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The Virulence Transcriptional Activator AphA Enhances Biofilm Formation by *Vibrio cholerae* by Activating Expression of the Biofilm Regulator VpsT[▽]

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***Vibrio cholerae* is the agent of the severe diarrheal disease cholera, and it perpetuates in aquatic reservoirs when not in the host. Within the host's intestines, the bacteria execute a complex regulatory pathway culminating with the production of virulence factors that allow colonization and cause disease. The ability of *V. cholerae* to form biofilms is thought to aid its persistence in the aquatic environment and passage through the gastric acid barrier of the stomach. The transcriptional activators VpsR and VpsT are part of the biofilm formation-regulatory network. In this study, we screened a *V. cholerae* genomic library in *Escherichia coli* cells containing a P_{vpsT}-*luxCDBAE* transcriptional fusion reporter and found that a plasmid clone containing the *aphA* gene activates the expression of *vpsT* in *E. coli*. AphA is a master virulence regulator in *V. cholerae* that is required to activate the expression of *tcpP*, whose gene products in turn activate all virulence genes including those responsible for the synthesis of the toxin-coregulated pilus (TCP) and cholera toxin through the activation of *toxT*. AphA has a direct effect on the *vpsT* promoter, as gel shift experiments demonstrated that AphA binds to the *vpsT* promoter region. Furthermore, *V. cholerae aphA* mutants exhibit significantly lower levels of *vpsT* expression as well as reduced biofilm formation. AphA thus links the expression of virulence and biofilm synthesis genes.**

Vibrio cholerae is the causative agent of the devastating diarrheal disease cholera. Human infection normally begins with the oral ingestion of food or water contaminated with *V. cholerae*. Bacteria that survive the acid shock of the stomach proceed to penetrate the mucus layers of the intestinal epithelium, which leads to the production of an array of virulence factors that facilitate adherence and colonization. The coordinate expression of *V. cholerae* virulence genes results from the activity of a cascading system of regulatory factors (25). Many virulence genes are controlled directly by ToxT. The transcription of *toxT* is regulated by the ToxRS and TcpPH proteins in response to environmental signals (10). The additional transcriptional regulators AphA and AphB function together to activate *tcpPH* expression (16, 17, 32). The expression of *aphA* is repressed by quorum sensing through the master quorum-sensing regulator HapR (18).

Between epidemics, *V. cholerae* cells live in natural aquatic habitats in association with various plankton and zooplankton, often in the form of biofilms. Various studies suggested that biofilm-mediated attachment to abiotic surfaces may be important for *V. cholerae* survival in the environment (37, 38, 41). Biofilm formation in *V. cholerae* is a multistep developmental process that is controlled by several regulatory pathways (37, 42). The surface attachment of *V. cholerae* activates the transcription of the *vps* (*Vibrio* polysaccharide synthesis [VPS]) genes, which are responsible for the synthesis of the VPS exopolysaccharide (13, 28, 41). VPS production is critical for biofilms, as a deletion in any *vps* gene completely abolishes biofilm formation. The regulation of VPS synthesis in *V. cholerae* is very complex. Environmental signals such as monosaccharides, nucleosides, indole, and osmolarity have been identified as being activators of *vps* gene transcription and biofilm formation (11, 13, 29, 31). VpsT and VpsR are positive regulators of *vps* expression (1, 34, 39). VpsT and VpsR positively autoregulate their own expression and also form a complex regulatory network by positively regulating each other's expression (1, 40). Quorum sensing negatively regulates biofilm formation, as mutations in the master quorum-sensing regulator HapR increase *vps* expression and biofilm formation (8, 40, 43). HapR directly represses the expression of *vpsT* (36) and also regulates the expression of *vpsR* and one of the *vps* genes (35, 40). Recently, it was reported that a hybrid sensor histidine kinase, VpsS, uses components of the quorum-sensing pathway to modulate biofilm formation (30). Moreover, the transcription of genes involved in biofilm formation is enhanced by the cytoplasmic signaling molecule cyclic di-GMP (c-di-GMP) and negatively regulated by the cyclic AMP (cAMP)-cAMP receptor protein (CRP) regulatory complex (5, 12, 19–22, 34).

Bacterial biofilms have been demonstrated to be clinically significant for many human pathogens because biofilm-associated bacteria are less susceptible to host immune responses and antimicrobial agents (3). Biofilm formation is also important for *V. cholerae* pathogenesis. Several lines of evidence suggested that *V. cholerae* may enter hosts in the form of biofilms. The concentration of *V. cholerae* required to induce symptomatic cholera is estimated to be approximately 10^4 to 10^6 total cells, a concentration easily achieved by bacteria within biofilms accumulated on biotic or abiotic surfaces (2).

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Evidence to support this was provided when the number of cholera cases in a Bangladeshi village dramatically declined following crude water filtration through sari cloth (2). Furthermore, free-living *V. cholerae* bacteria are highly sensitive to low pH, while biofilm-associated *V. cholerae* bacteria are more acid resistant, resulting in the hypothesis that this increased resistance to acid may promote survival during passage through the stomach (43). However, it is unclear how *V. cholerae* regulates its biofilm formation during infection. In this study, we performed a genetic screen and found that the virulence activator AphA enhances biofilm formation through the activation of *vpsT* expression, which provides a link between the pathways of virulence factor production and biofilm formation.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. All *V. cholerae* strains used in this study were derived from E1 Tor C6706 (33) and were propagated in Luria broth (LB) medium containing appropriate antibiotics at 37°C. For biofilm formation, test tubes containing *V. cholerae* cultures were incubated at 22°C without shaking. Transcriptional fusion reporters were constructed by cloning promoter sequences of the genes of study into pBBR-lux, which contains a promoterless *luxCDABE* reporter (9), digested with BamHI and SacI. The Lux reporter containing a deletion of the putative AphA binding site TATACA in the *vpsT* promoter region was constructed by overlapping PCR and cloned into plasmid pBBR-lux as described above. The plasmids containing P_{tac}-*aphA or* P_{tac}-*aphB* were constructed by cloning *aphA* and *aphB* coding sequences into pMal-c2x (New England Biolabs) digested with NdeI and HindIII (the *malE* gene is replaced by *aphA* or *aphB*). The plasmid containing P_{BAD}-*aphA* was constructed by cloning *aphA* coding sequences into pBAD24 (7) digested with EcoRI and HindIII. The plasmid overexpressing recombinant AphA was constructed by cloning the *aphA* coding sequences into pET32a (EMD Biosciences) digested with NdeI and XhoI. The resulting plasmid that produces the AphA-His_6 C-terminal fusion was then introduced into *Escherichia coli* strain BL21(DE3) (Promega). In-frame deletions of *aphA*, *vpsT*, *vpsR*, *lrp*, and *cdgC* were constructed by cloning the regions flanking the target genes into suicide vector pWM91 containing a *sacB* counterselectable marker (27). The resulting plasmids were introduced into *V. cholerae* by conjugation, and deletion mutants were selected for double homologous recombination events.

Screening for *vpsT* activators in *E. coli*. The *V. cholerae* genomic library was constructed by cloning partially HincII-digested *V. cholerae* genomic DNA fragments into pEZ-Seq-Amp (Lucigen). *E. coli* DH5α transformants containing library plasmids and the plasmid harboring P_{vpsT}-*luxCDABE* were spread onto LB agar plates containing the appropriate antibiotics. Bright colonies were restreaked, and plasmids were purified for sequencing.

Measurement of transcriptional expression using Lux reporters. Bacterial cultures were grown without shaking at 22°C for 24 h, and the luminescence of either planktonic cells or biofilm-associated cells was read by using a Bio-Tek Synergy HT spectrophotometer and normalized for growth against the optical density at 600 nm (OD_{600}). Lux expression is reported as light units/OD_{600}.

Gel retardation assays. *E. coli* BL21(DE3) cells containing a plasmid overexpressing C-terminal His-tagged AphA were grown at 37°C and induced with 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) at 16°C. The AphA-His_6 protein was purified through nickel columns according to the manufacturer's instructions (Qiagen). PCR products containing various lengths of the *vpsT* promoter region and the full *tcpP* promoter region were digested with EcoRI and end labeled by using [α-^{32}P]dATP and the Klenow fragment of DNA polymerase I. Binding reaction mixtures contained 0.1 ng DNA and His_6-AphA proteins in a buffer containing 10 mM Tris-HCl (pH 7.9), 1 mM EDTA, 1 mM dithiothreitol (DTT), 60 mM KCl, and 30 mg/ml calf thymus DNA. After 20 min of incubation at 37°C, samples were size fractionated by using 5% polyacrylamide gels in 1× TAE buffer (20 mM Tris-acetate, 1 mM EDTA [pH 8.5]). The radioactivity of free DNA and AphA-DNA complexes was visualized by using a Typhoon 9410 variable model imager (Molecular Dynamics).

Biofilm formation assays. *V. cholerae* strains were grown on LB agar plates overnight and resuspended in LB at an OD_{600} of ~0.6. A 1:100 dilution of this suspension was then inoculated into 1 ml of LB in glass test tubes. Biofilms were formed by allowing these cultures to stand at room temperature. At the time points indicated, culture supernatants were removed, and biofilms were washed in LB medium. Biofilm formation was quantified by use of crystal violet as described previously (44).

Infant mouse colonization assays. The infant mouse colonization assay was performed as previously described (6), by inoculating approximately 10^5 *V. cholerae* cells per mouse into 6-day-old suckling mice. After a period of colonization, intestinal homogenates were collected, and the ratio of the two strains was determined by plating onto LB agar containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and normalized against the ratio of the two strains grown *in vitro*.

RESULTS AND DISCUSSION

Identification of AphA as a positive regulator of *vpsT*. *V. cholerae* biofilm formation requires a number of transcriptional regulators. The expression of one of the key regulators, VpsT, is known to be controlled by a number of regulatory components, such as quorum sensing (42), c-di-GMP (21), the biofilm activator VpsR (40), and osmolarity (31). To further investigate whether additional regulation is involved in *vpsT* transcription, we constructed a P_{vpsT}-*luxCDABE* transcriptional fusion on a plasmid. We found that *V. cholerae* strains containing this plasmid produced light (data not shown), but *E. coli* cells with the P_{vpsT}-*luxCDABE* plasmid were dim (Fig. 1A, left two panels). We reasoned that a *V. cholerae* factor necessary for the activation of *vpsT* may not be present in *E. coli* to induce its expression. Thus, we introduced a *V. cholerae* genomic library into *E. coli* strains containing P_{vpsT}-*luxCDABE* plasmids and screened for bright transformants. Of approximately 50,000 transformants screened, 11 bright colonies were isolated (Fig. 1A, right two panels). The plasmid from each isolate (pEMF0) was purified, and analysis by restriction enzyme digestion indicated that they all contained a similar 7.2-kb insertion and were possibly siblings of one original clone. DNA sequencing revealed that this fragment covered the *V. cholerae* genome region of VC2643 to VC2649 (Fig. 1B). Although most of the genes in this region encode products involved in bacterial metabolism, VC2647 encodes a key virulence activator, AphA. AphA is required for the induction of another virulence regulator, TcpP (32), and it also regulates the expression of a number of other genes, including a gene encoding penicillin amidase (15) and genes encoding enzymes involved in acetoin biosynthesis (14).

To test whether AphA can activate *vpsT* expression in *E. coli*, we constructed a plasmid containing P_{tac}-*aphA* (pEMF6). In the absence of IPTG, the level of *vpsT* expression was low (data not shown). The overexpression of *aphA* in the presence of IPTG (100 μM, to induce the P_{tac} promoter) induced *vpsT* expression to levels similar to those when *aphA* was expressed from pEMF0 (Fig. 1C). This construct could also activate *tcpP* expression in *E. coli* (data not shown). These data suggest that AphA plays a role in the regulation of *vpsT* expression, at least in *E. coli*. In its previously described role of activating the *tcpPH* promoter, AphA acts cooperatively with its partner protein, AphB (16). To determine whether AphB could play a role in the activation of *vpsT*, we constitutively expressed *aphB* and found that AphB was unable to drive *vpsT* expression (Fig. 1C), indicating that AphB cannot promote *vpsT* activity and that AphA may be the primary activator.

AphA binds directly to the *vpsT* promoter region. To investigate whether AphA activation of *vpsT* is direct or acts through another regulator present in *E. coli*, we purified AphA

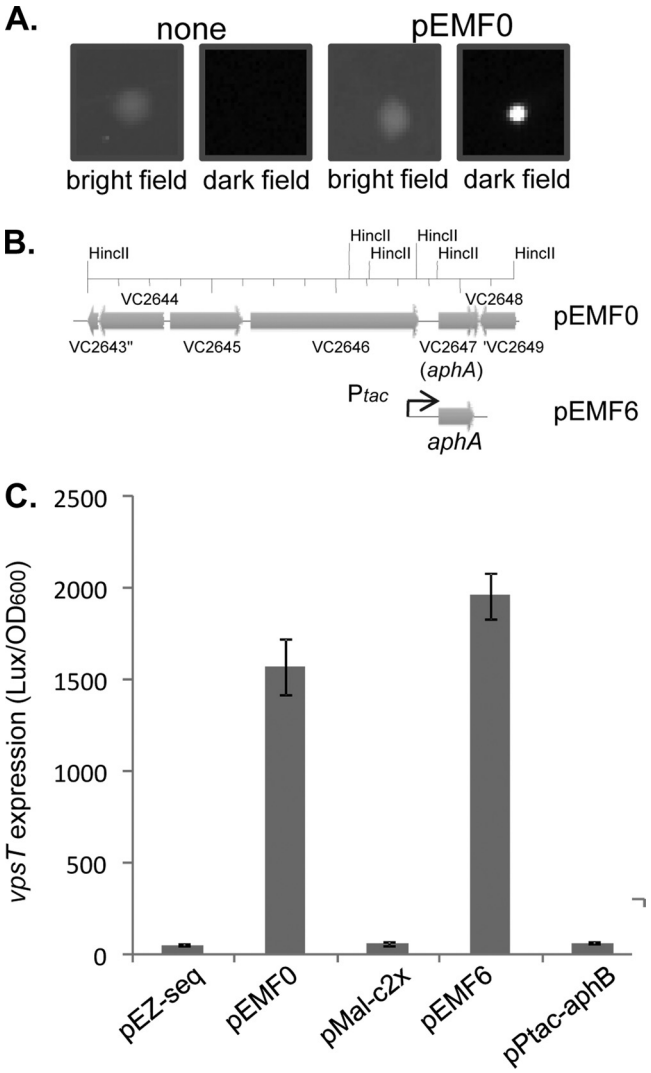

FIG. 1. AphA is required to induce *vpsT* in *E. coli* cells. (A). Luminescence production of *E. coli* DH5α colonies containing the P_{vpsT}-*luxCDABE* plasmid and *V. cholerae* genomic library plasmids. Pictures of colonies were taken either in light or in the dark. (B) Genetic organization of a *V. cholerae* fragment (in pEMF0) that activates *vpsT* expression. pEMF6 was constructed by cloning *aphA* into the pMal-c2x vector (New England Biolabs). (C) Luminescence of *E. coli* DH5α strains containing P_{vpsT}-*luxCDABE* with vector controls, pEMF0, pEMF6, or a plasmid overexpressing *aphB*. Cells were grown in the presence of 0.1 mM IPTG in LB at 37°C for 8 h, and luminescence was measured by using a Bio-Tek plate reader and normalized against the OD_{600}. The results are the averages of data from three experiments. Error bars indicate standard deviations.

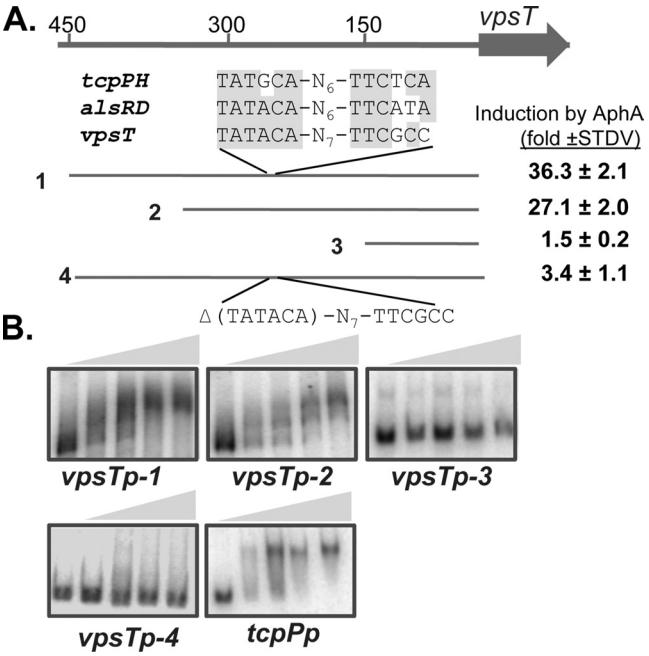

FIG. 2. AphA binds to the *vpsT* promoter region to regulate *vpsT* gene expression. (A) The *vpsT* locus and putative AphA-binding-site sequences in the promoter. Numbers are relative to the translational start of *vpsT*. The four promoter regions indicated were PCR amplified and cloned into pBBR-lux containing a transcriptional Lux reporter (9). The induction of *vpsT* by AphA was calculated by comparing Lux expression levels with pMal-c2x (vector control) or with pEMF6 (P_{tac}-*aphA*) in the presence of 0.1 mM IPTG. STDV, standard deviation. (B) Gel shift assays using purified AphA-His_6 and DNA as shown above or the *tcpP* promoter as a control. Protein concentrations used in the gel shift assay (shown as shaded triangles) were 0, 10, 20, 40, and 80 ng/reaction mixture.

as a His_6 fusion. Recombinant AphA is functional, as it could activate both *tcpP* and *vpsT* in *E. coli* (data not shown). We then performed electrophoretic mobility shift assays (EMSAs) using AphA-His_6 and various lengths of *vpsT* promoter DNA (Fig. 2A). We also included *tcpP* promoter DNA as a control. Figure 2B shows that purified AphA-His_6 was able to shift the two large *vpsT* promoter fragments as well as control *tcpP* DNA with similar affinities. All of these mobility shifts could be inhibited by the addition of unlabeled DNA, indicating that the binding of AphA to these DNA sequences is specific (data not shown). AphA was unable to shift the shortest *vpsT* promoter fragment encompassing 150 bp upstream of the *vpsT* translational start site, suggesting that the AphA binding site is located between 150 and 450 bp upstream of the *vpsT* gene. Consistent with the gel shift data, AphA could not induce *vpsT* expression when the 150-bp fragment was fused with the *luxCDABE* reporter in *E. coli* DH5α cells (Fig. 2A). Of note, we did not rule out the possibility that this fragment may be too small to include all the regulatory components of the *vpsT* promoter. However, by searching the *vpsT* promoter region for the AphA binding site identified previously (14), we found that a similar region is located around 240 bp upstream of *vpsT*. We deleted TATACA from the *vpsT* promoter sequences and found that AphA failed to activate *vpsT* expression (measured by the Lux reporter fusion) (Fig. 2A) and that AphA proteins could not bind to the mutated *vpsT* promoter region (*vpsT*p-4) (Fig. 2B). Further study is required to confirm that AphA binds to this sequence on the *vpsT* promoter. Taken together, these data suggest that AphA directly regulates *vpsT* expression.

AphA regulates *vpsT* expression and biofilm formation in *V. cholerae*. We next examined the effect of natively produced AphA on *vpsT* expression and biofilm formation in *V. cholerae*. We first compared *vpsT* expression by monitoring light production in strains containing wild-type *aphA* or an *aphA* in-

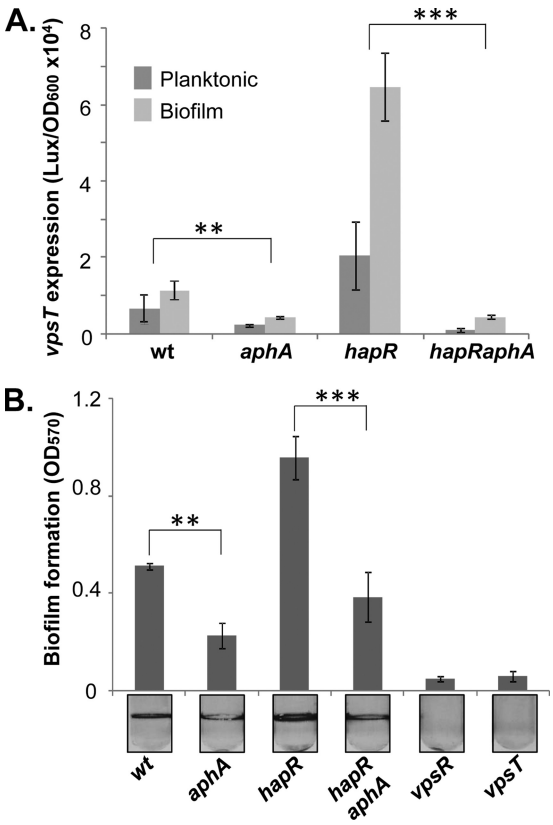

FIG. 3. AphA modulates *vpsT* expression and biofilm formation in *V. cholerae*. Different *V. cholerae* strains containing *vpsT-luxCDABE* plasmids were grown without shaking in LB for 24 h. Both planktonic and biofilm cells were withdrawn. (A) Luminescence was measured, and data were normalized against the OD_{600}. (B) The biofilm mass was determined by staining surface-attached cells with crystal violet and dimethyl sulfoxide (DMSO) quantitation. The results are the averages of data from three experiments. Error bars indicate standard deviations. The Student *t* test for *P* value was performed (**, *P* value of <0.05; ***, *P* value of <0.01). wt, wild type.

frame deletion with the P_{vpsT}-*luxCDABE* plasmid. The level of expression of *vpsT* was higher in biofilm-associated cells than in planktonic cells (Fig. 3A), consistent with previous findings that the *vps* gene expression level is elevated in biofilm cells compared to planktonic cells (1). The *aphA* mutants exhibited low *vpsT* expression levels in both planktonic and biofilm-associated cells (approximately one-third of the *vpsT* expression level in the wild type; Student *t* test *P* value of <0.05) (Fig. 3A and Table 1), and the overexpression of *aphA* in *aphA* mutants restored *vpsT* expression (Table 1), indicating that AphA is involved with *vpsT* regulation in *V. cholerae*. In addition, we tested the effect of AphA on *vpsT* expression in *hapR* mutants, as the expression of *aphA* itself is repressed by quorum sensing through the master quorum-sensing regulator HapR (18). We found that the effects of AphA on *vpsT* in both planktonic and biofilm cells became more prominent (Fig. 3A). We reason that since the *aphA* expression level is higher in *hapR* mutants, the deletion of *aphA* in the *hapR* mutant background produces a greater effect on *vpsT* expression.

It was previously reported that the expression of *vpsT* is regulated by a number of factors, including VpsR and c-di-GMP (42). Interestingly, the expression of *aphA* is activated by VpsR and the leucine-responsive regulatory protein (Lrp) (23). To determine whether VpsR and Lrp regulations of *vpsT* act through the activation of *aphA*, we compared P_{vpsT}-*luxCDABE* expression levels in *vpsR* mutants in the presence or absence of overexpressed AphA (Table 1). The level of expression of *vpsT* in *vpsR* mutants was very low, and the overexpression of AphA only slightly increased *vpsT* expression levels. On the other hand, the overexpression of AphA could restore *vpsT* expression in *lrp* mutants. These data suggest that VpsR is a major positive regulator of *vpsT* expression. In addition, unlike data from a previous report (22), a deletion in *cdgC*, encoding a GGDEF-EAL domain protein, did not affect *vpsT* expression (Table 1). It is possible that the difference in strain background and rugose/smooth phenotypes results in these discrepancies.

Because VpsT is an essential activator for *vps* genes, a reduction of *vpsT* expression levels will lead to a decrease in biofilm formation. We thus measured the biofilm-forming capacities of different *V. cholerae* strains. The deletion of either *vps* activator (VpsR or VpsT) abolished biofilm formation in this *V. cholerae* strain (C6706 background) (Fig. 3B). The amount of biofilm formed by *aphA* mutants was significantly smaller than that formed by $aphA^+$ strains. However, biofilm formation was not completely abolished in *aphA* mutants as it was in *vps* mutants (Fig. 3B and data not shown). This finding together with the observation that the overexpression of AphA in the *vpsR* mutant could not restore *vpsT* expression (Table 1) suggest that AphA plays a modulating role in *V. cholerae* biofilm formation.

Temporal requirements of AphA during *V. cholerae* biofilm formation. To further understand the physiological role of the AphA regulation of biofilm formation, we first examined the expression of *aphA* in planktonic and biofilm-associated cells using a plasmid containing P_{aphA}-*luxCDABE*. Figure 4A shows that the *aphA* expression level in both the wild type and *hapR* mutants was significantly higher in biofilm-associated cells than in planktonic cells. Interestingly, since HapR represses *aphA* expression (24) and is present in higher levels in biofilm cells than in planktonic cells, the elevated level of expression of *aphA* in biofilms suggests that additional regulatory components are involved with *aphA* regulation in biofilms. Previous studies showed that environmental conditions such as temper-

TABLE 1. Effects of AphA and other regulatory components on *vpsT* expression

Genotype[b]	Avg (SD) *vpsT* expression level (light units/OD_{600} [10^3])[a]	
	Vector	pBAD-aphA
Wild type	6.1 (0.4)	15.7 (0.5)
aphA	2.1 (0.03)	7.1 (0.04)
vpsR	0.092 (0.02)	1.6 (0.02)
lrp	2.3 (0.99)	11.7 (2.5)
cdgC	7.0 (1)	13.9 (0.4)

[a] Values presented are the averages of three replicates. The numbers in parentheses indicate standard deviations. Cultures were grown at 22°C without shaking for 24 h. A total of 0.1% arabinose was added to the cultures. Luminescence was measured by using planktonic cells.

[b] The wild type and the various in-frame deletion mutants indicated containing the P_{vpsT}-*luxCDABE* reporter were introduced with either the vector control (pBAD24) or an *aphA* overexpression plasmid (pBAD-aphA).

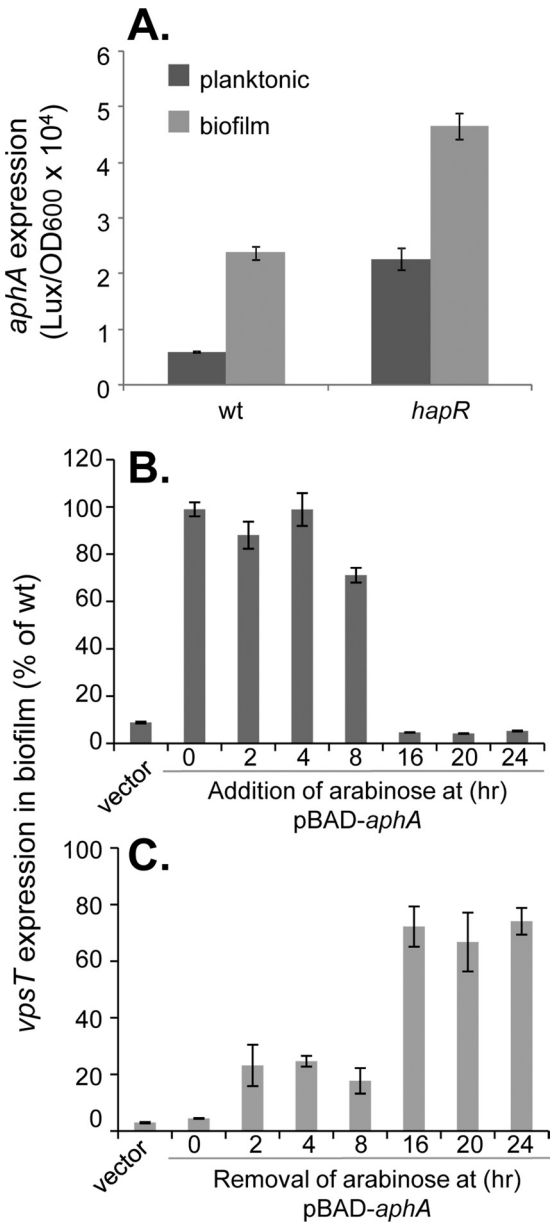

FIG. 4. Temporal requirements of AphA in *V. cholerae* biofilm formation. (A) Expression of *aphA* in planktonic and biofilm-associated cells. The wild type and *hapR* mutants containing P_{aphA}-*luxCDABE* plasmids were grown in LB at 22°C for 24 h, and the luminescence of planktonic and biofilm cells was measured and normalized against the OD_{600}. (B and C) *vpsT* expression in biofilm-associated cells of *aphA* mutants containing pBAD-*aphA* plasmids. The *aphA* mutants containing pBAD24 were used as a vector control. The level of *vpsT* expression was compared to that of wild-type bacteria containing the P_{vpsT}-*luxCDABE* plasmid. (B) The strains were inoculated in LB in the absence of arabinose, 0.1% arabinose was then added to the cultures at the time indicated, and growth continued for a total of 24 h at 22°C. (C) The strains were inoculated in LB in the presence of 0.1% arabinose. At the time points indicated, the culture supernatants were removed and replaced with equal volumes of fresh LB. After a total of 24 h of incubation, luminescence was measured and normalized against the OD_{600}. The data were then compared with the *vpsT* expression level (Lux/OD_{600}) of the wild-type strain in biofilms incubated for 24 h at 22°C and are presented as a percentage of wild-type *vpsT* expression levels. The results are averages of data from three experiments. Error bars indicate standard deviations.

ature and pH do not alter *aphA* expression in LB medium (32). Throughout its natural cycle of infection, however, *V. cholerae* encounters many various environments that present unique challenges for bacterial survival and colonization. Therefore, it is possible that there is an unknown condition that may alter *aphA* expression and affect biofilm formation at some point in time during infection. We thus tested the temporal effect of AphA on *vpsT* expression by using *V. cholerae aphA* mutants containing an inducible P_{BAD}-*aphA* plasmid. This strain formed biofilms similar to that of the wild type when 0.1% arabinose was included in the medium (data not shown). We then added or removed arabinose at different time points during biofilm development and measured *vpsT* expression in biofilms over a 24-h incubation period. When induced with arabinose up to 8 h after the beginning of incubation, we were able to restore *vpsT* expression levels to wild-type levels. In contrast, when pBAD-*aphA* was induced later during incubation, little expression of *vpsT* was observed (Fig. 4B). Similarly, the early removal of arabinose (up to 8 h postinoculation) resulted in lower *vpsT* expression levels (Fig. 4C). Taken together, these data suggest that AphA and, therefore, VpsT are required during the earlier stages of biofilm formation. *aphA* is expressed at a low cell density in LB, but how *aphA* is regulated temporally under certain environmental conditions and in host intestines is currently unclear and will be further investigated.

Roles of AphA-regulated biofilm formation in the infant mouse colonization model. Biofilm formation was previously shown to be important for *V. cholerae* survival in aquatic environments and resistance to gastric acids during passage through the host stomach (43). It is unclear, however, whether *V. cholerae* biofilm formation-related genes are important during infection. We used the infant mouse model to test the colonization abilities of *aphA*, *vpsT*, and *vps* mutants. The *aphA* mutant was profoundly defective in colonization (data not shown), consistent with a previous report showing that AphA is required to activate virulence genes and subsequently colonize the intestines (17). Both *vpsT* and *vps* mutants that abolished biofilm formation *in vitro* (Fig. 3B and data not shown) could colonize infant mice as well as the wild type (data not shown), suggesting that VPS-dependent biofilm formation may not be critical for the colonization of the infant mouse. Since *V. cholerae* infection of infant mice does not replicate some naturally occurring human cholera symptoms (e.g., watery diarrhea), this model may not be suitable for analyzing *in vivo* biofilm formation. Further studies using other animal models are required to elucidate the relationship between virulence gene induction and biofilm formation controlled by the shared regulator AphA.

In this study, we demonstrate that the indispensable virulence gene regulator AphA is also capable of promoting *V. cholerae* biofilm development through the direct stimulation of the *vpsT* promoter. This adds another regulatory component to the already complex regulatory pathway of biofilm formation. Throughout its natural cycle of infection, *V. cholerae* encounters several various environments that present challenges for bacterial survival and colonization. Endurance in hostile environments is facilitated by encapsulation within the matrix of a protective biofilm structure, and host colonization is aided by the expression of various virulence factors activated by a signal transduction cascade. AphA serves as a vital con-

nection between these two processes. It is possible that during initial infection, *V. cholerae* cells utilize AphA to upregulate virulence factor production through the activation of *tcpP* expression, facilitating the bacterial colonization of intestinal surfaces. Simultaneously, AphA also enhances *V. cholerae* biofilm formation in the host, leading to resistance to various antimicrobial agents. Although biofilm formation apparently is not important for *V. cholerae* colonization in the infant mouse model, a recent report (4) indicated that stool samples collected from cholera patients contain a heterogeneous mixture of biofilm-like aggregates and free-swimming planktonic cells of *V. cholerae*. An estimation of the relative infectivity of these different forms of *V. cholerae* cells suggested that the enhanced infectivity of *V. cholerae* shed in human stools (26) is due largely to the presence of biofilm-like structures. *aphA* expression is repressed by quorum sensing at a high cell density (18). This repression presumably happens late in *V. cholerae* infection and may help bacteria exit the host (44). However, since AphA is required during the early stages of biofilm formation (Fig. 4), quorum-sensing repression of *aphA* expression may not affect biofilm structures. On the other hand, once *V. cholerae* bacteria come out of the host, the derepression of *aphA* in planktonic *V. cholerae* cells (becoming low-cell-density states) may help initiate biofilm formation in the environment. Intriguingly, the expression of *aphA* itself is also activated by the biofilm formation regulator VpsR (23). VpsR and VpsT also positively autoregulate their own and each other's expressions (1, 40). It may be interesting to further study how these positive regulatory loops contribute to the success of *V. cholerae* as a human pathogen and a colonizer of the aquatic environment.

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