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The *Vibrio cholerae vieSAB* Locus Encodes a Pathway Contributing to Cholera Toxin Production

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The genes encoding cholera toxin (CT), *ctxAB*, are coregulated with those for other *Vibrio cholerae* virulence factors by a cascade of transcriptional activators, including ToxR, TcpP, and ToxT. Additional regulators that modulate expression of *ctxAB* during infection were recently identified in a genetic selection. A transposon insertion in *vieS*, the sensor kinase of the VieSAB three-component signal transduction system, resulted in failure to induce expression of a *ctxA*-recombinase fusion during murine infection. To determine which components of the VieSAB system are essential for CT regulation, *ctxAB* transcript levels were assessed by RNase protection assay in various *vieSAB* in-frame deletion mutants after growth in vitro under virulence gene inducing conditions. A threefold reduction in *ctxAB* transcript levels was observed for the Δ*vieSAB* strain; consistent with this, the Δ*vieSAB* strain produced twofold less CT protein than the wild type, and this defect was complementable in *trans*. These results suggest that the VieSAB three-component system is required for full activation of the *ctxAB* operon during in vitro growth as well as during infection. The VieSAB system may regulate *ctxAB* expression indirectly by affecting production of ToxT, because decreased *toxT* transcript levels were observed in the Δ*vieSAB* strain.

The gram-negative bacterium *Vibrio cholerae* requires both colonization of the small intestinal epithelium, mediated by the toxin-coregulated pilus (TCP), and production of cholera toxin (CT), an ADP-ribosylating toxin, to cause the severe diarrheal disease cholera. CT and TCP are regulated by a cascade of transcription factors: these include, but are not limited to, ToxR and TcpP, which are both membrane-localized proteins of the OmpR transcriptional activator family that sense environmental signals and cooperatively induce expression of ToxT, an AraC family transcriptional regulator (5, 15, 24). Both ToxT and ToxR activate transcription of the *ctxAB* operon, which encodes the A and B subunits of CT (3, 29). ToxT also controls expression of the major pilin subunit TcpA (5), but induction of transcription of *tcpA* differs from that of *ctxA* during an infection with respect to both timing and dependence on colonization. Specifically, *tcpA* is induced prior to *ctxA*, and *ctxA* induction is dependent upon successful colonization, because it does not occur in a *tcpA* mutant background (16). These observations suggest that regulation of *ctxA* is complex and may require the action of additional regulatory factors.

In vivo positive regulators of *ctxA* have previously been identified by transposon mutagenesis coupled with recombination-based in vivo expression technology (RIVET) (18). RIVET uses a transcriptional fusion between the promoter of interest and *tnpR*, which encodes a resolvase, and a *res-tet-res* cassette located elsewhere on the chromosome. When the promoter is active, TnpR is produced and acts at the *res* sites to excise the tetracycline resistance (Tc^r) marker. Transposon insertion mutants that fail to induce expression of a *ctxA*::*tnpR* fusion during infection of the infant mouse remain tetracycline resistant and were selected on this basis. Among the mutants identified by this method was a transposon insertion in *vieS*, which encodes the sensor kinase of the three-component *vieSAB* signal transduction system (18).

One family of bacterial signal transduction systems consists of two components: a membrane-localized sensor histidine kinase and a DNA-binding response regulator. The kinase activity of the sensor is stimulated in response to a particular environmental signal, and phospho-transfer to the response regulator alters its affinity for DNA (25). The VieSAB system differs from conventional two-component signal transduction systems, because it encodes two putative response regulators, VieA and VieB. The *vieSAB* genes are encoded adjacent to each other and in the same transcriptional orientation on the *V. cholerae* large chromosome, but genetic evidence suggests they are differentially expressed. Both *vieS* and *vieA* are expressed during in vitro growth, but *vieS* expression is constitutive, while *vieA* expression is VieA dependent (17). Induction of *vieB*, in contrast, occurs only during infection and requires successful colonization mediated by TCP (16; Sang Ho Lee and Andrew Camilli, unpublished data). Because different response regulators are expressed during in vivo and in vitro growth, the VieSAB system has the potential to differentially regulate *ctxA* expression under these two conditions. VieA appears to be a typical response regulator, because it contains a helix-turn-helix domain for DNA binding in addition to the phosphoreceiver domain. VieB, however, contains a phosphoreceiver domain, but lacks any recognizable DNA binding motif.

Because only one other member of the two-component signal transduction family, the response regulator VarA, has been implicated in *V. cholerae* virulence gene regulation (28) and the cognate sensor kinase for this regulator has not been identi-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype and/or phenotype	Source or reference
Strains		
E. coli		
DH5α*λpir*	F⁻ Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1* λ::*pir*	10, 14
Sm10*λpir*	*thi recA thr leu tonA lacY supE* RP4-2-Tc::Mu λ::*pir*	Laboratory strain
AC-E479	Sm10*λpir*(pSL479) Ap^r	16
AC-E481	Sm10*λpir*(pSL481) Ap^r	16
AC-E482	Sm10*λpir*(pSL482) Ap^r	This work
AC-E1078	Sm10*λpir*(pSL148) Ap^r	This work
AC-E1082	Sm10*λpir*(pSL108) Ap^r	This work
AC-E1211	Sm10*λpir*(pAT1211) Ap^r	This work
V. cholerae El Tor biotype		
AC-V66	C6709-1 *lacZ*::*res-tet-res* Sm^r Tc^r	2
Bah-2	E7946 Δ*attRS* Sm^r *lacZ*	22
AC-V282	C6709-1 Δ*vieSAB* Sm^r Tc^r	17
AC-V279	AC-V66 Δ*vieAB* Sm^r Tc^r	17
AC-V494	AC-V66 Δ*toxT* Sm^r Tc^r	16
AC-V752	AC-V66 *vieS*::mTn*5Kn2* Sm^r Tc^r Kn^r	18
AC-V765	AC-V66 Δ*vieSAB* Sm^r Tc^r	This work
AC-V323b	AC-V66 Δ*vieB* Sm^r Tc^r	This work
AC-V1079	AC-V66 Δ*vieS* Sm^r Tc^r	This work
AC-V1221	AC-V66 Δ*vieA*-HTH Sm^r Tc^r	This work
AC-V553	AC-V66 *ctxA*::*tnpR135* Sm^r Tc^r Ap^r	16
AC-V1023	AC-V752 *ctxA*::*tnpR135* Sm^r Tc^r Ap^r	18
AC-V1083	AC-V323b *ctxA*::*tnpR135* Sm^r Tc^r Ap^r	This work
AC-V1097	AC-V66 *ctxA*::*tnpR168* Sm^r Tc^r Ap^r	This work
AC-V1098	AC-V752 *ctxA*::*tnpR168* Sm^r Tc^r Ap^r	This work
AC-V1151	AC-V1079 *ctxA*::*tnpR135* Sm^r Tc^r Ap^r	This work
AC-V1093	AC-V765 *tcpA*::*tnpR135* Sm^r Tc^r Ap^r	This work
AC-V1117	AC-V66 pMMB67EH Sm^r Tc^r Ap^r	This work
AC-V1110	AC-V765 pMMB67EH Sm^r Tc^r Ap^r	This work
AC-V1118	AC-V66 pAT1114 Sm^r Tc^r Ap^r	This work
AC-V1120	AC-V765 pAT1114 Sm^r Tc^r Ap^r	This work
Plasmids		
pIVET5	*oriR6K mobRP4 tnpR-lacZY* Ap^r	2
pSL479	pIVET5::*tcpA′*::*tnpR135*	16
pSL481	pIVET5::*ctxA′*::*tnpR135*	16
pSL482	pIVET5::*ctxA′*::*tnpR168*	This work
pCVD442	*oriR6K mobRP4 sacB* Ap^r	6
pSL108	pCVD442::Δ*vieB*	17
pSL148	pCVD442::Δ*vieS*	This work
pAT1211	pCVD442::Δ*vieA*-HTH	This work
pCR-Script	f1(+) *ori* Ap^r	Stratagene
pMMB67EH	IncQ broad-host-range cloning vector, Ap^r	21
pAT1113	pCR-Script::*vieSAB*	This work
pAT1114	pMMB67EH::*vieSAB*	This work
pGEM-T	f1 *ori* Ap^r	Promega
pAT853	pGEM-T::*′ctxA′*	This work
pAT854	pGEM-T::*′ctxB′*	This work
pAT856	pGEM-T::*′rpoB′*	This work
pDSM701	pGEM-T::*′toxT′*	This work

fied, study of the VieSAB three-component system may provide insight into the inducing signals that activate virulence factor expression as well as contribute to understanding the differential regulation of *ctxA* and *tcpA* during infection. In this report, we demonstrate that the VieSAB system is required for full CT expression both in vitro and in vivo and suggest that regulation may be indirect through modulated ToxT expression.

MATERIALS AND METHODS

Growth conditions. Bacteria were grown in Luria-Bertani (LB) broth with aeration at 37°C and maintained at −80°C in LB broth containing 30% glycerol. To induce *ctxA* expression, bacteria were grown in AKI broth (13). For counterselection of the *sacB*-containing pCVD442 plasmid, bacteria were grown at 30°C on LB agar lacking NaCl and supplemented with 10% sucrose. Antibiotics were used at the following concentrations unless otherwise noted: streptomycin, 100 μg/ml; ampicillin, 50 μg/ml; tetracycline, 3 μg/ml; kanamycin, 50 μg/ml.

Plasmid and strain construction. All strains and plasmids used in this study are listed in Table 1. All oligonucleotide primers used are listed in Table 2. Plasmid pSL482, which encodes a transcriptional fusion between *ctxA* and a ribosome-binding site mutant of *tnpR*, *tnpR168* (16), was constructed by inserting a *ctxA′* fragment (bp −516 to +53) into pIVET5 immediately upstream of *tnpR168*. The *ctxA′* fragment was amplified in a PCR from AC-V66 genomic DNA by using *Taq* polymerase and primers CtxF1 and CtxR1, digested with *Xba*I and *Bgl*II and ligated into similarly digested pIVET5::*tnpR168*.

TABLE 2. Sequences of primers used in this study

Primer	DNA sequence (5′→3′)[a]
CtxF1	GCTCTAGATCGAGTCAGAGCAATCCGAG
CtxR1	GAAGATCTGCATATGAAAATGATGATAA
VieSF4	GCGAAAAGGAAATGGTATGAA
VieSR4	AGCTGTTTTTTGATCATAATACTGCAATGGTGACTGT
VieSF5	CACCATTGCAGTATTATGATCAAAAAAACAGCTCAGTG
VieSR5	TGATACTGATGAAGCCACTGT
AHTHF1	GCAGCTTTCCGTCAACATC
AHTHR1	CTCTCGGTACTACTCTTCTTGTTGCGCCTGT
AHTHF2	CAAGAAGAGTAGTACCGAGAGTTATGGCTG
AHTHR2	TCCCCAACCGATAAGCAC
F18	AATTGAGCAGTGTGGCTTGCAT
AHTHR3	TGTTCCGATTCCGCTTGTC
F13	TCGTCGTCAAAGCGAATACTT
R14	CTGGTGCGGGCAGTCATAGAGT
F36	CAACAACAGAGTGGTTTG
VieSR1	ACATGCATGCTCTTAGCTTGAGCTTCAGGC
LSABF	TAGTCAGGTACCAGCAGTAATTGGACGAGCGAAAAGG
LSABR	TAGTCAGCATGCTTCAGAACAAAACCGAGGCAATG
CTAF	CTGTTAAACAAAGGGAGCATT
CTAR	GTGGGCACTTCTCAAACTAAT
CTBF	ATGCACATGGAACACCTCAA
CTBR	GCATGAGGCGTTTTATTATTC
RpoBF	CAGTTTGGGTCACTTATCAGC
RpoBR	ATTGGAAATACAGAACGAAAA
ToxTF	TTCAATTATCTTACTCAA
ToxTR	ACAAATATCTGCCCAACG
SP6	GCACCCCAGGCTTTACAC
T7	GGGTTTTCCCAGTCACGA

[a] Restriction enzyme sites are underlined.

The plasmid pAT1113 was constructed by cloning a 7.8-kbp fragment that includes the entire *vieSAB* locus and approximately 800 bp upstream of the putative VieS start codon into the *Srf*I site of pCR-Script (Stratagene). The *vieSAB* locus was amplified by PCR from AC-V66 genomic DNA with the primers LSABF and LSABR, which contain *Kpn*I and *Sph*I restriction sites at their 5′ ends, respectively. The PCR was performed with a 10:1 mixture of *Taq* and *Pfu* polymerases and extensions of 6 min plus 15 s per cycle at 68°C. The 7.8-kb insert was removed from pAT1113 by digestion with *Kpn*I and *Sph*I, gel purified, and subsequently ligated into similarly digested pMMB67EH to generate pAT1114.

Plasmids for generating gene deletions in *V. cholerae* were constructed in pCVD442, which encodes the *sacB* gene for counterselection (6). Plasmids pSL148 and pAT1211, used to generate in-frame deletions of *vieS* and *vieA*-HTH, respectively, were constructed by splicing by overlap extension (SOE) PCR (23). For plasmid pSL148, upstream and downstream fragments of approximately 600 bp each were amplified by PCR from AC-V66 genomic DNA by using *Pfu* polymerase and primer pairs vieSF4 and vieSR4 and vieSF5 and vieSR5, respectively. Primers vieSR4 and vieSF5 were designed with complementary sequence at their 5′ ends, allowing the upstream and downstream fragments to be annealed together, and amplified by PCR with primers vieSF4 and vieSR5. The resulting 1.2-kbp product was ligated into *Sma*I-digested pCVD442 by blunt-end ligation. For plasmid pAT1211, upstream and downstream fragments of approximately 900 bp were amplified by PCR from AC-V66 genomic DNA with *Pfu* polymerase and the primer pairs AHTHF1 and AHTHR1 and AHTHF2 and AHTHR2, respectively. The upstream and downstream fragments were annealed together and amplified by PCR with primers AHTHF1 and AHTHR2. The resulting 1.8-kbp product was treated with *Taq* polymerase and ligated into pGEM-T (Promega). The 1.8-kbp insert was removed from pGEM-T by digestion with *Sal*I and *Sph*I and ligated into similarly digested pCVD442.

Strains AC-V323b, AC-V1079, and AC-V1221, harboring in-frame deletions of *vieB*, *vieS,* and the *vieA* helix-turn-helix domain, respectively, were constructed by allelic exchange in the AC-V66 background. Plasmids pSL108 (Δ*vieB*), pSL148 (Δ*vieS*), and pAT1211 (Δ*vieA*-HTH) were conjugated into AC-V66 by mating with the appropriate *Escherichia coli* Sm10λ*pir* strain, as previously described (17). After one passage in LB broth in the absence of antibiotic selection, sucrose-resistant colonies were screened for deletion alleles by colony PCR with the primer pairs F13 and R14 for Δ*vieB*, F36 and vieSR1 for Δ*vieS*, or F18 and AHTHR3 for Δ*vieA*-HTH. Strains carrying transcriptional fusions between the *ctxA* promoter and *tnpR135* or *tnpR168* were constructed by mating the *V. cholerae* strains AC-V66, AC-V752, AC-V323b, and AC-V1079 with *E. coli* strain AC-E481 or AC-E482, respectively, followed by selection for Sm^r Ap^r exconjugates in which the suicide plasmid had integrated into the *V. cholerae* chromosome by a single crossover in the *ctxA* promoter region. Strain AC-V1093, carrying the *tcpA*::*tnpR135* transcriptional fusion, was constructed by mating the Δ*vieSAB* strain AC-V765 with the *E. coli* strain AC-V479 and selecting for Sm^r Ap^r exconjugates. The *V. cholerae* strain AC-V765 was constructed by transduction of the *res-tet-res* cassette from AC-V66 into the Δ*vieSAB* strain AC-V282 by using the CP-T1ts phage, as previously described (11).

V. cholerae strains AC-V1110, AC-V1117, AC-V1118, and AC-V1120 carrying the low-copy-number vector pMMB67EH or the derivative pAT1114 were generated by electroporation. Strains AC-V66 and AC-V765 were made electrocompetent by growing bacteria to mid-log phase in LB broth, washing the cells twice with ice-cold 2 mM $CaCl_2$, and freezing them at −80°C in 10% glycerol. Electrocompetent bacteria were transformed with 20 ng of plasmid DNA at 2.1 kV in a 0.2-cm-diameter cuvette (Bio-Rad) and grown for 1 h at 37°C with shaking prior to selection.

Transcription induction assay. Induction of transcriptional fusions to *tnpR* during infection of the small intestine of infant mice was measured as previously described (16). Strains carrying the *ctxA*::*tnpR* fusions were grown overnight at 37°C with shaking in LB broth containing streptomycin (50 μg/ml), ampicillin (30 μg/ml), and tetracycline (1 μg/ml). Cultures were diluted 10^{-3} in LB broth, and ~10^6 CFU was intragastrically inoculated into 5-day-old CD-1 mice. At various times postinoculation, the small intestines were removed and homogenized in LB broth containing 20% glycerol, and bacteria were recovered by plating serial dilutions on LB broth containing streptomycin. The resulting colonies were replica plated to LB broth containing tetracycline to determine the percentage of Tc^s CFU.

RPAs. RNase protection assays (RPAs) were performed with total RNA isolated from strains grown in AKI broth (13). Briefly, cultures of each strain grown overnight in LB broth were diluted 10^{-3} into 10 ml of AKI broth in 25-ml glass culture tubes. After 4 h of growth under static conditions at 37°C, 3 ml of the culture was removed to a fresh 18-ml glass tube and shifted to growth with shaking at 37°C for an additional 3 h. RNA was harvested from 1 ml of cells with the RNeasy kit (QIAgen) as previously described (20). Riboprobe templates for *ctxA*, *ctxB*, *rpoB*, and *toxT* were generated by PCR amplification from AC-V66

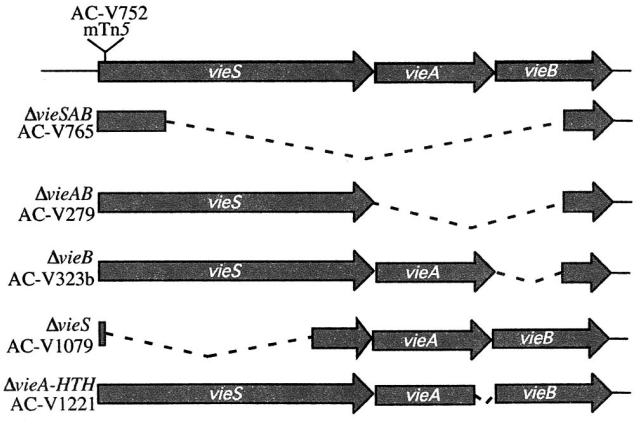

FIG. 1. Genetic organization of the *V. cholerae vieSAB* locus. The site of the mTn*5* insertion in strain AC-V752 is indicated at the top. Dashed lines indicate deleted sequences. Relevant strains isogenic with AC-V66 are listed next to the corresponding deletion.

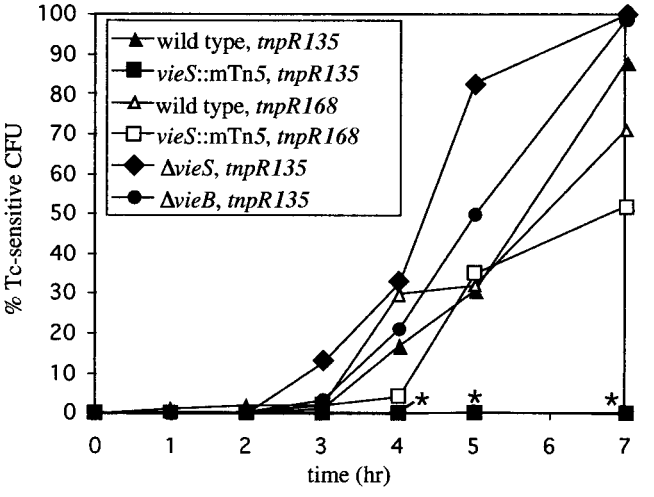

FIG. 2. Induction kinetics of the *ctxA*::*tnpR* fusion in various *vieSAB* mutant strain backgrounds. Infant CD-1 mice were intragastrically infected with the indicated strains, and the percentage of Tc^s CFU, indicated on the *y* axis, was determined by replica plating of bacteria isolated from intestinal homogenates generated at various times postinoculation, indicated on the *x* axis. The data shown are the mean of multiple experiments. Data at 4, 5, and 7 h were tested for statistical significance with an unpaired *t* test. Asterisks denote strains with reduced induction of the gene fusion ($P < 0.05$). Strains AC-V553, AC-V1023, AC-V1083, and AC-V1151 carry the *ctxA*::*tnpR135* fusion, indicated by solid symbols. Strains AC-V1097 and AC-V1098 carry the *ctxA*::*tnpR168* fusion, indicated by open symbols.

genomic DNA with *Taq* polymerase and the following primer pairs: CTAF and CTAR (287 bp), CTBF and CTBR (300 bp), RpoBF and RpoBR (211 bp), and ToxTF and ToxTR (409 bp). The PCR products were ligated to pGEM-T (Promega) to generate plasmids pAT853, pAT654, pAT856, and pDSM701. Insert orientation was determined by PCR, and templates for in vitro transcription were generated by PCR from the plasmids by using the appropriate reverse primer and either the T7 or SP6 primer. Riboprobes were synthesized by in vitro transcription with the Maxiscript kit (Ambion) and 50 μCi of [^{32}P]UTP (NEN) and gel purified on 4% denaturing polyacrylamide gels. RPAs were performed with the RPAII kit (Ambion) according to the manufacturer's instructions with 1 to 2 μg of total sample RNA. Products were separated on 4% denaturing polyacrylamide gels and exposed to Kodak phosphor screens. Data were collected with a phosphorimager and analyzed with the ImageQuant program (Molecular Dynamics).

Western blot analysis. Western blots were performed with culture supernatants from strains grown in AKI broth, as described above. Culture supernatants were filter sterilized with 0.45-μm-pore-diameter Durapore filters (Millipore) and subsequently concentrated approximately 10-fold with YM10 spin columns (Centricon) (5,000 × *g*, 1.5 h). The Sigma Micro Protein Determination kit was used to measure total protein in concentrated supernatants. Whole-cell extracts were prepared by pelleting equivalent numbers of cells based on optical density at 600 nm (OD_{600}) and boiling cells in 1× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) buffer for 5 min. Equivalent amounts of total protein were separated on SDS-PAGE gels (12% polyacrylamide) and electrophoretically transferred to nitrocellulose membranes (Hybond). The CT-A and -B subunits were detected simultaneously with a mixture of rabbit polyclonal antisera against the purified proteins and the ECL enhanced chemiluminescence detection system (Amersham Pharmacia). For quantitative Western blots, known amounts of purified CT-B subunit (List Biological Laboratories) were added to concentrated supernatants from strain Bah-2. Reactive bands were detected by exposure to X-ray film (Kodak) and quantitated by measuring light transmittance on a Kodak Image Station 440. A standard curve was generated for the purified CT-B samples relating band intensity to the known CT-B protein concentration. The equation of this line was used to determine the concentration of the B subunit in culture supernatants from observed band intensities.

RESULTS

VieSAB affects *ctxA* induction in vivo. We previously reported that an mTn*5* transposon insertion in *vieS* (Fig. 1), which is the putative sensor kinase of the VieSAB three-component signal transduction system, prevents induction of *ctxA* expression in the infant mouse model of cholera (18). As shown in Fig. 2, *ctxA* expression is induced in the wild-type C6709-1 background beginning 3 to 4 h after introduction into the animal (solid triangles). In contrast, *ctxA* induction is never observed in the *vieS*::mTn*5* background, even after 24 h of infection (solid squares [data not shown]). In these experiments, *ctxA* expression was analyzed in a qualitative manner by using a transcriptional fusion between the *ctxA* promoter and the severe-ribosome-binding-site mutant, *tnpR135*. To determine whether disruption of *vieS* completely inhibits *ctxA* transcription or merely reduces expression to a level undetectable with the *tnpR135* allele, we utilized a second ribosome-binding-site mutant, *tnpR168*, which is translated more efficiently than the *tnpR135* allele, allowing lower levels of transcription initiation to be detected (16). The *ctxA*::*tnpR168* transcriptional fusion was constructed and introduced into wild-type and *vieS*::mTn*5* strain backgrounds containing the *res-tet-res* cassette to generate strains AC-V1097 and AC-V1098, respectively. Infant mice were inoculated with strains AC-V1097 and AC-V1098, and bacteria isolated from small intestine homogenates were tested for induction of *ctxA* expression over time by measuring the percentage of Tc^s CFU. As shown in Fig. 2, transcription initiation at the *ctxA* promoter could be detected in the *vieS*::mTn*5* mutant by using the *tnpR168* allele, although expression was marginally delayed and reduced in comparison to that of the wild-type control (compare open triangles and open squares). When strains were analyzed for induction of the *ctxA*::*tnpR* fusions after 7 h of growth in vitro in AKI broth (a CT-inducing condition), similar results were obtained; induction of *ctxA* transcription in the *vieS*::mTn*5* background was never observed with the *tnpR135* reporter, but was wild type with the *tnpR168* reporter (data not shown). These results suggest that transcription does still occur from the *ctxA* promoter in a *vieS*::mTn*5* mutant background during growth in

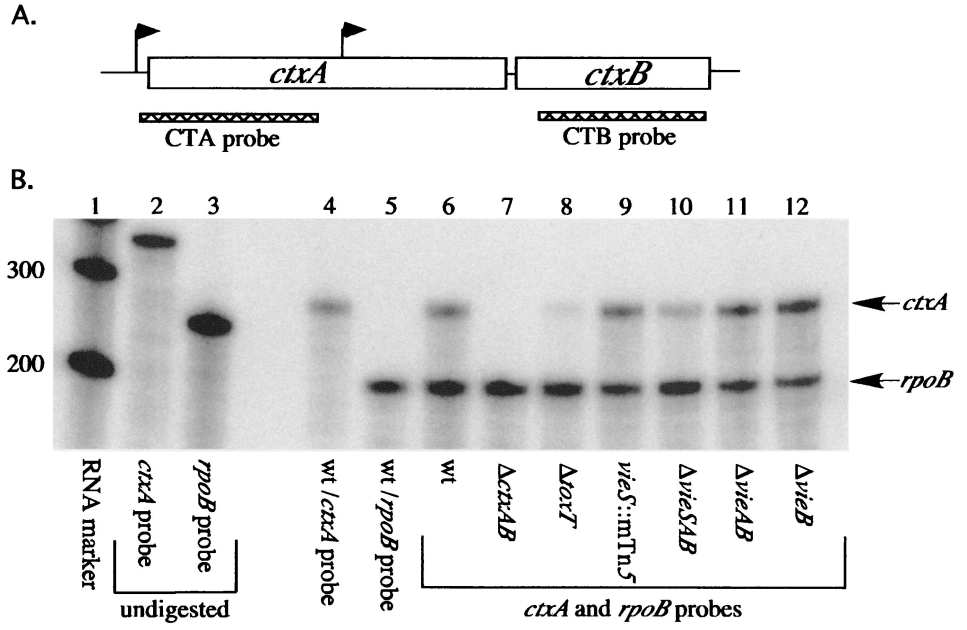

FIG. 3. RPA for *ctxAB* transcript in various *vieSAB* mutant strain backgrounds. (A) Genetic organization of the *V. cholerae ctxAB* operon. Putative promoters are indicated by arrows. Probes designed to detect the *ctxA* and *ctxB* portions of the message are indicated by hatched bars drawn to scale. (B) RPA for *ctxA* message. Total RNA (1 μg) isolated from strains grown under the AKI inducing condition for 7 h as described in Materials and Methods was analyzed by using the *ctxA*-specific probe. A probe against *rpoB* was included as an internal loading control. Lanes: 1, RNA marker; 2 and 3, undigested *ctxA* and *rpoB* probes, respectively; 4 and 5, AC-V66 RNA *ctxA* probe only and *rpoB* probe only, respectively. Lanes 6 to 12 all contain both *ctxA* and *rpoB* probes. Lanes: 6, AC-V66; 7, Bah-2; 8, AC-V494; 9, AC-V752; 10, AC-V765; 11, AC-V279; 12, AC-V323b. wt, wild type. Protected bands of the expected sizes are indicated by arrows to the right. The sizes of the molecular weight markers in base pairs are given on the left. The blot shown is representative of three independent experiments.

vivo, but that the frequency of transcription initiation is reduced compared to that in the wild type.

To determine which specific components of the VieSAB system are required for *ctxA* induction in vivo, a *ctxA*::*tnpR135* transcriptional fusion was introduced into strains harboring various in-frame deletions in the *vieSAB* locus (Fig. 1). Strains AC-V1079 and AC-V1083, which contain the Δ*vieS* and Δ*vieB* mutations, respectively, were constructed. These strains both carry the *ctxA*::*tnpR135* fusion integrated at the *ctxA* locus and remain Tc^r during growth in LB broth due to inactivity of the fusion. Both the Δ*vieS* and Δ*vieB* strains exhibit wild-type induction of *ctxA* expression during growth in vivo, suggesting that neither VieS nor VieB alone is essential for activation of *ctxA* expression under this condition (Fig. 2). The observation that the Δ*vieS* and *vieS*::mTn*5* mutants do not have the same phenotype with respect to *ctxA* induction suggests that the mutations have different effects on expression of the downstream *vieA* and *vieB* genes.

Surprisingly, exconjugates produced by matings between AC-E481 carrying the *ctxA*::*tnpR135* reporter and strains AC-V765, AC-V279, and AC-V1221, which harbor the Δ*vieSAB,* Δ*vieAB*, and Δ*vieA*-HTH mutations, respectively, were consistently Tc^s after mating and colony purification on LB plates. These results suggest that *ctxA* transcription is induced in these backgrounds during growth on LB broth, which is normally a noninducing condition for *ctxA* expression. The results also indicate that the DNA binding activity of VieA specifically is required for proper regulation of *ctxA* transcription under these conditions. Since it was not possible to construct strains containing both the *ctxA*::*tnpR135* reporter and an intact *res-tet-res* cassette in the Δ*vieSAB,* Δ*vieAB*, or Δ*vieA*-HTH strain background, we could not assess whether these mutations affect *ctxA* expression during in vivo growth by using the RIVET assay.

Transcription of *ctxAB* in vitro is reduced in a Δ*vieSAB* background. Since suitable strains containing the *ctxA*::*tnpR* reporter could not be constructed, RPAs were performed to directly assess whether in-frame deletions in the *vieSAB* locus affect *ctxAB* transcription under the in vitro inducing condition in AKI broth. Because only modest differences in *ctxA* transcript levels were observed in initial experiments, an internal control transcript was probed in all subsequent experiments to allow normalization of *ctxA* transcript levels. The *rpoB* gene, which encodes the β subunit of RNA polymerase, was chosen for the internal control, because *rpoB* is expressed fairly constitutively, autoregulated at the transcriptional level (7), and has previously been used with success as a control in Northern blotting experiments (C. Squires, personal communication). Steady-state levels of *rpoB* transcript were stable during logarithmic-phase growth under the culture conditions we studied, and there was no detectable difference in *rpoB* transcript levels between RNA isolated from wild-type cells grown in LB broth or under AKI growth conditions (data not shown). In addition, the *rpoB* probe did not interfere with detection of the *ctxA* transcript (Fig. 3B, compare lanes 4 and 6).

RPAs were performed with 1 μg of total RNA isolated from the wild type and each *vieSAB* mutant strain grown under the AKI inducing condition for 7 h. Two negative controls were

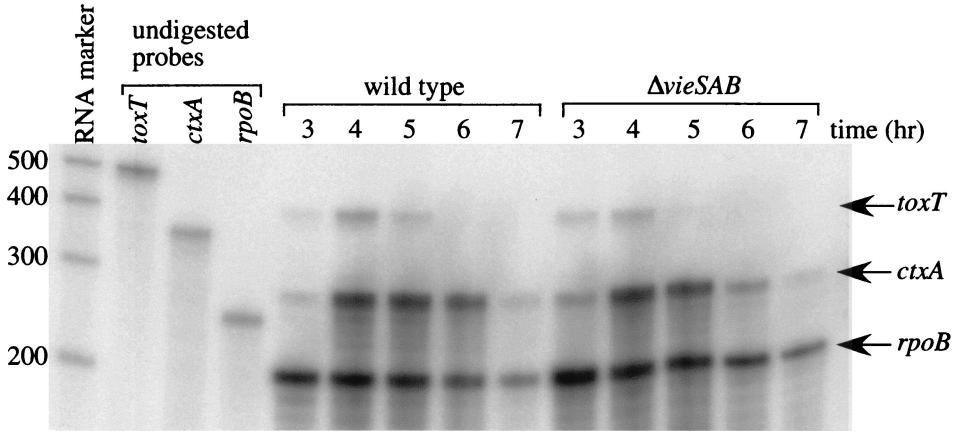

FIG. 4. Time course RPA to detect *ctxA* and *toxT* transcripts in the Δ*vieSAB* strain background. Strains AC-V66 (wild type) and AC-V765 (Δ*vieSAB*) were grown under the AKI inducting condition, and RNA was isolated at hourly time points as described in Materials and Methods. Total RNA (2 μg) from each time point was hybridized with radiolabeled riboprobes against *ctxA*, *toxT*, and *rpoB*. Protected bands of the expected sizes are indicated by the arrows to the right. The sizes of the molecular weight markers in base pairs are given on the left. The data shown are representative of two independent experiments.

included in these experiments: Bah-2, an El Tor strain in which CTXΦ has been deleted and is thus *ctxAB* (22); and AC-494, a strain isogenic with AC-V66 in which the helix-loop-helix domain of ToxT, a known positive regulator of *ctxA*, has been deleted (16). The growth of all mutant strains was identical to that of the wild type under AKI conditions, as assessed by measuring the OD_{600} at hourly intervals (data not shown). When the *ctxA* riboprobe was used (Fig. 3A), an ~287-bp protected band was observed in the wild type and all *vieSAB* mutant strains (Fig. 3B). This band corresponds to the *ctxA* transcript, because it is absent in RNA isolated from Bah-2 and reduced in intensity in RNA isolated from strain AC-494 (Fig. 3B, lanes 7 and 8). Because the *ctxA* transcript was observed in RNA isolated from all *vieSAB* mutant strains, the VieSAB system is not essential for induction of *ctxA* transcription in vitro in AKI broth. However, in three independent experiments, decreased *ctxA* transcript levels were observed in RNA isolated from the Δ*vieSAB* strain AC-V765 in comparison to that of the wild-type control (Fig. 3B, lanes 6 and 10). The *ctxA* band intensity was measured for each strain and normalized to the intensity of the *rpoB* internal control. When values for the *vieSAB* mutant strains were compared to those for the wild-type control, an approximately threefold reduction in *ctxA* transcript was consistently observed for the Δ*vieSAB* mutant strain AC-V765 (0.383 ± 0.101). This difference was determined to be significant by an unpaired *t* test ($P < 0.001$). There were no significant differences in *ctxA* transcript levels for any of the other *vieSAB* mutants tested, including the *vieS*::mTn*5* strain AC-V752 and the Δ*vieS* strain AC-V1079 (Fig. 3B) (data not shown).

Although the genes for *ctxA* and *ctxB* are generally considered to be transcribed as an operon, differential transcription of the *ctxB* gene has previously been reported to occur from a promoter within the *ctxA* gene (9) (Fig. 3A). To test for possible differential transcriptional regulation of *ctxA* and *ctxB* by the VieSAB system, a second probe was designed to detect the *ctxB* transcript (Fig. 3A). The results obtained in RPAs with the *ctxB* riboprobe were essentially identical to those observed with the *ctxA* probe (data not shown). The *ctxB* transcript was absent or diminished in RNA prepared from the Bah-2 and AC-V494 negative controls. In two independent experiments, an approximately threefold reduction in the amount of *ctxB* transcript was observed for the Δ*vieSAB* strain AC-V765, which was significantly different from the level in the wild type by an unpaired *t* test (0.388 ± 0.092, $P < 0.001$). Significant differences in the concentration of *ctxB* transcript from the wild type were not observed for any other *vieSAB* mutant strain tested (data not shown). These results suggest that the VieSAB system is required for wild-type expression of both *ctxA* and *ctxB* in AKI broth and that the *ctxA* and *ctxB* genes are coregulated under the experimental conditions tested.

VieSAB system is required for maintenance of *ctxAB* transcription. It has previously been reported that *ctxA* transcription is initiated in El Tor *V. cholerae* after 5 h of growth under AKI conditions and continues to be detectable by primer extension analysis after up to 10 h of growth (19). Failure to detect wild-type levels of *ctxAB* transcript in a Δ*vieSAB* strain background after 7 h of growth under the AKI inducing condition could be explained in several ways. The Δ*vieSAB* mutation might (i) delay the timing of *ctxA* transcription induction, (ii) reduce the level of *ctxA* transcription throughout growth, or (iii) shorten the time of maintenance of *ctxA* transcription after the initial induction. To distinguish among these possibilities, time course RPAs were performed with RNA isolated from AKI cultures of AC-V66 and AC-V765 (Δ*vieSAB*) at hourly intervals. Wild-type levels of *ctxA* transcript were observed at h 3 to 5 for the Δ*vieSAB* strain (Fig. 4), suggesting that the VieSAB system is not required for the initial induction of transcription from the *ctxA* promoter. In two independent experiments, however, a dramatic reduction in *ctxA* transcript was observed at 6 h for the Δ*vieSAB* strain, although *rpoB* transcript levels paralleled those of the wild type (Fig. 4). When band intensities were quantitated as described above, the amount of *ctxA* transcript at 6 h was reduced approximately 10-fold in the Δ*vieSAB* mutant in comparison to that in the wild type (0.123 ± 0.051, $P < 0.05$). Similar to previous exper-

iments, at 7 h, we observed only a twofold reduction in the level of *ctxA* transcript in the Δ*vieSAB* mutant compared to the wild type (0.48 ± 0.05, $P < 0.05$); the 10-fold difference detected at 6 h was not sustained, because the concentration of *ctxA* transcript in the wild type declined dramatically between the two time points. These data suggest that the VieSAB system is not required for the initial induction of *ctxA* transcription or peak levels of transcript accumulation but rather is necessary for maintenance of *ctxA* transcription after induction.

VieSAB affects *toxT* transcription. There are at least two ways in which the VieSAB system could affect *ctxAB* transcription. Either one of the response regulators VieA or VieB might bind directly to the *ctxA* promoter to activate transcription or the system might be required for wild-type expression of ToxT, a positive regulator of *ctxA* (3, 5, 12). To assess whether the VieSAB system might act indirectly on *ctxAB* by affecting *toxT* transcription, we included in the time course RPA experiments a riboprobe to detect *toxT* transcripts. If the VieSAB system affects *ctxAB* transcription indirectly through ToxT, reduced levels of *toxT* transcript should be observed. Previous work has shown that ToxT transcripts are detectable by primer extension at 4 to 5 h during growth under AKI conditions (19). In RNA isolated from the wild-type strain AC-V66, a protected band of 409 bp corresponding to the *toxT* transcript was detectable by RPA at 3 to 5 h (Fig. 4). In RNA isolated from the Δ*vieSAB* mutant strain AC-V765, *toxT* transcripts were also observed at 3 and 4 h of growth, but by 5 h, there was virtually no detectable transcript (Fig. 4). When *toxT* band intensities were quantitated and normalized to the *rpoB* control, a reproducible threefold decrease in *toxT* transcript was observed for the Δ*vieSAB* strain at 5 h (0.359 ± 0.102, $P < 0.05$). The levels of *toxT* transcript were not significantly different from those of the wild type at earlier time points. These data suggest that decreased ToxT expression may be responsible for reduced *ctxAB* transcription in the Δ*vieSAB* mutant. To test whether the reduced *toxT* transcript levels might also affect expression of another ToxT-regulated gene, *tcpA*, we utilized the RIVET system. A *tcpA*::*tnpR135* fusion was introduced into the Δ*vieSAB* mutant background to generate strain AC-V1093, which was subsequently tested for resolution both in vitro under the AKI condition and in vivo. The Δ*vieSAB* mutation did not affect induction of the *tcpA*::*tnpR135* fusion under either condition (data not shown).

Western analysis confirms reduced CT production in a Δ*vieSAB* background. To test whether the decreased *ctxAB* transcript levels observed for the Δ*vieSAB* strain AC-V765 are relevant at the protein level, Western blotting was performed. The wild-type strain, AC-V66, and the Δ*vieSAB* strain, AC-V765, were grown for 7 h under the AKI inducing condition, and culture supernatants were analyzed for the presence of both the A and B subunits of CT. A reproducible decrease in the amount of both subunits was observed for the Δ*vieSAB* strain background in comparison to the wild type (Fig. 5). In this assay, the *toxT* mutant strain AC-494 produced no detectable CT, and consistent with the RPA data, mutants carrying other in-frame deletions in the *vieSAB* locus produced wild-type levels of CT protein (data not shown). Reduced CT production by the Δ*vieSAB* mutant could not be attributed to a defect in secretion, because CT was undetectable in whole-cell extracts by Western blotting (data not shown).

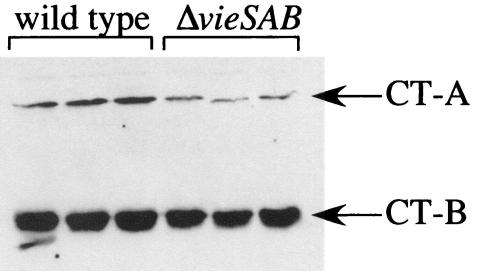

FIG. 5. Western blot for CT. Strains AC-V66 (wild type) and AC-V765 (Δ*vieSAB*) were grown under the AKI inducing condition for 7 h, and CT was detected in equivalent amounts of total protein from concentrated, filtered supernatants by using a mixture of rabbit polyclonal antisera against the purified A and B subunits of CT. The experiment was performed in triplicate with independent cultures, and the results of all cultures are shown.

To quantitate the difference in CT protein production between the wild-type and Δ*vieSAB* strains, quantitative Western blots were performed. Known amounts of the CT-B subunit were added to concentrated supernatants of strain Bah-2, which is deleted for *ctxAB*, and band intensities on the Western blot were used to generate a standard curve. The quantities of CT-B subunit in concentrated culture supernatants from strains AC-V66 and AC-V765 were calculated from band intensities according to the standard curve. While the wild-type strain AC-V66 produced 0.20 ng of CT-B subunit per μg of total supernatant protein, the Δ*vieSAB* strain AC-V765 produced approximately 65% of this amount (0.13 ng of CT-B per μg of total supernatant protein). This result is consistent with the approximately twofold reduction in *ctxAB* transcript observed by RPA.

The Δ*vieSAB* mutation can be complemented in *trans*. Since the defects in *ctxAB* transcription and CT protein production were observed only for the Δ*vieSAB* in-frame deletion mutant, it is formally possible that strain AC-V765 harbors a secondary mutation that affects expression of *ctxAB*. To demonstrate that loss of VieSAB causes decreased CT production by strain AC-V765, we complemented the Δ*vieSAB* mutation by providing the *vieSAB* locus in *trans* on plasmid pAT1114. This construct contains the *vieSAB* locus cloned on the stable, low-copy-number vector pMMB67EH. Both pAT1114 and pMMB67EH were introduced into the wild-type and Δ*vieSAB* strain backgrounds by electroporation, and the resulting strains were tested for production of CT under the AKI inducing condition by Western blotting of culture supernatants. Growth of plasmid-containing strains in AKI broth was indistinguishable from that of the wild type, as determined by measuring the OD_{600} at hourly intervals (data not shown). In addition, replica plating of bacteria isolated from AKI cultures to medium containing ampicillin demonstrated that both plasmids were maintained in >99% of the population, even in the absence of antibiotic selection. Reduced CT protein production was observed for the Δ*vieSAB* strain containing the pMMB67EH vector backbone (Fig. 6, lanes 1 and 2). The Δ*vieSAB* strain carrying the pAT1114 complementing plasmid, however, produced wild-type levels of CT protein (Fig. 6, lane 3). These data indicate that the Δ*vieSAB* in-frame deletion in strain AC-V765 is responsible for the defect in CT protein production.

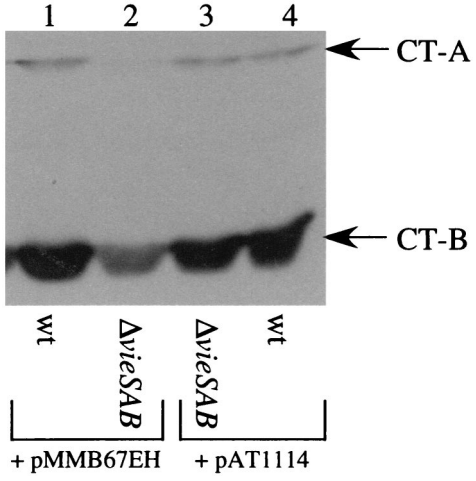

FIG. 6. Complementation of the Δ*vieSAB* mutation in *trans*. Strains were grown under the AKI inducing condition for 7 h, and the CT-A and -B subunits were detected by Western blotting as described in the legend to Fig. 5. The A subunit was evident in lane 2 upon longer exposure of the blot. Lanes: 1, AC-V1117; 2, AC-V1110; 3, AC-V1120; 4, AC-V1118. wt, wild type.

DISCUSSION

Bacterial pathogens often control expression of virulence factors in response to environmental conditions with two-component signal transduction systems (8, 25). In this report, we demonstrate that the *V. cholerae* VieSAB three-component signal transduction system is required for full expression of CT, the major pathogenicity factor of this diarrheal pathogen. The VieSAB system differs from conventional two-component systems, because, in addition to the VieS sensor kinase, it encodes two putative response regulators, VieA and VieB (17).

The VieSAB system was identified as a putative positive regulator of *ctxAB* by a selection strategy to isolate transposon insertion mutants that fail to induce expression of a *ctxA*::*tnpR* fusion during growth in the infant mouse small intestine (18). In this selection, a mutant was obtained with an mTn*5* insertion near the 5′ end of the *vieS* gene. By using a *ctxA*::*tnpR168* fusion, which has increased translational activity relative to the *tnpR135* allele, we were able to detect induction of *ctxA* in the *vieS*::mTn*5* mutant background. These results suggest that signaling through the VieSAB system is required for wild-type induction of *ctxA* expression during infection but is not absolutely essential. Consistent with this, in preliminary studies, we were not able to detect any significant difference between the wild-type and *vieS*::mTn*5* strains in fluid accumulation assays in suckling mice (data not shown), although this assay (1) is not particularly sensitive.

Genetic evidence suggests that *vieA* may be expressed from a promoter distinct from the *vieS* promoter (17). The in vivo phenotype of the *vieS*::mTn*5* mutant could therefore be due to expression of VieA in the absence of VieS and should be reproduced by an in-frame deletion of *vieS*. An in-frame deletion of *vieS* was constructed that removes the periplasmic, transmembrane, and histidine kinase domains, but leaves intact approximately 600 bp of sequence upstream of *vieA*. However, no detectable differences in *ctxAB* expression were observed between the wild-type and Δ*vieS* strains in any assay, either in vivo or in vitro. There are several possible explanations for the discrepancy between the Δ*vieS* and *vieS*::mTn*5* mutant phenotypes. The mTn*5* transposon insertion in *vieS* may be polar on the downstream *vieA* and *vieB* genes, leading to a reduction in *ctxA* transcription. This hypothesis is not favored because both *vieA* and *vieB* are believed to be expressed from unique promoters (17). A second possibility is that the putative *vieA* promoter has been deleted in the Δ*vieS* strain, but is still intact in the *vieS*::mTn*5* mutant, allowing production of the VieA response regulator in the latter strain background. Finally, it is possible that transcription from the Kn^r gene promoter on the mTn*5* transposon reads into the *vieS* gene, allowing production of a truncated VieS protein with altered signaling properties from an alternative translation start site.

Since we were not able to use RIVET to study *ctxA* induction in several *vieSAB* mutant strains, we instead examined directly whether in-frame deletions in the *vieSAB* locus affect *ctxA* transcription in vitro by RPA. An approximately threefold reduction in *ctxAB* transcript and a nearly twofold reduction in CT protein were observed for a Δ*vieSAB* strain, but other deletion mutations in the *vieSAB* locus had no effect on CT production. The defect observed for the Δ*vieSAB* strain was due to the in-frame deletion and not a secondary mutation, because we could complement the defect in *trans*. Although we observed a defect in *ctxA* transcription for the *vieS*::mTn*5* strain by using RIVET, we did not detect any difference in the level of *ctxAB* message for this strain by RPA. It is possible that the ribosome-binding-site mutants of TnpR used in this study are sufficiently sensitive that we were able to detect a very minor reduction in *ctxA* transcription that was not observable by RPA.

Because a defect in CT expression is observed only when all components of the VieSAB system are deleted, there must be cross talk between VieSAB and another two-component system or other phosphodonor. One protein that might be involved in cross talk with VieSAB is VarA. VarA encodes a response regulator homologous to GacA, the global regulator of virulence factors in *Pseudomonas aeruginosa*. VarA is believed to act upstream of TcpP/H and ToxT to regulate *ctxA* expression (28). The cognate sensor kinase responsible for phosphorylation of VarA has not been identified. VarA may be phosphorylated by VieS, and this positive regulation could compensate for the loss of positive regulation by VieA in the Δ*vieAB* strain background. Similarly, it is possible that VieA can be phosphorylated by the cognate sensor for VarA and that this phosphorylation can compensate for loss of phosphorylation by VieS in a Δ*vieS* strain background. By this reasoning, reduced *ctxA* expression would be observed only if both *vieS* and *vieA* were deleted, as in the Δ*vieSAB* strain.

The VieSAB system may regulate *ctxAB* expression by affecting transcription of *toxT*, because reduced amounts of *toxT* transcript were observed in the Δ*vieSAB* mutant in comparison to the wild type by RPA. Since the initial induction of both *ctxA* and *toxT* is like that of the wild type in a Δ*vieSAB* strain background, signaling through ToxR, which sits atop the virulence gene regulatory cascade, is likely not affected by mutations in the VieSAB system. Decreased production of ToxT protein in the Δ*vieSAB* mutant may prevent this strain from

maintaining wild-type levels of *ctxA* transcription after the initial induction, as observed in time course RPAs. One limitation of using RPAs to examine transcriptional regulation is that the assay measures steady-state levels of RNA and cannot distinguish between differences in promoter activity or RNA degradation, although we favor a role for VieSAB in promoter activity. We attempted to confirm that ToxT protein levels are reduced in the Δ*vieSAB* mutant by assessing the transcription of *tcpA*, another gene positively regulated by ToxT, by using the RIVET system. Induction of the *tcpA*::*tnpR135* fusion was not affected by the Δ*vieSAB* mutation, suggesting either that there is no difference in the level of ToxT or that the amount of ToxT protein, although reduced, is still sufficient to activate *tcpA* transcription. Consistent with a reduction in expression of CT, but not TcpA, caused by deletion of *vieSAB*, the Δ*vieSAB* strain is not reduced in colonization of the infant mouse small intestine (17). It is known that TcpA is absolutely essential for colonization in this model (27), whereas CT is dispensable (26).

All RPAs presented in this work utilized an internal control probe against *rpoB* to confirm that equivalent amounts of RNA were analyzed. This probe should be useful as an internal control for any experiment, because it does not interfere with the ability of other probes to hybridize with the complementary mRNA. In addition, the medium in which *V. cholerae* is grown does not affect the level of *rpoB* transcript, because similar amounts were observed in RNA isolated from wild-type cells grown in LB or AKI broth. Finally, expression of *rpoB* is autoregulatory, so mutations in other regulatory systems should not affect *rpoB* transcription (7). One potential limitation of this internal control is that the level of *rpoB* transcript is growth rate dependent. As cells reach the end of exponential growth in AKI broth (7 h), the amount of *rpoB* transcript decreases relative to the total amount of RNA in the cell. Thus, to analyze a mutant compared to the wild type with this control, it is essential to harvest RNA from cells that are in the same growth phase. Because mutations in the *vieSAB* locus did not affect the growth rate of *V. cholerae* under the conditions tested, use of the *rpoB* control is reasonable.

While this work confirms that the VieSAB system contributes to regulation of CT expression, it raises many new questions. Although we have provided some evidence that the system regulates *toxT* expression, further work is needed to determine which response regulator is important for this function and to determine whether it acts directly at the *ctxA* promoter, the *toxT* promoter, or perhaps another promoter upstream of ToxT in the regulatory cascade. The VieA response regulator is the most likely candidate to regulate *ctxAB* expression, because differences in *ctxA* expression were observed under in vitro conditions in which *vieB* is not expressed (17).

The environmental signal or signals that VieS senses to upregulate expression of CT are also unclear. The AKI condition used to induce expression of the ToxR regulon in El Tor *V. cholerae* is a complex condition with many signals that could potentially upregulate toxin production, including nutrient availability, oxygen availability, and pH. The VieSAB system shares some homology with the BvgAS two-component signal transduction system of *Bordetella pertussis*, which regulates expression of virulence genes, including pertussis toxin. While in vitro conditions that inhibit the activity of the BvgAS pathway have been identified (growth at 25°C or in medium containing magnesium sulfate or nicotinic acid), the signals that activate this system during growth in the host have not been identified (4). A conserved motif search of VieS indicates that the protein contains two motifs similar to bacterial extracellular solute-binding motifs that bind amino acids. This observation suggests that the kinase activity of VieS may be activated by the binding of particular amino acids to the periplasmic domain. We have preliminary evidence to suggest that in the classical biotype of *V. cholerae*, CT protein production stimulated by the addition of amino acids to minimal medium is abrogated when the VieSAB system is inactivated (Anna D. Tischler and Andrew Camilli, unpublished data). We plan to continue studying this system by using the classical background to determine what specific amino acid or acids activate the VieS sensor kinase. Presumably, this amino acid inducing signal is also present in the complex AKI medium, which is an inducing condition for El Tor biotype *V. cholerae*, as well as in the small intestine, to allow for full induction of CT production.

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