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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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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.

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This article is a direct contribution from a Fellow of the American Academy of Microbiology.

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*

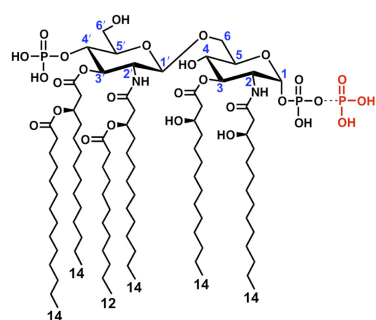
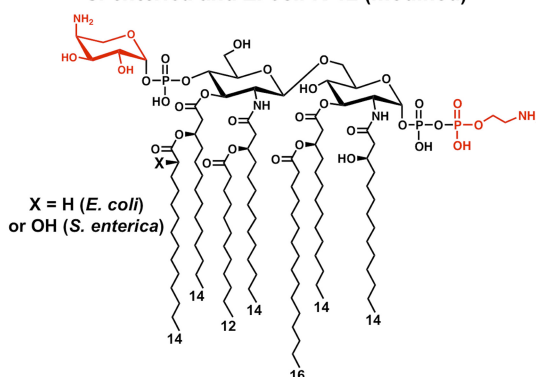
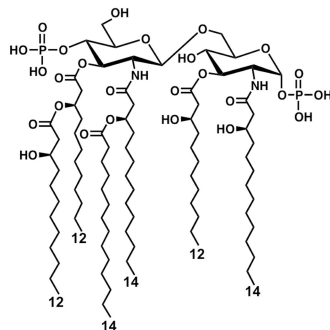
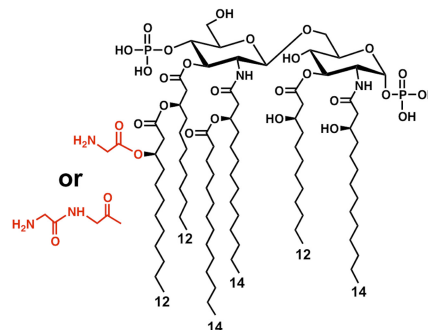
S. enterica* and *E. coli* K-12 (unmodified)**S. enterica* and *E. coli* K-12 (modified)*****V. cholerae* (unmodified)****Exact mass: 1757.2*****V. cholerae* (glycine modified)****Exact mass: 1814.2 or 1871.2**

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 bac-

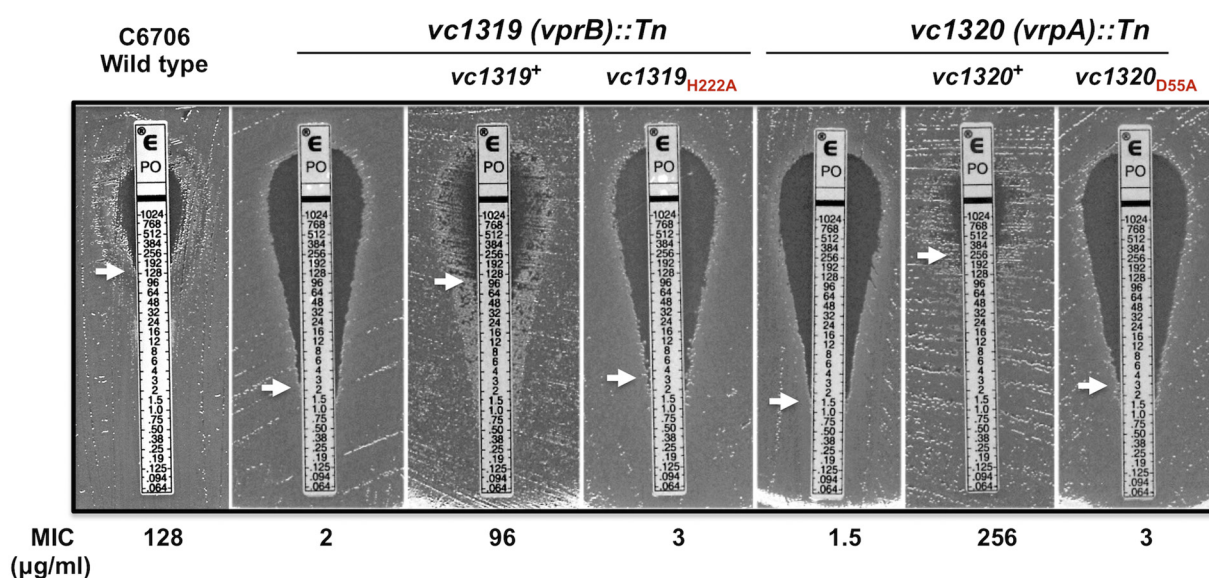
TABLE 1 MIC of polymyxin against *V. cholerae* strains

Mutated gene	Gene description	MIC ($\mu\text{g/ml}$)	
		Mutant	Complemented strain
None	Wild-type C6706	128	
<i>vc0212</i>	<i>lpxN</i> , lipid A biosynthesis, Kdo ₂ -(lauroyl)-lipid IV _A acyltransferase	1.5	128
<i>vc0224</i>	LPS biosynthesis glycosyltransferase	1.5	192
<i>vc0239</i>	LPS biosynthesis, putative glycosyltransferase	6	12
<i>vc1319</i>	<i>vprB</i> , sensor histidine kinase	2	96
<i>vc1320</i>	<i>vprA</i> , DNA binding response regulator	1.5	256
<i>vc1578</i>	<i>almF</i> , amino acid carrier protein F	0.5	12
<i>vc1579</i>	<i>almE</i> , amino acid ligase protein E	0.75	128
<i>vc1981</i>	Rhomboid family serine protease	16	128
<i>vc2728</i>	<i>gspI</i> , type II secretion system	8	48
<i>vc2731</i>	<i>gspF</i> , type II secretion system	32	128
<i>vc2732</i>	<i>gspE</i> , 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 glycylation 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 $\mu\text{g/ml}$) grown in Luria broth (LB) medium. Eleven transposon mutants exhibited limited growth (optical density at 600 nm [OD₆₀₀] 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 $\mu\text{g/ml}$, whereas all selected



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 $\mu\text{g/ml}$) (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 glycylation. 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 *almE*

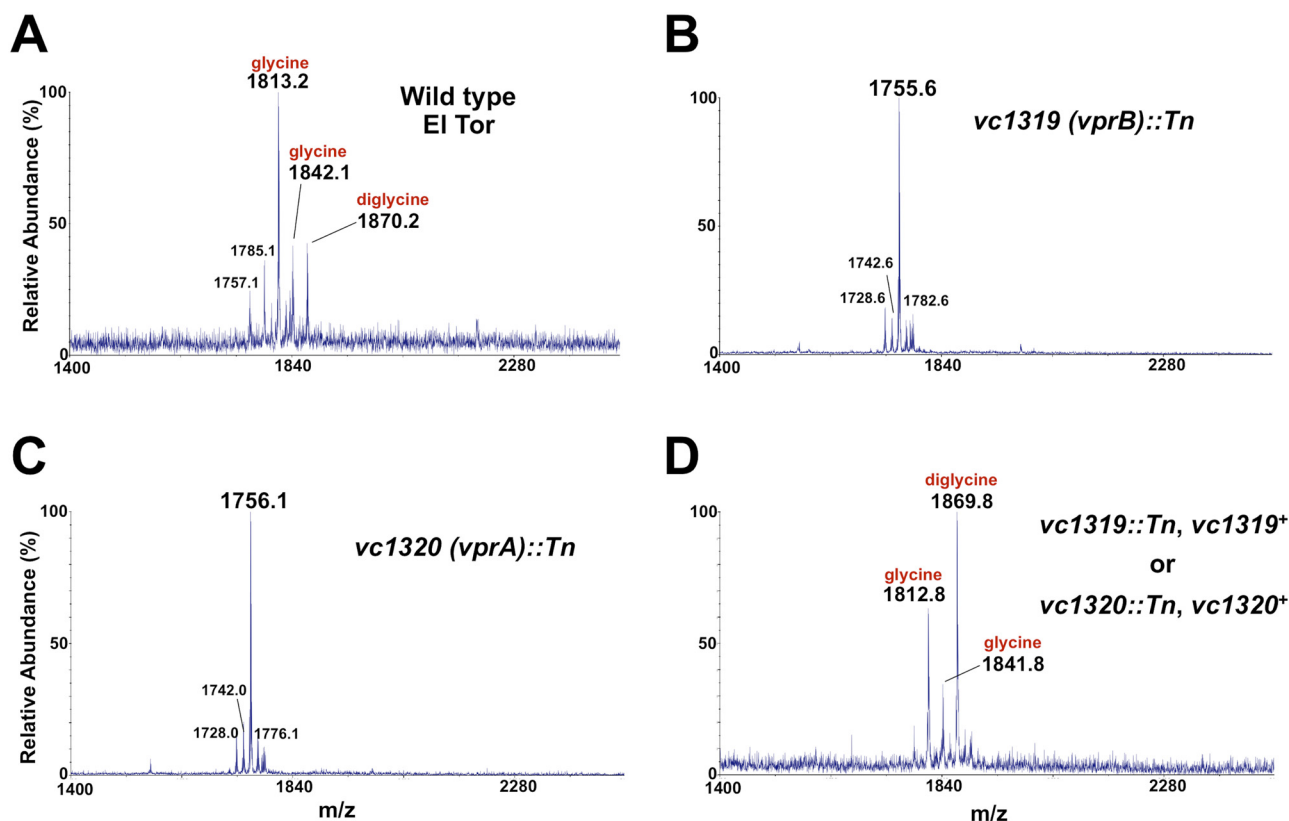


FIG 3 Glycine-lipid A modification is dependent on the VprA-VprB two-component system. Lipid A species from wild-type, *vprA*, *vprB*, *vprA*⁺, and *vprB*⁺ strains were analyzed by MALDI-TOF mass spectrometry. Both glycine (*m/z* 1,813.2)- and diglycine (*m/z* 1,870.2)-modified lipid A were present in 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 ³²P-*P*_{almE} decreased as increasing concentrations of VprA-His₆ were added (Fig. 4B), indicating that VprA binds to the promoter region of *almE*. VprA binding to ³²P-*P*_{almE} was unaffected by nonspecific DNA and was minimized if 10-fold excess of unlabeled *P*_{almE} was included in the assay (Fig. 4B).

Complex formation between the *P*_{almE} and VprA-His₆ was also verified by EMSA (Fig. 4C). VprA-His₆ retards the mobility of ³²P-*P*_{almE} in the gel mobility shift assay compared to ³²P-*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-His₆ binding and therefore reduces the degree of ³²P-*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 ³²P-*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 glycylation.

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 resis-

tance 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., Mg²⁺, Fe³⁺), 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 μ

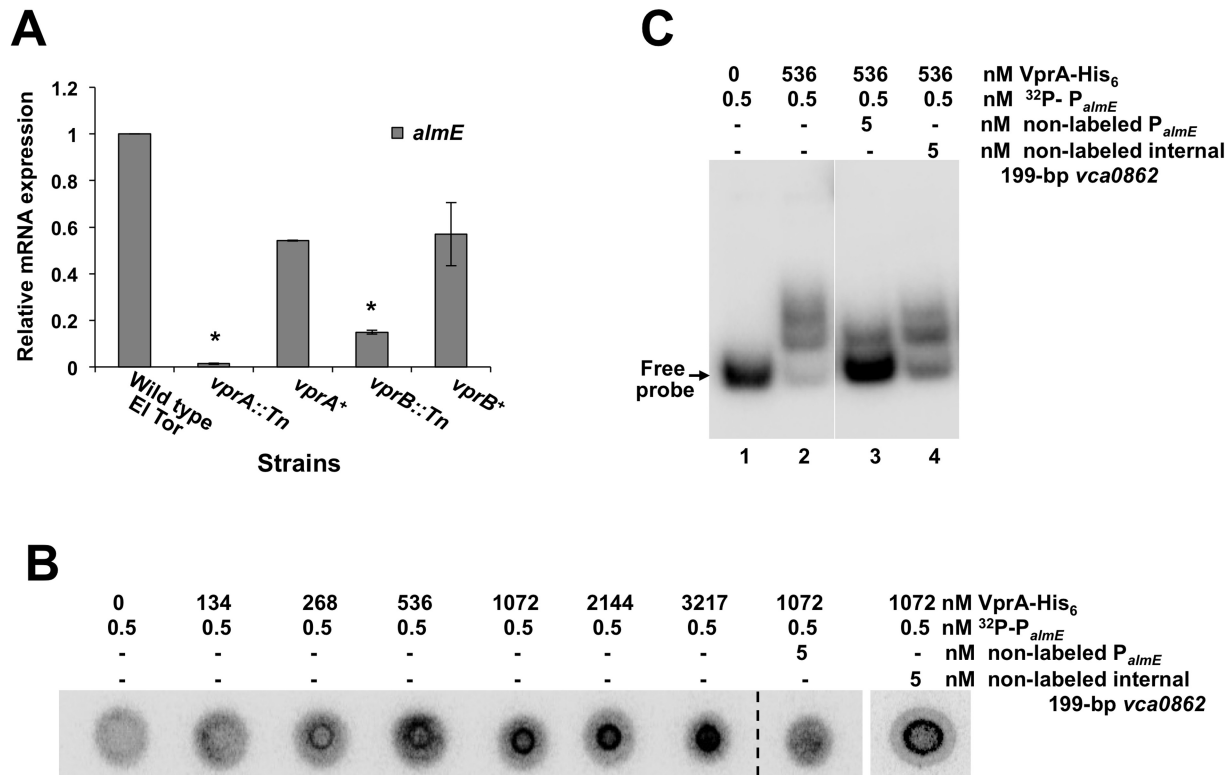


FIG 4 The VprA-VprB two-component system directly regulates *almE* synthesis. (A) The VprA-VprB two-component system is required for synthesis of the *almEFG* operon. Expression of *almE* was determined by qPCR in wild-type, *vprA::Tn*, *vprA::Tn*, *vprB*⁺, and *vprA*⁺ strains grown in LB. All cells 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-His₆ 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-His₆ promote binding to the P_{almE} region, restricting the radial complex dispersion of the ³²P-labeled promoter region of *almE* (³²P- P_{almE}). Total mobility of the double-stranded ³²P- P_{almE} without protein served as a negative control (far left). Reduction in protein-³²P-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-His₆- 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 Fe³⁺ (40 μ M), 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*

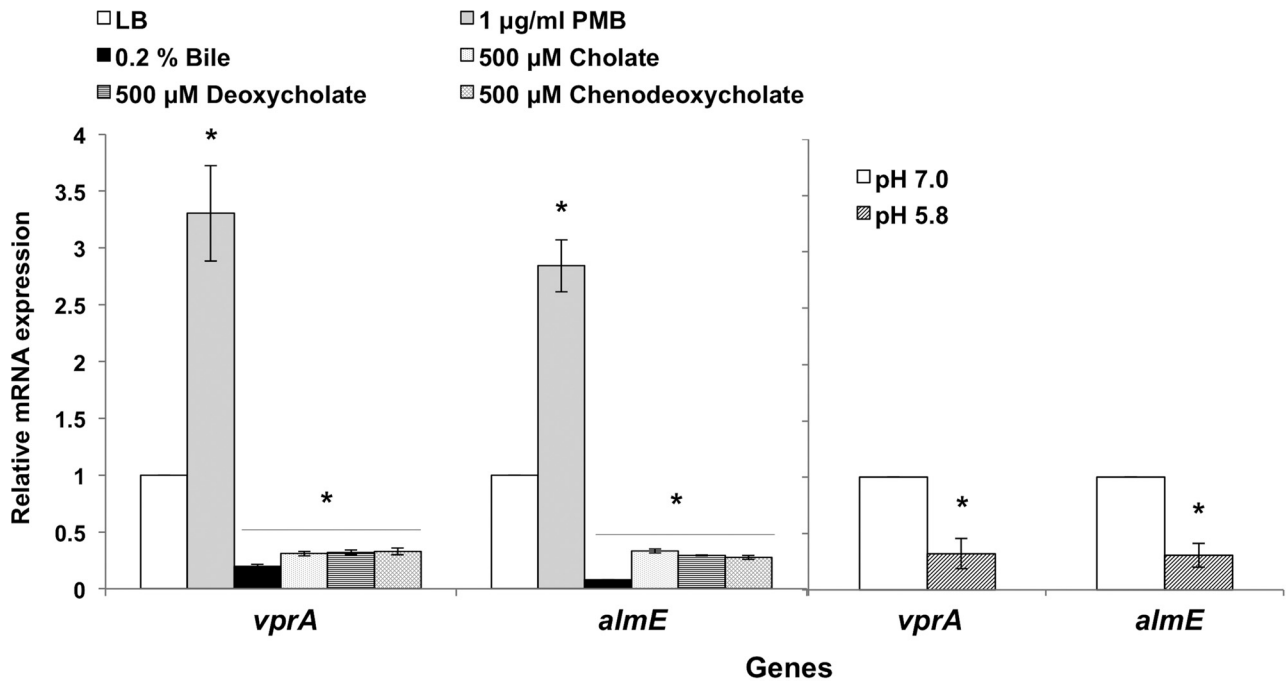


FIG 5 Changes in *vprA* and *almE* transcription during different growth conditions. Transcript levels of *vprA* and *almE* were determined in C6706 wild-type cells using qPCR. Cells were grown in LB medium or LB supplemented with 1 µg/ml polymyxin B, 0.2% bile, or specific bile components. For pH effect, 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 2.6×10^{-9}), and even more striking, the *almE* mutant exhibited a 363-fold fitness defect (P value of 7.4×10^{-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 $\Delta vprA$ strain (see Table S3 in the supplemental material). The $\Delta vprA$ 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 $\Delta vprA$ mutant showed complete loss of glycine/diglycine modification, identical to the C6706 *vprA*::Tn strain (Fig. 3). In mouse competition assays, the E7946 $\Delta vprA$ strain showed a 31-fold colonization defect (P value of 4.9×10^{-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

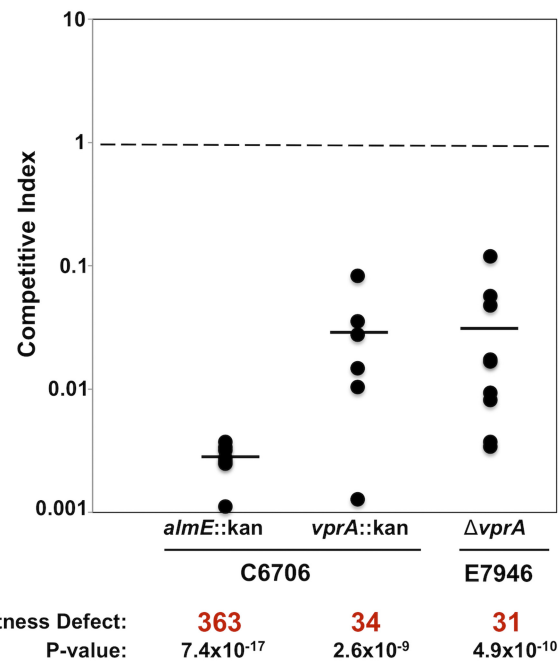


FIG 6 Glycine-dependent lipid A modification contributes to intestinal colonization. The ability of strains defective in glycine-lipid A modification to colonize the suckling mouse was evaluated by competition with the corresponding isogenic wild-type El Tor strain (E794

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, glycylation 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 phosphodonator 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 (

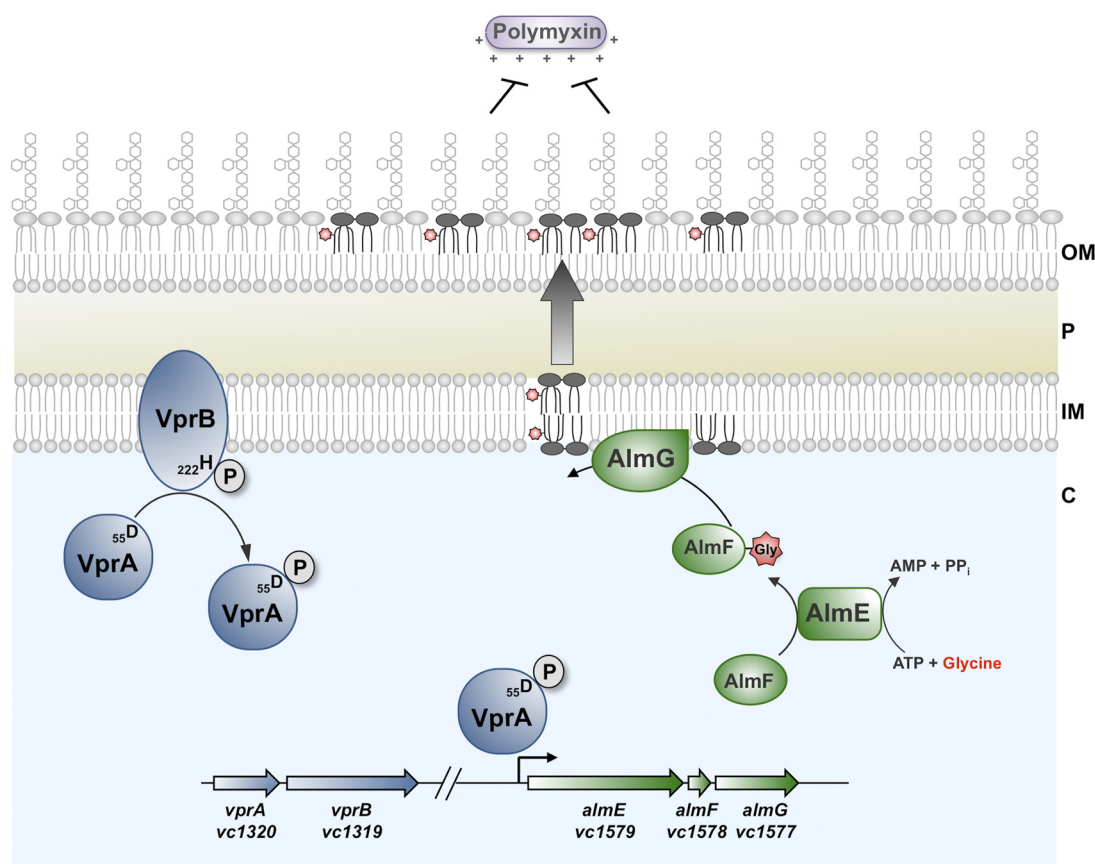


FIG 7 A model illustrating the regulation of lipid A modification of *V. cholerae* El Tor. The VprA-VprB two-component system is expressed in an operon. VprB likely functions as a sensor histidine kinase, which upon stimulation autophosphorylates at residue histidine (₂₂₂H) and transfers the phosphate group (represented by P) to a conserved aspartate residue (₅₅D) 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 μ M cholate, 500 μ M deoxycholate, 500 μ M chenodeoxycholate, 10 mM CaCl₂, 10 mM MgCl₂, or 40 μ M FeSO₄, 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 (NH₄)₂SO₄, 0.2% glucose, 1 mM MgSO₄, 1 mM CaCl₂, 0.015 mM FeSO₄, 0.075 mM thiamine, and 0.15 mM KH₂PO₄ (20, 66). In all cases, cells were grown with aeration at 37°C. *E. coli* strains were grown in LB

medium. Antibiotics streptomycin (100 μ g/ml), kanamycin (30 μ g/ml), and ampicillin (100 μ g/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

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-His₆ 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 His₆ 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 *vprA*His-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 λ pir 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-His₆ protein fusion. For overexpression of VprA-His₆, the *E. coli* host strain HMS174(DE3) carrying the plasmid pET-1320 was grown in LB broth supplemented with ampicillin. During exponential growth (OD₆₀₀ of 0.6 to 0.7), 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) was added to induce the expression of VprA-His₆. After 2 h of induction at 37°C, cells were harvested at 10,000 $\times$ g for 10 min at 4°C followed by one wash in 1 $\times$ 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/in² using a chilled pressure cell. The resulting crude cell extract was centrifuged at 10,000 $\times$ g for 15 min at 4°C. The cell extract supernatant was filtered through a 0.45- μ m-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 phen

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 μ g/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.

ACKNOWLEDGMENTS

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Correction for Herrera et al., The *Vibrio cholerae* VprA-VprB Two-Component System Controls Virulence through Endotoxin Modification

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Volume 5, no. 6, doi:10.1128/mBio.02283-14, 2014. In Results, in the paragraph headed “The VprA-VprB two-component system responds to polymyxin, bile, and mildly acidic pH” (PDF page 5), the fourth and fifth sentences should be modified to read as follows (revised text is indicated in bold): “Alternatively, when wild-type cells were exposed to divalent cations such as Ca²⁺ (10 mM) and Mg²⁺ (10 mM), *vprA* and *almE* synthesis was downregulated. **It has been reported that a small, but statistically significant, change in the expression of *vc1319-vc1320*, also known as *carS-R*, occurs during growth of *V. cholerae* in the presence of Ca²⁺ (77).** However, in the current work, statistically significant changes were observed only in *almE* transcripts (see Fig. S5A in the supplemental material), suggesting that *V. cholerae* moderately responds to divalent cations with a **predicted** reduction in glycine-lipid A modification.”

In Discussion (PDF page 8), the end of paragraph 5 should read as follows (revised text is indicated in bold): “Overall, this study establishes that the VprAB TCS positively regulates the *almEFG* operon to control glycine-lipid A modification (Fig. 7). **Furthermore, the current work is consistent with previous microarray data which show that *vc1319-vc1320* is involved in *alm* gene expression (77).**”

In References, due to the new text indicated above, the following reference should be added.

77. Bilecen K, Yildiz FH. 2009. Identification of a calcium-controlled negative regulatory system affecting *Vibrio cholerae* biofilm formation. *Environ Microbiol* 11:2015–2029. doi:10.1111/j.1462-2920.2009.01923.x.

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