RESEARCH ARTICLE

The *Vibrio cholerae* VprA-VprB Two-Component System Controls Virulence through Endotoxin Modification

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ABSTRACT **The bacterial cell surface is the first structure the host immune system targets to prevent infection. Cationic antimicrobial peptides of the innate immune system bind to the membrane of Gram-negative pathogens via conserved, surface-exposed lipopolysaccharide (LPS) molecules. We recently reported that modern strains of the global intestinal pathogen *Vibrio cholerae* modify the anionic lipid A domain of LPS with a novel moiety, amino acids. Remarkably, glycine or diglycine addition to lipid A alters the surface charge of the bacteria to help evade the cationic antimicrobial peptide polymyxin. However, the regulatory mechanisms of lipid A modification in *V. cholerae* are unknown. Here, we identify a novel two-component system that regulates lipid A glycine modification by responding to important biological cues associated with pathogenesis, including bile, mildly acidic pH, and cationic antimicrobial peptides. The histidine kinase Vc1319 (VprB) and the response regulator Vc1320 (VprA) respond to these signals and are required for the expression of the *almEFG* operon that encodes the genes essential for glycine modification of lipid A. Importantly, both the newly identified two-component system and the lipid A modification machinery are required for colonization of the mammalian host. This study demonstrates how *V. cholerae* uses a previously unknown regulatory network, independent of well-studied *V. cholerae* virulence factors and regulators, to respond to the host environment and cause infection.**

IMPORTANCE ***Vibrio cholerae*, the etiological agent of cholera disease, infects millions of people every year. *V. cholerae* El Tor and classical biotypes have been responsible for all cholera pandemics. The El Tor biotype responsible for the current seventh pandemic has displaced the classical biotype worldwide and is highly resistant to cationic antimicrobial peptides, like polymyxin B. This resistance arises from the attachment of one or two glycine residues to the lipid A domain of lipopolysaccharide, a major surface component of Gram-negative bacteria. Here, we identify the VprAB two-component system that regulates the charge of the bacterial surface by directly controlling the expression of genes required for glycine addition to lipid A. The VprAB-dependent lipid A modification confers polymyxin B resistance and contributes significantly to pathogenesis. This finding is relevant for understanding how *Vibrio cholerae* has evolved mechanisms to facilitate the evasion of the host immune system and increase bacterial fitness.**

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Vibrio cholerae is the etiological agent of cholera, a potentially lethal diarrheal disease that infects millions of people every year. There have been seven recorded cholera pandemics. The first six cholera pandemics were caused by the *V. cholerae* classical biotype. The current 7th pandemic is caused by the *V. cholerae* El Tor biotype that globally displaced the classical biotype during the end of the 20th century and represents the largest recorded evolutionary change in *V. cholerae* epidemiology (1, 2).

El Tor *V. cholerae* is endemic to many regions of the developing world, causing seasonal and sporadic outbreaks. *V. cholerae* lives in aquatic reservoirs and enters the human host orally through consumption of contaminated food or water. Following colonization of the small intestine, *V. cholerae* secretes cholera toxin, which causes the profuse and potentially lethal diarrhea characteristic of cholera disease (3). Modeling of cholera epidemics has implicated both environmental transfer and person-to-person transfer as important factors in disease spread (4, 5).

The human gastrointestinal tract possesses different barriers to impede colonization of pathogenic microorganisms such as *V. cholerae*. These include gastric acid barrier of the stomach, bile, and cationic antimicrobial peptides (CAMPs) produced by the host immune system (6, 7). While CAMPs vary in composition and structure, their bactericidal effect involves the electrostatic interaction between the peptide and the target outer membrane of Gram-negative bacteria (6, 8). This interaction allows the peptide to be inserted into the bilayer structure, leading to increased bacterial membrane permeability and ultimately bacterial lysis (8).

We previously reported that El Tor strains modify lipid A with glycine or diglycine residues to increase the net positive charge to prevent binding of CAMPs in *V. cholerae* (Fig. 1). In fact, glycine

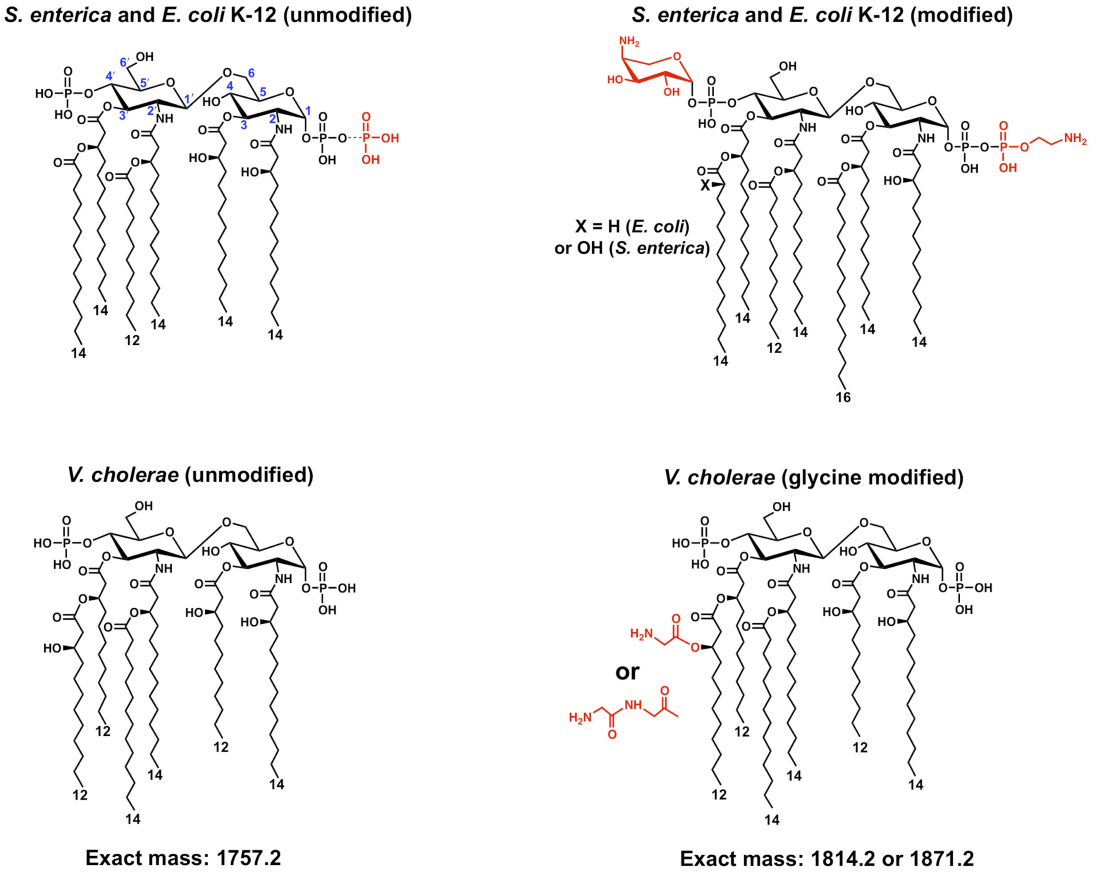

FIG 1 Comparison of the lipid A domains among *E. coli*, *S. enterica*, and *V. cholerae* LPS. The unmodified forms of *E. coli*, *S. enterica*, and *V. cholerae* lipid A are hexa-acylated and phosphorylated at the 1 and 4′ positions. The phosphate groups of *E. coli* and *S. enterica* lipid A can be modified with the amine-containing moieties L-4-aminoarabinose and phosphoethanolamine, shown in red. Also, a palmitate residue 16 carbons in length (shown in black) can be added to the 2 position, yielding a hepta-acylated structure. In *S. enterica*, a hydroxyl group is added to the 3′-linked secondary acyl chain at the 2 position, yielding a 2-OH myristate. *V. cholerae* can be modified with either glycine or diglycine residues attached to the 3-OH group of the secondary acyl chain attached at the 3′ position.

addition to the lipid A of O1 El Tor and O139 strains confers a high level of resistance to the CAMP polymyxin, whereas the O1 classical biotype, which lacks the glycine modification machinery, is sensitive to polymyxin (9). The difference was used to distinguish between the two pathogenic strains, but for 50 years the mechanism for this resistance was unknown. Our previous report identified 3 aminoacyl lipid modification (*alm*) genes, *almEFG*, which are required for glycine/diglycine addition in O1 El Tor strains and therefore responsible for their unique polymyxin resistance. These genes are cotranscribed, and disruption of any of the three results in an ~100-fold decrease in polymyxin resistance (9). Recently, our laboratory has elucidated the initial steps in the addition of glycine to lipid A via structural and biochemical studies (10). In summary, AlmF functions as a carrier protein and contains a phosphopantetheine residue. The *almE* gene encodes the glycine carrier ligase AlmE, which attaches a glycine residue to the phosphopantetheine group of AlmF, which in turn transfers the glycine moiety to the receptor protein AlmG. Ultimately, AlmG catalyzes the transfers of glycine to the hexa-acylated *V. cholerae* lipid A, promoting the resistance to polymyxin (see Fig. 7). Finally, expression of the *almEFG* system in the classical biotype results in a significant increase in polymyxin resistance. This demonstrates that lipid A modifications are evolving in ongoing bacterial pathogen pandemics and highlights the importance of examining lipid A structures.

Lipid A is synthesized via a well-conserved mechanism, known as the Raetz pathway (11). In model microorganisms such as *Salmonella* and *Escherichia coli*, the lipid A domain of LPS is a β-1′,6-linked disaccharide of glucosamine that is hexa-acylated and *bis*-phosphorylated. During growth in rich medium, a portion of lipid A contains a pyrophosphate group at the 1 position, yielding a *tris*-phosphorylated species (12, 13) (Fig. 1). The polysaccharide portion of LPS extends from the lipid anchor at the 6′ position of the distal sugar. In *E. coli* and *Salmonella*, modification of lipid A in response to CAMPs results in loss of the *tris*-phosphorylated lipid A species and addition of the positively charged residues phosphoethanolamine (pEtN) or L-4-aminoarabinose (Ara4N) (Fig. 1) (12, 14). Other modifications include the addition of a palmitate chain and, in *Salmonella*, the addition of a hydroxyl group to the 3′ secondary myristoyl chain (Fig. 1) (15–17).

To understand how *Vibrio* regulates its LPS structure and to reveal additional mechanisms of antimicrobial peptide resistance, we screened the O1 El Tor nonredundant transposon (Tn) library (18) for mutants with increased sensitivity to polymyxin B. From this analysis, a VC1320-VC1319 two-component system (TCS) was identified and shown to promote polymyxin resistance by

TABLE 1 MIC of polymyxin against *V. cholerae* strains

Mutated gene	Gene description	MIC (μg/ml)	
		Mutant	Complemented strain
None	Wild-type C6706	128	
vc0212	*lpxN*, lipid A biosynthesis, Kdo_2-(lauroyl)-lipid IV_A acyltransferase	1.5	128
vc0224	LPS biosynthesis glycosyltransferase	1.5	192
vc0239	LPS biosynthesis, putative glycosyltransferase	6	12
vc1319	*vprB*, sensor histidine kinase	2	96
vc1320	*vprA*, DNA binding response regulator	1.5	256
vc1578	*almF*, amino acid carrier protein F	0.5	12
vc1579	*almE*, amino acid ligase protein E	0.75	128
vc1981	Rhomboid family serine protease	16	128
vc2728	*gspI*, type II secretion system	8	48
vc2731	*gspF*, type II secretion system	32	128
vc2732	*gspE*, type II secretion system	1.0	128

controlling the regulatory pathway for lipid A modification. This VC1320-VC1319 (VprAB) TCS directly regulates expression of the *alm* operon, which encodes proteins essential for glycine modification of lipid A. Furthermore, mutants in lipid A glycinylation and its regulation display significantly reduced fitness during mammalian intestinal colonization. Despite a plethora of research on *E. coli* and *Salmonella* lipid A regulation, this is the first study to characterize the regulatory factors that control the *V. cholerae* lipid A structure. The VprAB TCS is distinct from those found in *E. coli* and *Salmonella*, and its role in endotoxin modulation promotes pathogenesis. This characterization identifies VprAB as a virulence regulatory system that functions alongside studied *V. cholerae* virulence networks such as the ToxR regulon (19). Beyond determining new virulence factors in *V. cholerae*, this work validates that LPS remodeling and its regulation play an important role in promoting bacterial survival in the mammalian host.

RESULTS

Identification of polymyxin-sensitive mutants in *V. cholerae* O1 El Tor. To identify the regulatory mechanisms required for antimicrobial resistance, we screened the *V. cholerae* O1 El Tor nonredundant transposon library for mutants increased in sensitivity to the CAMP polymyxin B (18). We identified mutants that were susceptible to subinhibitory concentrations of polymyxin (50 μg/ml) grown in Luria broth (LB) medium. Eleven transposon mutants exhibited limited growth (optical density at 600 nm [OD_{600}] of <0.2) after 24 h (Table 1). All identified mutant strains showed growth rates similar to that of the wild type in the absence of polymyxin B (data not shown). Further analysis determined the MIC using gradient polymyxin B E-strips in LB agar medium to confirm the antimicrobial peptide-related phenotype (Table 1 and Fig. 2). Wild-type El Tor (strain C6706) is highly resistant to polymyxin B, displaying a MIC of 128 μg/ml, whereas all selected

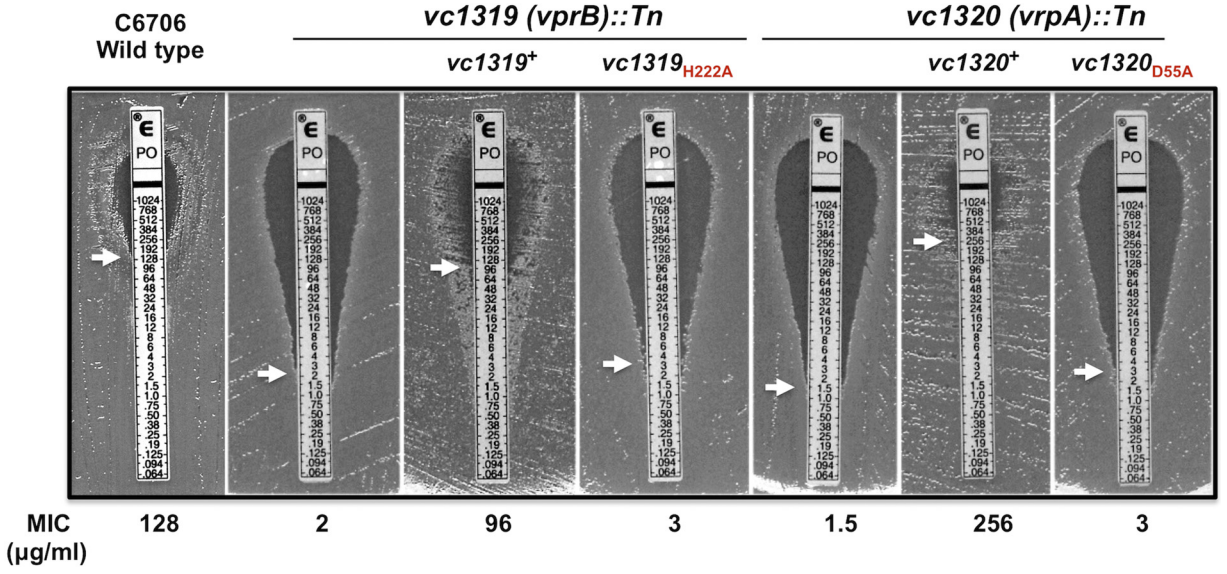

FIG 2 The *vc1320-vc1319* (vprA-vprB) two-component system plays a role in polymyxin resistance. The MIC of polymyxin was determined in candidate mutants carrying empty pWSK29 plasmid or pWSK29 constructs (see Table S2 in the supplemental material) expressing the wild-type (+) or mutated alleles (subscript) in *trans*. The MIC value is indicated with a white arrow. Data are representative of three biological repetitions.

mutants showed a drastic reduction in resistance (Table 1). Some of the identified genes were previously reported to be required for *V. cholerae* El Tor polymyxin resistance, thus validating our screening method (9, 20). For example, the screen identified *vc1579* (*almE*) and *vc1578* (*almF*) (Table 1), which are part of the *alm* operon (*vc1579-78-77*) and are required for the addition of glycine and diglycine residues to *V. cholerae* lipid A (Fig. 1; see also Fig. 7) (9). It is important to note that *vc1577* (*almG*) was not identified in this screen, because the Tn insertion occurs in the last 1% of the coding region, most likely resulting in a functional but truncated gene product. Additionally, the *vc0212* (*lpxN*) gene was identified (Table 1), which encodes the hydroxyacyltransferase responsible for the addition of a 3-hydroxylaurate (3-OH C12:0) to the primary acyl chain linked at the 3′ position of *Vibrio* lipid A (20). The 3-OH group of this secondary fatty acid serves as the attachment site for glycine/diglycine (Fig. 1) and is required for polymyxin resistance (9). Accordingly, these mutants exhibited drastic reduction in resistance to polymyxin (Table 1), as previously reported by our laboratory (MIC of 0.5 to 1.5 μg/ml) (9, 20).

Mutants in genes *vc2728* (*gspI*), *vc2731* (*gspF*), and *vc2732* (*gspE*) also exhibited sensitivity to polymyxin (Table 1). These genes are included in a transcriptional unit of 10 genes which encode components of the *V. cholerae* type II secretion system (T2SS) (21). Previous studies have demonstrated that *V. cholerae* T2SS mutants exhibit compromised outer membrane integrity and increased sensitivity to antimicrobial peptides (22). Additional transposon mutants include genes that have not been previously associated with polymyxin resistance. The gene *vc0224* encodes a putative LPS glycosyltransferase, and nearby the *vc0239* gene was also identified, with an 85- and 21-fold reduction in MIC, respectively (23). Analysis of LPS of these strains by SDS-PAGE indicated truncation of the core oligosaccharide of *V. cholerae* LPS (see Fig. S1 in the supplemental material). Complementation analyses confirmed that these genes contribute to polymyxin resistance (Table 1). Mutants with only partial complementation (*vc2728*, *vc0239*, and *vc1578*) may indicate that the transposon insertion induces polar effects on downstream genes.

The *vc1319* and *vc1320* genes identified in the screen are predicted to be cotranscribed in an operon according to ProOpDB analysis (23). The *vc1319* gene encodes a putative sensor histidine kinase and *vc1320* a putative response regulator, which suggests they comprise a two-component system (TCS). The *vc1320-vc1319* operon is widely conserved among *Vibrio cholerae* strains, including classical and El Tor biotypes (see Table S1 in the supplemental material). *vc1319* and *vc1320* mutants exhibited a 64- and 85-fold reduction in polymyxin B MIC values, respectively (Table 1, Fig. 2). Complementation of either mutation restored wild-type resistance or greater, indicating that each gene is required for resistance to polymyxin B (Table 1, Fig. 2).

The *vc1320-vc1319* operon is required for lipid A glycinylation. The addition of glycine or diglycine moieties to lipid A (Fig. 1) improves resistance to polymyxin by >100-fold (9). Our polymyxin sensitivity screen suggested a possible link between the *vc1320-vc1319* operon and the modification of lipid A. To evaluate this further, lipid A species from El Tor strain C6706 and derived strains were isolated and evaluated using matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry. Lipid A from the wild type exhibited a minor peak at *m/z* 1,757.1, characteristic of unmodified hexa-acylated lipid A in *V. cholerae* biotype El Tor, and predominant peaks at *m/z* 1,813.2 and 1,870.2 corresponding to glycine and diglycine addition to lipid A (Fig. 3A) (9). In the absence of functional genes *vc1319* or *vc1320*, mutant strains exhibited a major peak at *m/z* 1,755.6 and 1,756.1 (Fig. 3B and C) and loss of peaks representative of glycine-modified lipid A molecules. Both glycine addition and diglycine addition were observed in complemented strains (Fig. 3D). These results confirm that Vc1319 and Vc1320 are required for lipid A modification, and given their homology to a prototypical TCS, these proteins may regulate expression of the glycine modification machinery.

Vc1320-Vc1319 (VprA-VprB) directly regulates expression of the *almEFG* operon. TCSs form regulatory switches where a phosphotransfer reaction between two proteins, the histidine kinase and the response regulator, is required to regulate downstream events (24). We hypothesized that Vc1319-Vc1320 forms a cognate TCS that regulates polymyxin resistance, and therefore mutation of conserved amino acids predicted to be TCS phosphorylation sites should affect polymyxin resistance (24, 25). Thus, highly conserved residues in Vc1319-Vc1320 consistent with phosphotransfer activity were identified through multiple-sequence alignment with proteins in related TCS and mutated (see Fig. S2 in the supplemental material). Histidine 222 (H_{222}) of Vc1319 and aspartate 55 (D_{55}) of Vc1320 (see Fig. S2A and S2B) were both mutated to alanine, and polymyxin MIC assays were evaluated. In each case, expression of the single-site alanine variant allele at otherwise wild-type levels resulted in a 42-fold reduction in polymyxin B MIC (Fig. 2). These results, along with what has been described in other TCSs, indicate that H_{222} in the Vc1319 histidine kinase and D_{55} in the Vc1320 response regulator are the essential phosphorylation sites. This result suggests that Vc1320-Vc1319 is a signaling pair that promotes resistance to polymyxin, here named the VprA-VprB (*Vibrio* polymyxin resistance) two-component system.

To verify that VprAB regulates lipid A modification via the control of *almEFG* synthesis, we evaluated the relative expression of *almE* in different mutant backgrounds. Quantitative PCR (qPCR) analysis showed that expression of the *almE* transcript decreased 70-fold in the *vprA* mutant and 6.7-fold in the *vprB* mutant relative to *almE* expression in the wild type (Fig. 4A). The level of *almE* transcripts could be restored in both *vprB* and *vprA* mutants when the corresponding *vpr* gene was complemented in *trans*. While *almE* expression was only partially recovered, it was sufficient to provide resistance to polymyxin at wild-type levels (Fig. 2).

An essential step in defining a TCS is identifying genes it directly regulates. To evaluate whether the VprA response regulator directly binds to the promoter region of *almE* (P_{almE}), we generated a C-terminally His_6-tagged VprA protein (VprA-His_6) and assayed its ability to bind to P_{almE}. VprA-His_6 successfully complemented the *vprA* mutant, restoring polymyxin resistance (see Table S2 in the supplemental material), and was purified by nickel affinity chromatography (see Fig. S3 in the supplemental material). A ^{32}P-labeled nucleotide probe consisting of the ~130-bp upstream region of the *almE* gene (^{32}P-P_{almE}) was used for DNA binding experiments, including differential radial capillary action of ligand assay (DRaCALA) (26) and gel mobility shift assays (electrophoretic mobility shift assay [EMSA]).

DRaCALA measures protein-DNA binding interactions by measuring the radial capillary action of DNA spotted on a nitrocellulose membrane. DNA-protein complexes show less mobility

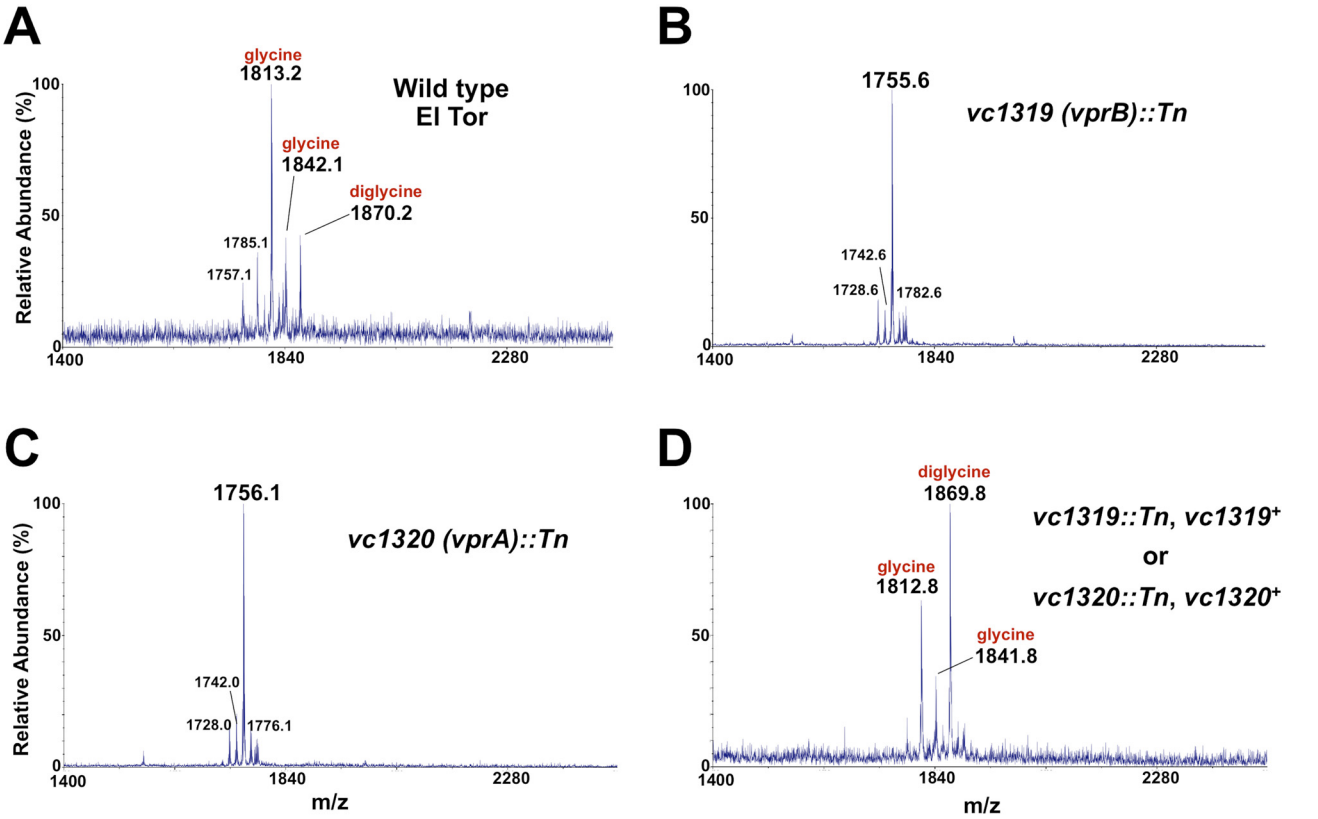

FIG 3 Glycine-lipid A modification is dependent on the VprA-VprB two-component system. Lipid A species from wild-type, *vprA*, *vprB*, *vprA*$^+$, and *vprB*$^+$ strains were analyzed by MALDI-TOF mass spectrometry. Both glycine (*m/z* 1,813.2)- and diglycine (*m/z* 1,870.2)-modified lipid A were present in the wild type (A); however, similar species were absent in *vprA* and *vprB* mutants. The major lipid A found in either *vprB* (*m/z* 1,755.6) (B) or *vprA* (*m/z* 1,756.1) (C) mutants is the hexa-acylated, *bis*-phosphorylated lipid A lacking glycine modifications. (D) Complementation restored glycine modification in both *vprA* and *vprB* mutants. Minor peaks at *m/z* 1,842.1 and 1,841.8 arise from glycine-modified lipid A having an acyl chain that is two additional carbons in length compared with that of the major observed ion.

on the membrane than unbound DNA. In our experiment, the mobility of ^{32}P-P_{almE} decreased as increasing concentrations of VprA-His_6 were added (Fig. 4B), indicating that VprA binds to the promoter region of *almE*. VprA binding to ^{32}P-P_{almE} was unaffected by nonspecific DNA and was minimized if 10-fold excess of nonlabeled P_{almE} was included in the assay (Fig. 4B).

Complex formation between the P_{almE} and VprA-His_6 was also verified by EMSA (Fig. 4C). VprA-His_6 retards the mobility of ^{32}P-P_{almE} in the gel mobility shift assay compared to ^{32}P-P_{almE} alone (Fig. 4C, lanes 1 and 2). The nature of this specific binding was confirmed by adding 10-fold-excess unlabeled P_{almE} to the assay. The unlabeled DNA competes for VprA-His_6 binding and therefore reduces the degree of ^{32}P-P_{almE} shifting (Fig. 4C, lane 3). A nonspecific sequence competitor was also used in 10-fold excess without affecting the formation of the VprA ^{32}P-P_{almE} complex (Fig. 4C, lane 4). Together, these results demonstrated that VprA directly binds to the promoter region of the *almEFG* operon concordant with the system's role in lipid A glycinylation.

Interestingly, in *Salmonella*, the lipid A modification regulatory pathway also includes genes that build or modify LPS structural components, like O-antigen polysaccharides and core (27–29). We therefore evaluated whether the VprAB TCS also influenced LPS assembly, as a truncation in the LPS structure might contribute to the observed phenotypes for polymyxin resistance and colonization. The LPS profile was compared between wild-type, *vprA*, *vprB*, and *almE* mutant strains using SDS-PAGE. Similar LPS migration patterns were observed in *vprA* and *vprB* mutants and in the wild type, suggesting that mutations in the VprAB regulatory pathway do not impact LPS assembly (see Fig. S4 in the supplemental material).

The VprA-VprB two-component system responds to polymyxin, bile, and mildly acidic pH. Two-component systems found in *S. enterica* and *E. coli* that are involved in LPS modification respond to different environmental signals, such as antimicrobial peptides, presence of metals (e.g., Mg^{2+}, Fe^{3+}), and changes in pH (30–32). Therefore, to identify environmental cues that the VprAB TCS responds to, the levels of *vprA* and *almE* transcripts were evaluated in *V. cholerae* grown in medium supplemented with specific extracellular signals. Wild-type cells grown in LB medium in the presence of a sublethal polymyxin B concentration (1 μg/ml) significantly upregulated *vprA* and *almE* transcripts, suggesting that in response to environmental CAMPs, El Tor cells can elicit a protective response (Fig. 5). Alternatively, when wild-type cells were exposed to divalent cations such as Ca^{2+} (10 mM) and Mg^{2+} (10 mM), *vprA* and *almE* synthesis were downregulated. Statistically significant changes were observed only in *almE* transcripts (see Fig. S5A in the supplemental material), suggesting that *V. cholerae* moderately responds to divalent

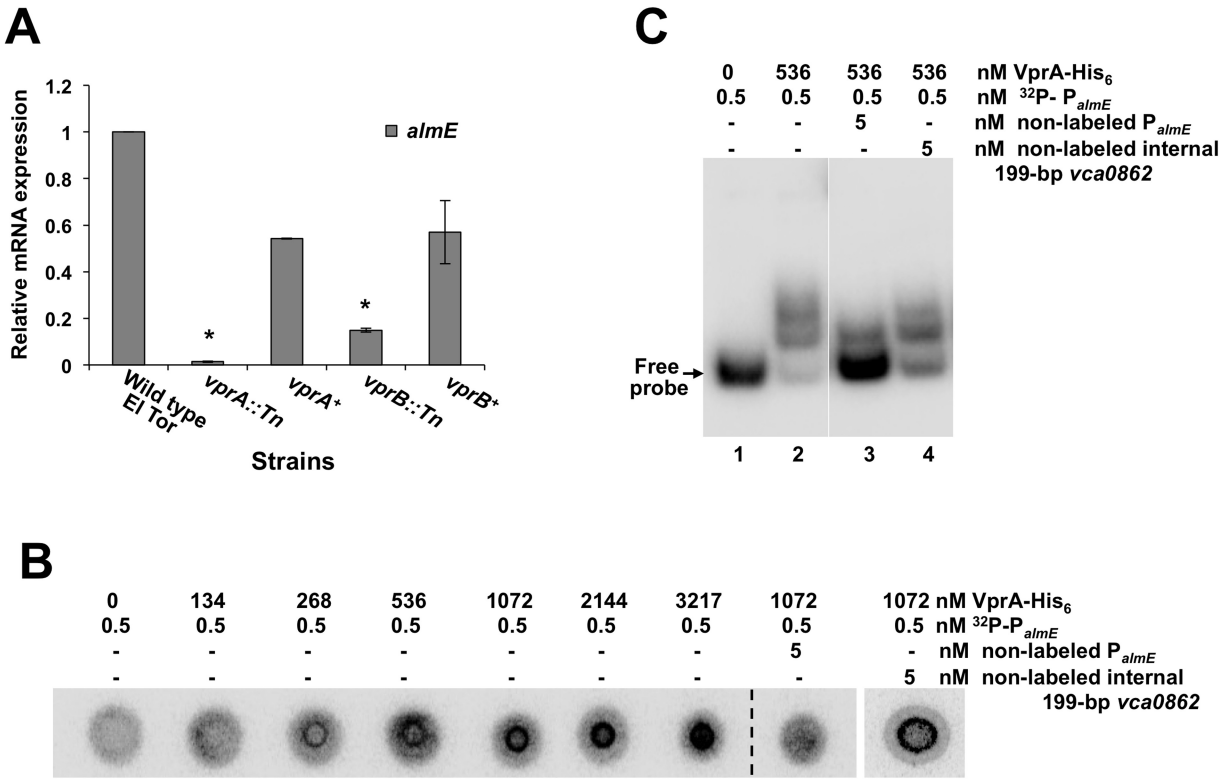

FIG 4 The VprA-VprB two-component system directly regulates *almE* synthesis. (A) The VprA-VprB two-component system is required for synthesis of the *almEFG* operon. Expression of *almE* was determined by qPCR in wild-type, *vprA*::Tn, *vprA*::Tn, *vprB*$^+$, and *vprA*$^+$ strains grown in LB. All cells were harvested during log phase, followed by total RNA extraction. The expression of each gene was normalized using *gyrA* as the reference gene. The data show the average from three biological repetitions. *, statistical significance was calculated using a *t* test ($P < 0.05$). (B and C) The transcriptional regulator VprA-His$_6$ binds the promoter of *almE* (P_{almE}). (B) Differential radial capillary action of ligand assay (DRaCALA) was used to demonstrate that increasing concentrations of VprA-His$_6$ promote binding to the P_{almE} region, restricting the radial complex dispersion of the ^{32}P-labeled promoter region of *almE* (^{32}P-P_{almE}). Total mobility of the double-stranded ^{32}P-P_{almE} without protein served as a negative control (far left). Reduction in protein-^{32}P-DNA binding is observed when a specific competitor is included in the reaction, but the DNA binding is restored in the presence of a nonspecific competitor (far right). (C) Electrophoretic mobility shift assay (EMSA). A VprA-His$_6$-P_{almE} binding complex is observed as a shifted migration (lane 2) compared to free probe (lane 1). A specific competitor decreased the observed shift intensity (lane 3), whereas a nonspecific competitor allowed formation of the DNA-protein complex (lane 4).

cations with a concomitant reduction in glycine-lipid A modification. Wild-type cells grown in the presence of iron Fe^{3+} (40 μM), however, did not show a significant change in the level of either *vprA* or *almE* transcripts (see Fig. S5A). With respect to pH, wild-type cells grown under mildly acidic conditions using pH 5.8-buffered LB medium showed a 3.0-fold decrease in *vprA* and *almE* synthesis compared to that of pH 7.0-buffered LB (Fig. 5). These observations suggest that the VprAB TCS may be responding to changes in the environment beyond antimicrobial peptides.

Conditions that mimic the lumen of the intestine are particularly relevant environmental cues to study in *V. cholerae*. Bile is a mixture of many molecules, such as saturated and unsaturated fatty acids, salts, and cholesterol, that is secreted into the lumen of the human intestine, aiding in the emulsification of lipids (33). The current model of *V. cholerae* virulence gene expression indicates that cells navigating the intestinal lumen, and therefore exposed to bile, consistently downregulate virulence gene expression (34, 35). It is not until the cells pass through the mucous layer coating the intestinal epithelia, and are therefore shielded from bile, that virulence gene expression is activated (34, 35). As *V. cholerae* has been shown to alter cell envelope components in response to bile, we examined the effect of crude bile on both *vprA* and *almE* regulation. Wild-type cells were exposed to a subinhibitory concentration of bile (0.2% bile) or specific bile salts. A strong downregulation of both *vprA* (5-fold) and *almE* (5.5-fold) transcripts was observed in the presence of crude bile or bile salts (Fig. 5). Further evaluation of cells lacking *vprA* or *vprB* genes confirmed that *almE* synthesis requires VprAB to respond to polymyxin, bile, and mildly acidic pH (see Fig. S5B, C, and D in the supplemental material). These data demonstrate that *vprAB* and *almEFG* operons are constitutively synthesized in rich medium, but key environmental cues can alter *almEFG* expression through a VprAB-dependent mechanism. Most significantly, the repression of *almE* in bile indicates it has a virulence gene expression profile and that the lipid A glycinylation system is a potential virulence factor *in vivo*.

Glycine-modified lipid A is required for intestinal colonization. Previous work has demonstrated that lipid A modification is an important virulence determinant for colonization of *Helicobacter pylori*, *Legionella pneumophila*, *Salmonella enterica*, and *Campylobacter jejuni* during mammalian infection (36–39). However, little is known regarding the role of *V. cholerae* lipid A modification in host colonization. To determine whether VprAB- and AlmE-dependent lipid A modifications are required for pathogen-

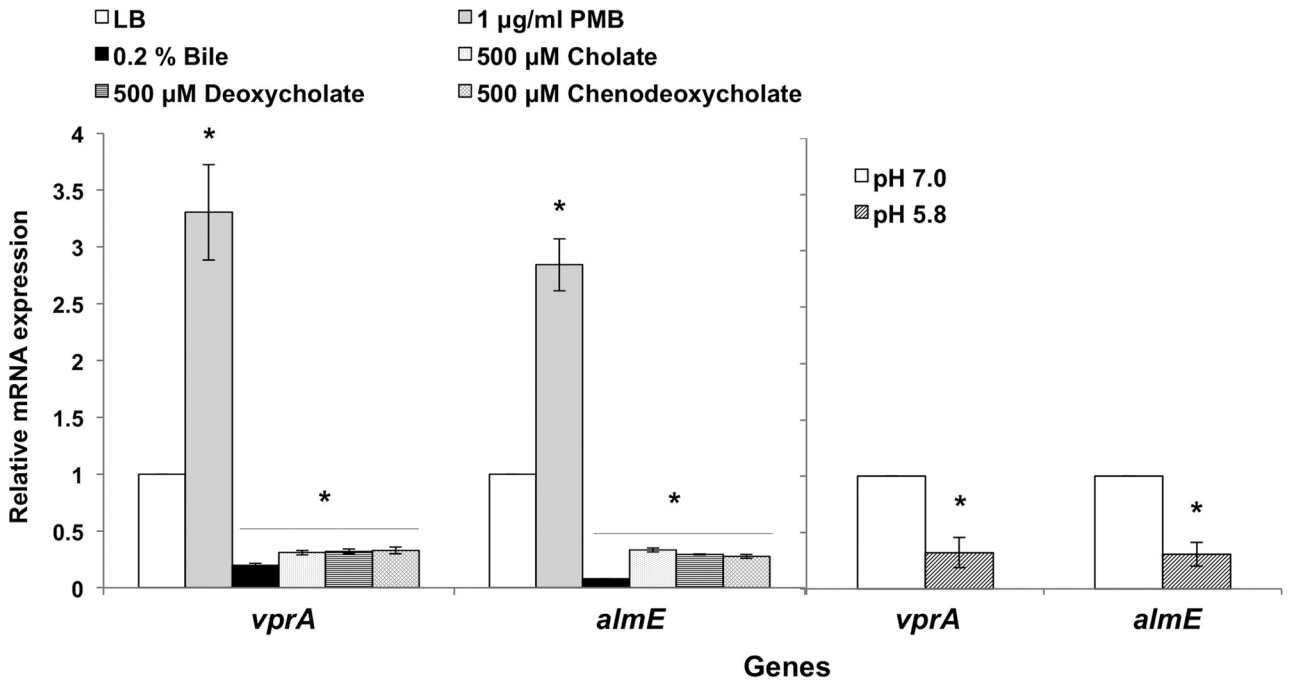

FIG 5 Changes in *vprA* and *almE* transcription during different growth conditions. Transcript levels of *vprA* and *almE* were determined in C6706 wild-type cells using qPCR. Cells were grown in LB medium or LB supplemented with 1 μg/ml polymyxin B, 0.2% bile, or specific bile components. For pH effect, buffered LB adjusted to pH 7.0 and 5.8 was used. Total RNA was isolated from cells at mid-exponential growth phase. Target gene expression was normalized to the *gyrA* gene. Error bars represent standard deviations from three biological replicates. *, statistical significance is indicated at *P* values of <0.05.

esis, the suckling mouse model was used to measure *V. cholerae* intestinal colonization. Transposon mutants of *vprA* and *almE* were competed against their wild-type background strain C6706 for their ability to colonize the suckling mouse small intestine. Remarkably, the *vprA* mutant exhibits a 34-fold fitness defect (*P* value of 2.6E−09), and even more striking, the *almE* mutant exhibited a 363-fold fitness defect (*P* value of 7.4E−17) in intestinal colonization (Fig. 6). These data demonstrate that the lipid A glycine modification machinery, as well as its regulating VprAB TCS, are important virulence factors *in vivo*.

Since different El Tor strains have shown various behaviors during mouse colonization (40), our *in vivo* competition assays were extended to the El Tor E7946 background. The *almEFG* operon was originally identified in the E7946 background, so examining the *in vivo* contribution of the newly identified VprAB TCS in that same background was of particular interest to us (9). We generated an unmarked nonpolar deletion in the transcriptional regulator, yielding the E7946 Δ*vprA* strain (see Table S3 in the supplemental material). The Δ*vprA* mutant exhibited a growth rate similar to that of the wild type and was sensitive to polymyxin (see Table S2 in the supplemental material), comparable to the C6706 *vprA*::Tn strain (Table 1). Also, the lipid A profile of the E7946 Δ*vprA* mutant showed complete loss of glycine/diglycine modification, identical to the C6706 *vprA*::Tn strain (Fig. 3). In mouse competition assays, the E7946 Δ*vprA* strain showed a 31-fold colonization defect (*P* value of 4.9E−10) (Fig. 6). For completeness, several attempts were made to obtain an unmarked deletion of *almE* or the complete *alm* operon in the E7946 background. However, we were unsuccessful after many attempts. Collectively, the colonization data support that VprAB-dependent regulatory mechanisms are involved in pathogenicity

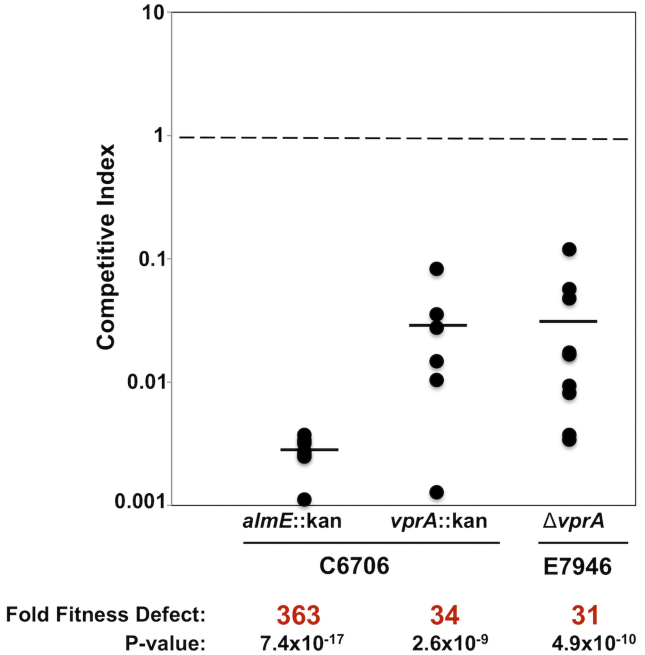

FIG 6 Glycine-dependent lipid A modification contributes to intestinal colonization. The ability of strains defective in glycine-lipid A modification to colonize the suckling mouse was evaluated by competition with the corresponding isogenic wild-type El Tor strain (E7946 or C6706). Each data point represents the ratio of mutant to wild-type bacteria after colonization of one mouse. A competitive index of 1, as indicated by a dashed line, is representative of no fitness defect. Significance ($P < 0.0001$) was determined by Student's *t* test.

and notably demonstrated the significance of *alm*-dependent lipid A modification during host colonization.

DISCUSSION

Despite elucidation of various antimicrobial peptide resistance mechanisms in bacteria, our knowledge of intrinsic and mutation-driven resistance of *Vibrio* to polymyxin is limited. We screened the El Tor C6706 ordered transposon mutant library for mutants sensitive to subinhibitory levels of polymyxin B. Our screen identified several genes previously known to participate in polymyxin B resistance and also revealed a number of uncharacterized genes. Many of the genes identified (*almE*, *almF*, *lpxN*, *vc0239*, and *vc0224*) are known to contribute to LPS biosynthesis and modification. Other genes (*vc2728*, *vc2731*, and *vc2732*) were part of the T2SS and are known to contribute to membrane stability (22, 41, 42). This finding is consistent with the importance of regulating LPS structure to maintain membrane stability, particularly in the presence of CAMPs (31, 43, 44). The gene *vc1981* was also identified and is a member of the rhomboid protease family showing structural similarity to *E. coli* GlpG. However, the role this protease plays in polymyxin resistance is unknown and is under investigation by our laboratory.

Our screen identified only a small repertoire of genes (Table 1) important for polymyxin B resistance, consistent with an equally limited number of polymyxin resistance determinants found in *Pseudomonas* (45). The screened mutant library does not include essential genes, however, and such genes likely contribute to the polymyxin resistance phenotype. Additionally, some transposon insertions may disrupt only nonessential domains of the gene (9). Although the mutant library screen has some limitations, it is remarkable how few genes contribute to polymyxin resistance. This may indicate the importance of the VprAB regulatory system as the main protective response to CAMPs. Also, glycinylation of lipid A is unique compared to the well-studied amino-arabinose modification system observed in *Salmonella*, *E. coli* (16, 46), *Pseudomonas* (47), *Yersinia* (48), *Burkholderia* (49), and *Bordetella* (50). Interestingly, glycine modification is found in the El Tor O1 strains dominating the current cholera pandemic but not in the classical *Vibrio* biotype, indicating that lipid A remodeling may be under evolutionary selection pressure in ongoing contagions.

This study identified a new two-component regulatory system, named VprAB, involved in antimicrobial peptide resistance. It is notable that this is the only putative TCS that was detected in this screen. The requirement of a phosphotransfer reaction between a sensor protein and its cognate response regulator (24, 25) offers support to the function of VprAB as a potential TCS. Supported by the fact that these residues are highly conserved among histidine kinase and response regulator orthologs (25) (see Fig. S2 in the supplemental material), we identified the amino acids involved in phosphorylation in $VprA_{D55}$ and $VprB_{H222}$ proteins, which were subsequently mutated to alanine (see Fig. S2). Expression of these mutated proteins, $VprA_{D55A}$ and $VprB_{H222A}$, resulted in the inability to restore the polymyxin resistance phenotype (Fig. 2). Although additional biochemical studies will be required, these facts strongly support the proposed functional association between VprA and VprB as cognate partners of a signal transduction system. Notably, no additional response regulators that could potentially integrate with the *vprAB* regulatory circuitry were identified in our polymyxin sensitivity screen (Table 1).

We demonstrated that VprAB is required for synthesis of the *almEFG* operon, whose gene products are responsible for glycine modification (9). The *almE* transcript, the first gene of the operon, is drastically downregulated in *vprA* and *vprB* mutants (Fig. 4A). A minimal expression level of *almE* transcript is observed in the *vprB* mutant. In this scenario, it is possible that VprA is autophosphorylated in the absence of its cognate kinase, using the small molecule phosphodonor acetyl phosphate (25, 51, 52). However, VprA autophosphorylation is probably low compared to the phosphorylation by its cognate sensor, resulting in both decreased *almE* synthesis and polymyxin resistance (Fig. 2). It is tempting to speculate that a minor level of phosphorylated VprA is required to control additional target genes under specific physiological conditions. For instance, VprAB TCS is conserved among *Vibrio* species (see Table S1 in the supplemental material); however, its role in polymyxin resistance in other species is unknown.

By complementation analysis, VprA and VprB expression in *trans* restored partial *almE* synthesis compared to the wild type (Fig. 4A). Although this lower level of transcription might be an effect of the low-copy-number plasmid, it was a sufficient amount of *vprA*-dependent *almE* synthesis to restore polymyxin resistance (Fig. 2). We further provide biochemical evidence that establishes a direct mechanistic link between VprA and its regulation of AlmE expression. VprA binds to the promoter of the *almEFG* operon (Fig. 4B and C), and mass spectrometry analysis showed that mutants of VprAB TCS are defective in their ability to modify their lipid A structure with glycine moieties (Fig. 3). Overall, this study establishes that the VprAB TCS positively regulates the *almEFG* operon to control glycine-lipid A modification (Fig. 7).

Vibrio typically inhabits aquatic ecosystems, most often associated with aquatic fauna before infection of the human host. Due to this wide range of conditions, *Vibrio* likely has evolved adaptive responses to various extracellular cues in order to promote survival and overall fitness during its infection cycle (53–55). Host environmental cues that regulate lipid A modification in *Vibrio cholerae* are relatively unknown. Therefore, our evaluation included some cues known to regulate lipid A modification in other microorganisms that might regulate the expression of *vprAB* and *almEFG*. Our finding reveals that *vprA* and *almE* are expressed constitutively in wild-type cells, and their levels of synthesis are significantly altered in the presence of specific stimuli. El Tor *V. cholerae* may maintain a basal level of glycinylated lipid A on their surfaces but still respond to CAMP-directed membrane disruption by upregulating VprAB-dependent genes.

Changes in pH are well-known environmental signals for bacteria (56, 57). Mildly acidic pH activates PhoPQ and PmrAB TCSs, promoting modification of lipid A (30, 58) in *S. enterica*. *Vibrio* is sensitive to growth in an acidic environment (pH 2.5) (59) but exhibits an adaptive response to survive in mildly acidic pH (pH 4.5) (60, 61). Bile is also a known environmental signal that downregulates the production of virulence factors in *Vibrio cholerae* (62–65). Transcription of *vprA* decreases in response to mildly acidic pH 5.8 and to 0.2% bile. Further, similar levels of downregulation are observed for *almE* transcription (Fig. 5). These transcriptional profiles support VprAB and AlmEFG as virulence factors in *V. cholerae*.

This is the first study to identify a lipid A modification regulatory pathway in *V. cholerae* and, importantly, to establish a link between lipid A modification and pathogenesis. Particularly, the recently identified *almEFG* lipid A modification operon has a striking contribution to colonization *in vivo*. The reduced intesti-

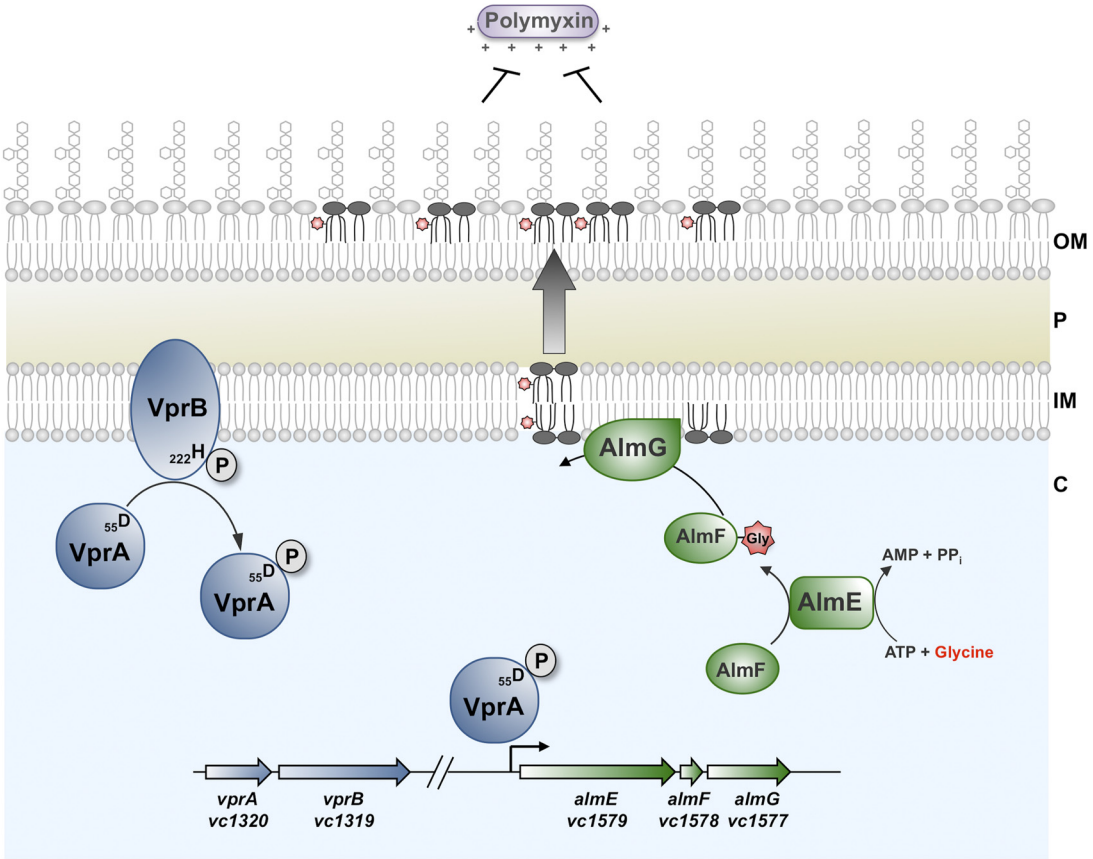

FIG 7 A model illustrating the regulation of lipid A modification of *V. cholerae* El Tor. The VprA-VprB two-component system is expressed in an operon. VprB likely functions as a sensor histidine kinase, which upon stimulation autophosphorylates at residue histidine ($_{222}$H) and transfers the phosphate group (represented by P) to a conserved aspartate residue ($_{55}$D) on the transcriptional regulator VprA. Phosphorylated VprA directly promotes expression of the *almEFG* operon. AlmE serves as an amino acid ligase and ligates a glycine residue (red star) to the small carrier protein AlmF. AlmF-glycine serves as the donor for the lipid A-modifying enzyme AlmG. Presumably, glycine addition increases the net positive charge of the bacterial surface providing resistance to the cationic antimicrobial peptide polymyxin. The diglycine (Fig. 1) likely arises from two successive additions of glycine to lipid A by AlmG. The addition of core oligosaccharide and O-antigen are not shown for simplicity. OM, outer membrane; P, periplasm; IM, inner membrane; C, cytoplasm.

nal colonization exhibited by *vprA* and *almE* mutants in two El Tor strains (Fig. 6) suggests that VprAB-dependent lipid A glycine modification is important for El Tor colonization of the mammalian host. The VprAB regulatory system likely regulates the charge of the cell surface to help protect against the host immune system, including CAMPs. Identifying other VprAB-regulated genes and their role in pathogenesis will be an exciting next step in studying *V. cholerae* pathogenesis.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *V. cholerae* O1 El Tor wild-type strains E7946 and C6706 and derived strains are listed in Table S3 in the supplemental material. *Vibrio* strains were grown in LB medium (Difco) or in LB supplemented with 0.2% Ox-bile (Sigma), 500 μM cholate, 500 μM deoxycholate, 500 μM chenodeoxycholate, 10 mM $CaCl_2$, 10 mM $MgCl_2$, or 40 μM $FeSO_4$, where applicable. When buffered LB was used, LB containing 100 mM HEPES or 100 mM Bis-Tris was adjusted to pH 7.0 or 5.8, respectively. *V. cholerae* was also grown in G56 minimal medium containing 45 mM HEPES (pH 7.5), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.2% glucose, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 0.015 mM $FeSO_4$, 0.075 mM thiamine, and 0.15 mM KH_2PO_4 (20, 66). In all cases, cells were grown with aeration at 37°C. *E. coli* strains were grown in LB medium. Antibiotics streptomycin (100 μg/ml), kanamycin (30 μg/ml), and ampicillin (100 μg/ml) were used when appropriate.

Standard DNA methods. Genomic DNA was extracted with an Easy DNA kit (Invitrogen). Plasmid DNA was obtained with a QIAprep Spin miniprep kit (Qiagen). PCR products were obtained using *Pfu* Turbo DNA polymerase (Stratagene) or TaKaRa *Ex Taq* DNA polymerase (TaKaRa). PCR products were purified from an agarose gel using a QIAquick PCR gel extraction kit (Qiagen). Custom DNA oligonucleotides (see Table S4 in the supplemental material) were purchased from Sigma-Aldrich. Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs Inc. (NEB). The QuikChange site-directed mutagenesis system (Stratagene) was used to introduce *in vitro* point mutations per the manufacturer's instructions. Nucleic acid quantification was carried out using NanoDrop 8000 (Thermo Scientific).

Plasmid constructs. For complementation analysis, the open reading frames containing an additional 20 bp upstream were amplified using primers indicated in Table S4 in the supplemental material and C6706 genomic DNA as the template. Most of the PCR products were digested with XhoI and XbaI restriction enzymes. In the case of *vc1319* (*vprB*), the PCR product was digested with EcoRI and BamHI, whereas the *vc1320* (*vprA*) reaction product was digested with HindIII and KpnI. Each of the digested PCR fragments was cloned into the low-copy-number plasmid pWSK29 (67). For the *vc2731* and *vc2732* genes, the open reading frames

carrying a ribosomal binding site from pET21a were cloned into pWSK29 at the BamHI and EcoRI sites. The resulting plasmids (see Table S3 in the supplemental material) were introduced into their respective *Vibrio* mutant backgrounds by electrotransformation.

For VprA-His_6 protein expression, the coding region of the *vprA* gene was amplified using primers vc1320-HisF-R (see Table S4) and C6706 genomic DNA as the template. The resulting 750-bp PCR product was digested with NdeI and HindIII restriction endonucleases and cloned into the expression plasmid pET21a (see Table S3). The generated plasmid pET-1320 results in expression of VprA with a C-terminal His_6 tag.

A DNA fragment encoding the C-terminally His-tagged VprA and carrying its native ribosome binding site (RBS) was generated using primers vprA-KpnIF and vprAHis-Hind3R (see Table S4). The PCR product was digested with KpnI and HindIII endonucleases and ligated into the medium-copy-number plasmid pBAD24, resulting in the plasmid pBAD-VprA. Expression plasmids pW1319 and pW1320 were subsequently modified by site-directed mutagenesis using the QuikChange kit (Stratagene) with primers listed in Table S4 in the supplemental material.

For construction of a suicide vector containing the unmarked deleted *vprA* gene, E7946 genomic DNA was used as the template followed by the splicing by overhang extension (SOE) PCR method (68). Approximately 500-bp flanking regions of *vprA* were amplified by PCR using upvprAF-R and downvprAF-R primers (see Table S4). The gene deletion included 675 bp of the 705 bp of the *vprA* coding region, retaining 12 bp at the 5′ end and the last 18 bp at the 3′ end. Upstream and downstream PCR products were annealed and amplified by SOE PCR using upvprAF-downvprAR primers (see Table S4). The resulting ~1,000-bp amplicon was subcloned into pGEM-T Easy vector (Promega). The DNA fragment containing the unmarked deleted gene was digested with ApaI and SpeI restriction enzymes and ligated into the counterselectable suicide plasmid pMW91 (69), generating the pWMvprA plasmid (see Table S3).

Construction of *vprA* deletion mutants. To construct *Vibrio cholerae* strains harboring the unmarked deletion of *vprA* (see Table S3), the suicide vector pWMVprA was conjugated from SM10 λpir into the E7946 strain as previously described (69, 70). Clean deletions were confirmed by PCR using genomic DNA from candidate mutants.

Expression and purification of C-terminal VprA-His_6 protein fusion. For overexpression of VprA-His_6, the *E. coli* host strain HMS174(DE3) carrying the plasmid pET-1320 was grown in LB broth supplemented with ampicillin. During exponential growth (OD_{600} of 0.6 to 0.7), 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to induce the expression of VprA-His_6. After 2 h of induction at 37°C, cells were harvested at 10,000 × *g* for 10 min at 4°C followed by one wash in 1× PBS buffer (pH 7.4). The resulting pellet was resuspended in cold binding buffer (20 mM HEPES [pH 7.4], 0.5 M NaCl, 20 mM imidazole) supplemented with complete EDTA-free protease inhibitor cocktail tablets (Roche). Cells were lysed by three cycles of French pressure at 30,000 lb/in^2 using a chilled pressure cell. The resulting crude cell extract was centrifuged at 10,000 × *g* for 15 min at 4°C. The cell extract supernatant was filtered through a 0.45-μm-pore-size filter (Millipore), transferred to a 1.0-ml HisTrap-FF affinity column (GE Healthcare), and purified using an AKTA FLPC purification system (GE Healthcare). Target protein was eluted with a linear gradient of elution buffer (20 mM HEPES, 0.5 M NaCl, 500 mM imidazole, pH 7.4). The resulting fractions containing purified protein were dialyzed against 20 mM HEPES (pH 7.4) and concentrated using an Amicon Ultra-15 centrifugal filter device with a 3,000-molecular-weight cutoff (Millipore). Proteins were evaluated using 4 to 12% NuPAGE Bis-Tris gels (Invitrogen) and visualized by Coomassie blue staining.

DNA-protein interaction assays. The 130-bp promoter region of *almE* (P_{almE}) was obtained by PCR amplification using primers Prom1579F-R (see Table S4 in the supplemental material) and C6706 genomic DNA as the template. This product was gel purified and digested at the 5′ end with EcoRI. The digested probe was purified using phenol:chloroform (71). Five pmol of EcoRI-digested probe was dephosphorylated and labeled with [γ-^{32}P]ATP (Amersham) using a DNA 5′-end-labeling system (Promega). Removal of unincorporated nucleotides was carried out using NucAway spin columns (Ambion, Inc.). The labeled product was diluted 1:10 for the binding assay. Likewise, probe without labeling was used as a specific competitor. For a nonspecific competitor, a region of 199 bp was obtained by PCR amplification using primers vca0862F-R (see Table S4). For cold competition, a 10-fold molar excess of unlabeled probe was added to the binding reaction.

The differential radial capillary action of ligand assay (DRaCALA) (26) reaction was performed by mixing VprA-His_6 protein in modified binding buffer containing 5 mM HEPES (pH 7.9), 40 mM KCl, 1 mM dithiothreitol (DTT), 0.1 mM EDTA, and 5% glycerol (72), preincubated for 10 min at room temperature before addition of 0.5 nM ^{32}P-DNA probe. The unlabeled DNA probe was included when necessary. A total of 20 μl of binding reaction mixture was incubated for 10 min before a 5-μl aliquot was loaded onto a nitrocellulose membrane. The dried membrane was exposed to a phosphorimager screen to visualize DNA binding. Binding reactions used for DRaCALA were also used for EMSA. A total of 10 μl of binding reaction was loaded onto a 6% retardation gel (Invitrogen), and the gel ran for 13 min at 300 V at a temperature lower than 30°C.

Total RNA isolation and quantitative RT-PCR. Overnight cell cultures were diluted 1:100 in LB medium and grown to an OD_{600} of 0.5. Cells were harvested, washed, diluted in fresh LB medium to an OD_{600} of 0.1, and grown to an optical density of 0.5. Cells were harvested, and total RNA was extracted with the SV Total RNA isolation system (Promega) followed by RQ1 RNase-free DNase (Promega) treatment to eliminate residual DNA contamination. cDNA synthesis was performed with the high-capacity cDNA reverse transcription (RT) kit (AB Applied Biosystems). The quantification of target gene by quantitative PCR (qPCR) was performed using 2× SYBR green PCR master mix (AB Applied Biosystems) and specific primers for each transcript (see Table S4 in the supplemental material). Data analysis was performed using the ABI 7900HT Fast real-time PCR system and Software Sequence Detection Systems (SDS) version 2.4 (AB Applied Biosystems). The relative expression ratio of the target transcript was calculated in comparison to the *gyrA* transcript as the reference gene following the Pfaffl method (73).

Screening for polymyxin-susceptible mutants and MIC determination. A 96-well microtiter plate assay was used to establish mutant susceptibility to polymyxin B. Each well, containing 200 μl of LB medium and supplemented with 50 μg/ml polymyxin, was inoculated by replica plate by using the C6706 nonredundant transposon insertion library (a total of 3,156 mutants) (18). Plates were incubated at 37°C with shaking at 250 rpm. After 16 h, the OD_{600} was read using a microplate reader (BioTek). Candidate mutants with decreased growth, determined by an OD_{600} of <0.2, were considered susceptible to polymyxin. The MIC was determined using Etest gradient polymyxin B strips (AB Biodisk). For this purpose, cells were grown overnight followed by a dilution of 1:100 in LB medium. Cells were grown to mid-exponential growth and diluted 1:10, and an aliquot was plated on LB agar. For complementation analysis, cells were plated on LB agar containing proper antibiotics and 0.2% arabinose when necessary. A polymyxin B Etest gradient strip was applied onto inoculated plates and incubated at 37°C, and the MIC was evaluated after 16 h.

Isolation of lipid A and mass spectrometry analysis. Cultures were grown at 37°C until an OD_{600} of 0.8 to 1.0. Bacteria were harvested at 10,000 × *g* for 10 min. Lipid A extraction was carried out by mild acidic hydrolysis as previously described (20, 74). Lipids were analyzed using a MALDI-TOF/TOF (ABI 4700 Proteomic Analyzer) mass spectrometer in the negative-ion linear mode as previously described (12).

***In vivo* colonization assay.** All animal studies were carried out under protocols approved by the Institutional Animal Care and Use Committee at the University of Texas at Austin in accordance with NIH guidelines. The suckling mouse colonization competition assay was performed with El Tor and derived mutant strains as previously described (75). Briefly, a 1:1 mixture of approximately 5 × 10^4 CFU mutant (*lacZ*$^-$) and 5 ×

10^4 CFU C6706 wild-type (*lacZ*$^+$) bacteria was intragastrically inoculated into the stomach of 5- to 6-day-old CD1 suckling mice (Charles River). An aliquot of the inoculum was serially diluted and plated onto selective LB agar containing streptomycin and 20 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) to verify the input ratio. After 16 h of colonization, the small intestine was removed and homogenized. Aliquots of the homogenates were plated on selective LB agar and incubated at 37°C, followed by the evaluation of the output ratio after 16 h. Competition index (CI) and statistical significance were determined as previously described (76).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.02283-14/-/DCSupplemental.

Figure S1, DOCX file, 0.4 MB.
Figure S2, DOCX file, 0.3 MB.
Figure S3, DOCX file, 0.3 MB.
Figure S4, DOCX file, 0.4 MB.
Figure S5, DOCX file, 0.2 MB.
Table S1, DOCX file, 0.01 MB.
Table S2, DOCX file, 0.01 MB.
Table S3, DOCX file, 0.03 MB.
Table S4, DOCX file, 0.02 MB.

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