDK9) was cultured under AKI conditions with the indicated NaCl concentrations for 5 h when β-galactosidase activity was quantified. (B and C) Growth analysis of *V. cholerae* Δ*ompR* mutants. Overnight LB cultures of *V. cholerae* WT, Δ*ompR*, ΔRND, and ΔRNDΔ*ompR* were diluted 1:10,000 in either fresh LB medium (0.172 M NaCl) (B) or LB medium containing a total concentration of 0.5 M NaCl (C) and cultured at 37°C with constant shaking in a microtiter plate reader. Growth was recorded as the OD_{600} every 30 min. Data indicate the average of at least three independent experiments performed in triplicate. (D and E) WT *V. cholerae* harboring an *ompR-lacZ* reporter plasmid was cultured in LB broth for 4 h when the indicated RND efflux substrates (D) or ethanol (E) was added to the culture medium. The cultures were then incubated with shaking for an additional hour when β-galactosidase activity was quantified. Data indicate the average ± SD of three independent experiments performed in triplicate. UT, untreated samples. *, $P < 0.01$; **, $P < 0.001$ relative to untreated determined by Dunnett's multiple-comparison test.

nonpolar compounds present in the AKI medium were accumulating in the RND efflux-deficient strain ΔRND and activating *ompR* transcription. To test this, we generated a depleted AKI medium by passing AKI broth through a Sep Pak C18 cartridge to deplete nonpolar and hydrophobic compounds from the medium. We then quantified *ompR* expression in WT and RND-negative strains harboring pDK9 (*ompR-lacZ*) following growth under AKI conditions in AKI broth and in the C18-depleted AKI medium. The results showed increased *ompR* expression in ΔRND during growth in AKI broth as expected (Fig. 6). Growth of the WT in the C18-depleted AKI medium did not affect *ompR* expression when compared to expression in the standard AKI medium. However, growth of ΔRND in the C18-depleted AKI medium alleviated the increase in *ompR* transcription observed in the standard AKI medium. To determine if the hydrophobic compounds from the AKI medium that were retained on the C18 column were responsible for *ompR* induction in ΔRND, we eluted the retained compounds from the C18 cartridges used to extract AKI and LB broth. We then determined if the respective eluates contained *ompR*-inducing compounds by adding them to LB broth cultures of ΔRND and WT and quantifying *ompR-lacZ* expression. The results showed that the addition of the AKI medium C18 column eluate, but not LB C18 column eluate, activated *ompR* expression in ΔRND, while neither eluate had an effect on *ompR* expression in the WT (Fig. 6). Collectively, these data suggest that hydrophobic and/or nonpolar compounds present in the AKI medium are responsible for increased *ompR* expression in the RND-negative strain. The fact that the C18-depleted AKI medium did not affect *ompR* expression in the WT indicated that this phenotype was RND dependent. Significantly, we also observed that the increase in *ompR* expression in ΔRND was not dependent on growth under AKI conditions (i.e., static growth followed by shaken
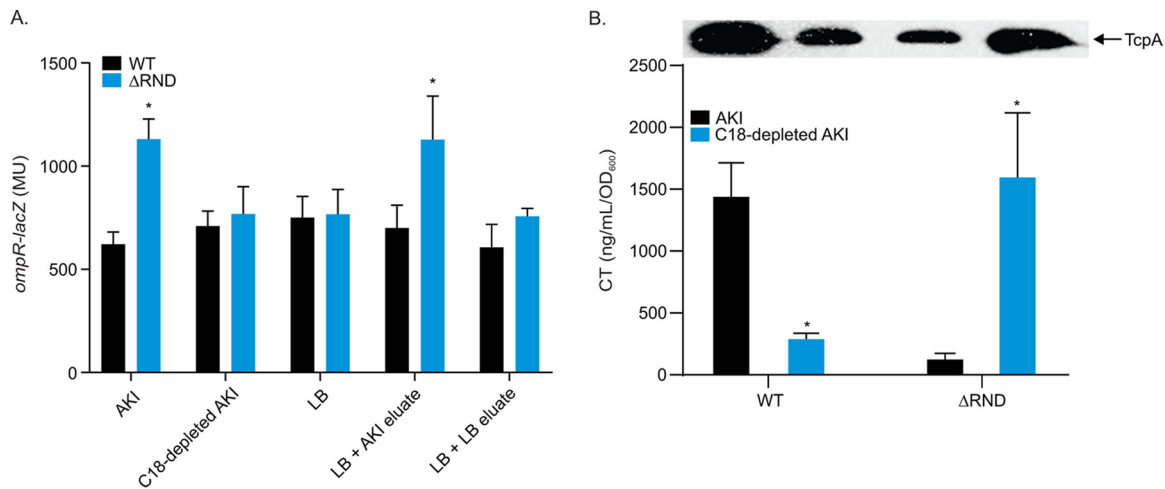
FIG 6 C18-depleted AKI medium abolishes the RND efflux-dependent induction of *ompR* expression in *V. cholerae*. (A) WT and ΔRND *V. cholerae* strains harboring an *ompR-lacZ* reporter plasmid (pDK9) were cultured in the indicated media for 5 h when *ompR-lacZ* expression was quantified. *, $P < 0.01$ relative to WT determined by a *t* test. (B) WT and ΔRND *V. cholerae* strains were cultured under AKI conditions in either an AKI medium or a C18-depleted AKI medium for 24 h when CT and TcpA production was assessed by GM1 ELISA and TcpA immunoblotting, respectively. Data represent the mean ± SD of three independent experiments. *, $P < 0.01$ relative to AKI, determined by *t* test. C18-depleted AKI medium and C18 column AKI medium eluates were prepared as described in Materials and Methods.

growth), as *ompR* expression was also enhanced in cultures grown in AKI broth under noninducing conditions (not shown). This observation, combined with the finding that *ompR* was not induced in ΔRND during growth in LB broth or T-medium (Fig. 2A), suggested that the *ompR*-inducing molecules were only present in the AKI medium. From these experiments, we conclude that hydrophobic and/or nonpolar compounds that are present in AKI medium, but not the LB medium, are responsible for *ompR* activation in ΔRND. Further, because this phenotype was RND efflux dependent, we infer that the inducing compounds are substrates for the *V. cholerae* RND efflux systems.

The observation that growth in C18-depleted AKI medium resulted in an RND efflux-dependent repression of *ompR* expression, coupled with the finding that OmpR is a virulence regulator, led us to explore if C18 depletion of the AKI medium also affected virulence factor production. To explore this, CT and TcpA production was quantified in WT and ΔRND strains cultured under AKI conditions in either standard AKI medium or a C18-depleted AKI medium. As previously illustrated, the ΔRND strain had reduced CT and TcpA production compared to WT when cultured in the standard AKI medium. Interestingly, growth of WT in the C18-depleted AKI medium resulted in a reduction in both CT and TcpA production (Fig. 6B). Inversely, growth of a ΔRND strain in the C18-depleted AKI medium resulted in an increase in both CT and TcpA production to levels relative to standard AKI-cultured WT. Collectively, these data suggest that the nonpolar and hydrophobic compounds that are extracted from the AKI medium by the Sep Pak C18 column play a role in both *ompR* expression and virulence factor production. The nature of these molecules will require further investigation.

DISCUSSION

V. cholerae is an inhabitant of the aquatic ecosystem that can colonize the human gastrointestinal tract to cause disease. The ability of *V. cholerae* to replicate in these two disparate ecosystems is dependent upon its ability to rapidly adapt to the changing environments it encounters. For example, upon host entry, *V. cholerae* must adjust to dramatic changes in temperature, pH, salinity, oxygen tension, and the presence of antimicrobial compounds. At the same time, colonization of the intestinal tract requires the expression of niche-specific genes (e.g., virulence factors). Prior to exiting the host, *V. cholerae* must also regulate the expression of genes that are important for transmission and dissemination (9–12). How all of these responses are integrated within the

dynamic environment in the host is poorly understood. What is clear is that periplasmic sensing systems play a critical role in the process. This includes ToxR, which regulates host entry; the Cad system, which contributes to acid tolerance; the CarRS TCS, which mediates antimicrobial peptide resistance; OscR, which regulates response to osmolality; and stress responses like the Cpx system that alleviate stress due to the presence of antimicrobial compounds in this host (1, 40–42, 49–51).

In this study, we interrogated the function of six regulatory genes on virulence factor production in *V. cholerae*. All of the tested regulatory genes were identified as being upregulated in an RND efflux-negative *V. cholerae* mutant (38). As RND-mediated efflux is required for virulence factor production, these induced regulatory genes represented potential efflux-dependent virulence repressors. We found that *ompR* contributed to repression of virulence factor production in the RND-null strain by repressing *aphB* expression. AphB is a key regulator in the ToxR virulence regulon (3). Previous studies have shown that AphB activity is modulated by low oxygen and acidic pH, but it was unknown whether expression of *aphB* was itself regulated (52). To our knowledge, OmpR is the first regulator shown to modulate *aphB* expression in *V. cholerae*. We further demonstrated that *ompR* was activated in response to membrane-intercalating compounds that are abundant in the host, suggesting that this regulatory circuit may be relevant *in vivo*.

Although the function of OmpR has been widely explored in *Enterobacteriaceae*, the function of the *V. cholerae* OmpR homolog has not been investigated previously. OmpR is known as an osmoregulator in *Enterobacteriaceae*. Expression of *ompR* is induced at high salt concentrations, and OmpR regulates transcriptional responses to alleviate osmotic stress (16, 53, 54). Herein we report that *V. cholerae ompR* was not induced in response to osmolality and that *ompR* was dispensable for growth at high salt concentrations. These findings were consistent with two previous studies on *V. cholerae* responses to osmolarity (50, 55), neither of which identified *ompR* as one of the genes to respond to increased osmolarity. In the latter study, OscR was identified as an osmoregulator which regulated motility and biofilm formation (50). We did not observe any effect of *ompR* on either of these two phenotypes (not shown), suggesting that OscR and OmpR function independently. Taken together, these results suggest that *V. cholerae* OmpR has evolved to respond to different environmental stimuli and fulfill new functions.

Bacterial regulatory networks evolve in response to evolutionary pressures placed on individual species, as they inhabit specific niches (56, 57). TCSs have been suggested to evolve under such selective pressures to respond to novel stimuli and regulate diverse target genes to meet the needs of specific bacterial species (58). EnvZ-OmpR is an example of this. While EnvZ-OmpR is ubiquitous in *Gammaproteobacteria*, its function appears to have evolved divergently in several bacterial species (20, 23, 24, 31, 59). Our results suggest that this divergent evolution has also occurred in *V. cholerae*. We speculate that the lifestyle of *V. cholerae*, which involves growth in murine environments and the human host gastrointestinal tract, has selected for OmpR to respond to novel stimuli and to fulfill a novel physiological role in *V. cholerae*. Sequence comparison of the *V. cholerae ompR* locus to the *E. coli ompR* locus supports this hypothesis. While *V. cholerae* OmpR is 83% identical in amino acid sequence to its *E. coli* homolog, the *V. cholerae* EnvZ sensor kinase is only 47% identical to its *E. coli* counterpart.

OmpR functioned as a repressor of virulence factor production, and its expression was induced by the addition of membrane-intercalating compounds such as detergents and ethanol. Further, our OmpR phosphomimic studies suggested that it was the phosphorylated form of OmpR that was responsible for virulence repression. This likely explains the upregulation of *ompR* in the RND-negative background, as cells lacking RND-mediated efflux are hypersensitive to membrane-intercalating compounds due to the RND mutant's diminished ability to actively efflux these compounds from within the cell (37, 46, 60). We speculate that virulence repression in the RND-null mutant resulted from the intracellular accumulation of nonpolar and hydrophobic molecules that are

present in AKI medium (e.g., fatty acids and detergent-like molecules). This hypothesis is supported by the finding that WT and RND-negative *V. cholerae* strains have comparable *ompR* expression when cultured in a C18 cartridge-depleted AKI medium. These molecules are likely the substrates of the RND transporters and thus accumulated in the RND-negative mutant, resulting in *ompR* induction and subsequent virulence repression. This is supported by the observations that OmpR contributes to virulence repression in an RND-negative strain and that growth of the RND-negative strain in the C18-extracted AKI medium attenuated *ompR* expression.

Bile salts are found at high concentrations in the lumen of the small intestine. The finding that *ompR* was activated in response to bile salts suggests the possibility that OmpR could contribute to spatial and temporal virulence regulation that has been observed *in vivo* (61). Following ingestion, *V. cholerae* enters the host small intestine, migrates to the intestinal epithelium, and activates virulence factor production while colonizing the intestinal epithelium. This suggests the possibility that high bile salt concentrations within the intestinal lumen may activate *ompR* to repress virulence factor production. Once *V. cholerae* traverses the mucus layer to colonize the epithelial surface, where bile salt concentrations are reduced, OmpR-mediated virulence repression would be alleviated. This tight regulation of virulence factor production is paramount to the pathogenic success of *V. cholerae*. It is interesting to speculate that *V. cholerae* OmpR is one of multiple factors that converge on the ToxR regulon to ensure that it is only expressed in the appropriate *in vivo* niche. It is noteworthy that bile salts and fatty acids have pleiotropic effects on the ToxR regulon. Fatty acids have been shown to negatively affect ToxT activity (62). Bile salts have been implicated in intermolecular disulfide bond formation in TcpP (63). Bile acids, fatty acids, and other detergent-like compounds also signal through ToxR to repress *aphA* (38, 64). Thus, there seems to be a coordinated response by *V. cholerae* to these environmental cues to effect virulence factor production.

The induction of *V. cholerae ompR* in response to nonspecific membrane-intercalating agents suggests that OmpR could also function as part of a generalized membrane stress response. Consistent with this, there is evidence that OmpR in *Enterobacteriaceae* may be a component of other stress response systems (65, 66). A conserved response to membrane stress in bacteria includes suppressing membrane protein production as a mechanism to alleviate envelope stress. Thus, OmpR-dependent virulence repression in *V. cholerae* could conceivably contribute to a membrane stress response because the ToxR regulon controls the expression of many membrane-bound and secreted proteins, including the two major outer membrane porins OmpU and OmpT (67). However, analysis of WT and Δ*ompR* whole-cell lysates by SDS-PAGE staining did not reveal any effect of OmpR on the production of OmpU and OmpT (not shown), which is consistent with the finding that OmpR did not affect *toxR* expression or protein production (Fig. 3 and data not shown). This contrasts what is observed in other bacterial species where OmpR regulates the expression of outer membrane porins (16–18, 21, 68).

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains EC100D*pir*+ and SM10λ*pir* were used for cloning and plasmid conjugation, respectively. *V. cholerae* strain JB58 was used as the WT in all experiments. Bacterial strains were routinely grown at 37°C in lysogeny broth or on LB agar. AKI growth conditions were used to induce *V. cholerae* virulence gene expression as previously described (69). The modified T medium was prepared as previously described (70). Antibiotics were used at the following concentrations: streptomycin (58), 100 μg/ml; carbenicillin (Cb), 100 μg/ml; and chloramphenicol (Cm), 20 μg/ml for *E. coli* and 2 μg/ml for *V. cholerae*. The C18-conditioned AKI medium was prepared as follows. Sep-Pak C18 cartridges (Waters) were preconditioned with 10 ml of 100% methanol followed by 10 ml of sterile double-distilled water (ddH$_2$O) before 50 ml of AKI broth was passed through the cartridge and the flowthrough collected and used as a conditioned AKI broth. Molecules that were retained on the C18 columns following the passage of LB or AKI broth medium were eluted from the column with 10 ml of 100% methanol. The eluates were concentrated by evaporation. The resulting residue was resuspended in a volume of LB broth that was identical to the volume of the extracted AKI broth and filter sterilized prior to use.

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

Strain, plasmid, or primer	Genotype, characteristics, or sequence (5'–3')	Source or reference
Strains		
E. coli		
EC100D*pir*+	F- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80d*lacZ*ΔM15 Δ*lacX74 recA1 endA1 araD139* Δ(*ara, leu*)7697 *galU galK* λ- *rpsL* (Str^R) *nupG pir*+	Epicenter
SM10λ*pir*	*thi-1 thr leu tonA lacY supE recA*::RP4-2-4-Tc::Mu Km^r (λ *pir*R6K)	67
V. cholerae		
JB58	01 El Tor strain N16961 Δ*lacZ* Sm^r	Lab collection
ΔRND	JB58 Δ*vexB* Δ*vexD* Δ*vexF* Δ*vexH* Δ*vexK* Δ*vexM*	37
DK243	JB58 Δ*ompR*	This work
DK246	ΔRND Δ*ompR*	This work
Plasmids		
pBAD33	Arabinose-regulated expression vector	39
pTB1	pBAD33 expressing VC0486	This work
pTB3	pBAD33 expressing VC1320-VC1319 (*carRS*)	This work
pTB5	pBAD33 expressing VC1081	This work
pTB7	pBAD33 expressing VC1638	This work
pTB9	pBAD33 expressing VC1825	This work
pTB11	pBAD33 expressing VC2714 (*ompR*)	This work
pDK14	pBAD33 expressing VC2714-D55E	This work
pDK15	pBAD33 expressing VC2714-D55A	This work
pTB15	pBAD33 expressing VC1320 (*carR*)	This work
pTL61T	Reporter plasmid for making transcriptional fusions to *lacZ*, Amp^r	72
pDK9	pTL61T containing the *ompR* promoter	This work
pXB192	pTL61T containing the *toxT* promoter	37
pXB193	pTL61T containing the *ctxAB* promoter	73
pXB194	pTL61T containing the *tcpA* promoter	73
pXB201	pTL61T containing the *toxRS* promoter	73
pXB202	pTL61T containing the *aphA* promoter	73
pXB203	pTL61T containing the *aphB* promoter	73
pXB266	pTL61T containing the *leuO* promoter	64
pWM91	Suicide vector used for allelic exchange, Amp^r	67
pWM91-Δ*ompR*	Suicide vector used for deletion of *ompR*	This work
Primers		
P-VC2714-F-XhoI	GGCTCGAGAACTCGATTGAGTATGAGAAAGG	
P-VC2714-R-XbaI	AATCTAGACCATGATCCCACCTAACTGTTGTTC	
VC2714-F1-XhoI	TTCTCGAGTGCGGCTTTGCTGTCGGCGAC	
VC2714-R1-BamHI	GCGGATCCCACCTTGGCTGCGATTGCTAAC	
VC2714-F2	ATGCGCGCTTGCGTTCCTGATGGTAAAGCCGCCAAC	
VC2714-R2	ACCATCAGGAACGCAAGCGCGCATCATCATCTACCAC	
VC2714-F-SacI	AAGAGCTCAACAGTTAGGTGGGATCATG	
VC2714-R-SmaI	TTCCCGGGCTAAAAGAAGTTAGTTGGCGGC	
aphA-F	GCAGAACCTTACCGTCTGCAA	
aphA-R	GCGTAATAAGCGGCTTCGATT	
aphB-F	ATCGGTGAAGTGAAAGACATTTTGG	
aphB-R	GATGTTGATGCAACTCTTCAGCAT	
ToxRS-F	CGTCAAAACGGTTCCGAAACG	
ToxRS-R	CGCGAGCCATCTCTTCTTCAA	
tcpP-F	TAGCCGGCATTACTCATGATCTAC	
tcpP-R	TTGTTATCCCCGGTAACCTTGC	
gyrA-F	CAATGCCGGTACACTGGTACG	
gyrA-R	AAGTACGGATCAGGGTCACG	

Plasmid and mutant construction. Oligonucleotides used in this study are listed in Table 1. Chromosomal DNA from the WT was used as the PCR template for cloning experiments. The *ompR-lacZ* reporter plasmid pDK9 was generated as follows. The *ompR* promoter region was amplified by PCR using the P-VC2714-F-XhoI and P-VC2714-R-XbaI oligonucleotide primers. The resulting amplicon was digested with XhoI and XbaI restriction endonucleases and ligated into a similarly digested pTL61T vector to generate the plasmid pDK9. The *ompR* expression vector pTB11 was created by amplifying *ompR* using the VC2714-F-SacI and VC2714-R-SmaI oligonucleotide primers. The resulting 766-bp fragment was digested with SacI and SmaI restriction endonucleases and ligated into similarly digested pBAD33 to generate pTB11. The other expression plasmids (pTB3, pTB5, pTB7, pTB9, and pTB15) were made in a similar manner. The OmpR aspartate 55 mutant expression plasmids were constructed as follows. Internal fragments (364 bp) of the *ompR* gene containing D55E and D55A mutations were custom synthesized by Integrated DNA Technologies. The resulting fragments were digested with PmlI and EcoRI before being

ligated into similarly digested pTB11 to generate plasmids pDK14 and pDK15. The *ompR* (VC2714) deletion construct was constructed as follows. Primer pairs *ompR*-F1/*ompR*-R2 and *ompR*-F2/*ompR*-R1 were used in separate PCRs with N16961 genomic DNA. The two resulting amplicons (~1.5 kb each) were collected and used as the template for the second-round PCR amplification with the flanking *ompR*-F1 and *ompR*-R1 PCR primers. The resulting ~3-kb amplicon was then digested with the SpeI and SmaI restriction endonucleases before being ligated into similarly digested pWM91 vector to generate pWM91-Δ*ompR*. pWM91-Δ*ompR* was then used to delete *ompR* through allelic exchange as previously described (37). All plasmids were validated via DNA sequencing.

Transcriptional reporter assays. *V. cholerae* and *E. coli* strains containing the indicated *lacZ* reporters were cultured under AKI conditions, in LB broth, or in a modified T medium. At the indicated times, aliquots were collected in triplicate, and β-galactosidase activity was quantified as previously described (71). The experiment quantifying *ompR* expression during growth under varying NaCl concentrations was performed as follows. WT strains harboring pDK9 were cultured under virulence factor-inducing conditions in AKI medium containing the indicated NaCl concentrations for 5 h. Culture aliquots were then collected in triplicate, and β-galactosidase production was assessed. The experiments quantifying gene expression responses to bile salts, deoxycholate, SDS, oxgall, indole, and ethanol were performed as follows. The indicated strains were grown in LB broth at 37°C with shaking for 4 h when the indicated compounds were added to the cultures. Thereafter, the cultures were then incubated with shaking for an additional hour before culture aliquots were collected in triplicate and β-galactosidase production was assessed. All of the transcriptional reporter experiments were performed independently at least three times.

Determination of CT and TcpA production. CT production was determined by ganglioside GM1 enzyme-linked immunosorbent CT assays as previously described by use of purified CT (Sigma) as a standard (37). The production of TcpA was determined by Western immunoblotting as previously described (10).

Growth curve experiments. Growth curves were generated in microtiter plates. Overnight cultures of WT, ΔRND, Δ*ompR*, and ΔRNDΔ*ompR* strains grown in LB broth were washed in phosphate-buffered saline (PBS) and then diluted 1:10,000 in fresh LB broth containing either 0.172 M NaCl or 0.5 M NaCl. Two hundred microliters of the diluted cultures was then aliquoted in triplicate wells of a 96-well microtiter plate. The microtiter plates were then incubated at 37°C with constant shaking, and the optical density at 600 nm (OD_{600}) was measured every 30 min using a BioTek Synergy microplate reader.

Quantitative real-time PCR. *V. cholerae* strains were grown under AKI conditions for 3.5 h when total RNA was isolated from the cultures using TRIzol (Invitrogen) per the manufacturer's directions. cDNA was generated from the purified RNA using the Maxima First Strand cDNA synthesis kit (Thermo). The expression levels of specific genes were quantified by amplifying 25 ng of cDNA with 0.3 μM primers using the SYBR green PCR mix (Thermo) on a StepOnePlus real-time PCR System (Applied Biosystems). The relative expression levels of genes in the mutant and WT cultures were calculated using the $2^{-\Delta\Delta CT}$ method. The presented results are the means ± standard deviations from three biological replicates, with each biological replicate being generated from three technical replicates. DNA gyrase (*gyrA*) was used as the internal control.

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