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Bile Acids and Bicarbonate Inversely Regulate Intracellular Cyclic di-GMP in *Vibrio cholerae*

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***Vibrio cholerae* is a Gram-negative bacterium that persists in aquatic reservoirs and causes the diarrheal disease cholera upon entry into a human host. *V. cholerae* employs the second messenger molecule 3′,5′-cyclic diguanylic acid (c-di-GMP) to transition between these two distinct lifestyles. c-di-GMP is synthesized by diguanylate cyclase (DGC) enzymes and hydrolyzed by phosphodiesterase (PDE) enzymes. Bacteria typically encode many different DGCs and PDEs within their genomes. Presumably, each enzyme senses and responds to cognate environmental cues by alteration of enzymatic activity. c-di-GMP represses the expression of virulence factors in *V. cholerae*, and it is predicted that the intracellular concentration of c-di-GMP is low during infection. Contrary to this model, we found that bile acids, a prevalent constituent of the human proximal small intestine, increase intracellular c-di-GMP in *V. cholerae*. We identified four c-di-GMP turnover enzymes that contribute to increased intracellular c-di-GMP in the presence of bile acids, and deletion of these enzymes eliminates the bile induction of c-di-GMP and biofilm formation. Furthermore, this bile-mediated increase in c-di-GMP is quenched by bicarbonate, the intestinal pH buffer secreted by intestinal epithelial cells. Our results lead us to propose that *V. cholerae* senses distinct microenvironments within the small intestine using bile and bicarbonate as chemical cues and responds by modulating the intracellular concentration of c-di-GMP.**

Vibrio cholerae is a Gram-negative bacterium responsible for the human diarrheal disease cholera. This bacterium primarily resides in marine reservoirs and associates with aquatic organisms by preferentially forming biofilms on chitinous surfaces (1–3). *V. cholerae* multiplies in the small intestine and upregulates two key virulence factors, toxin-coregulated pilus (TCP) and cholera toxin (CT), to cause severe and acute diarrhea. Modulation of the second messenger 3′,5′-cyclic diguanylic acid (c-di-GMP) by *V. cholerae* is thought to be one mechanism by which *V. cholerae* mediates the transition from life in aquatic environments to a virulent state in the human host. It is well documented that c-di-GMP regulates many bacterial phenotypes, including biofilm formation, motility, expression of virulence genes, and cell cycle progression (4–9). It has been proposed that the intracellular c-di-GMP concentration in *V. cholerae* is relatively high in aquatic environments, leading to a sessile biofilm-forming lifestyle, whereas in the human host, reduced c-di-GMP concentrations stimulate virulence factor expression (10). Due to the technical limitations of measuring intracellular c-di-GMP, this model has not been directly tested, and the specific environmental cues that regulate the transition between these two niches remain unknown.

c-di-GMP is synthesized by diguanylate cyclase (DGC) enzymes and hydrolyzed by c-di-GMP-specific phosphodiesterase (PDE) enzymes. All DGCs contain a conserved C-terminal GGDEF domain with a GG[D/E]EF active site motif and a highly variable N terminus that often carries conserved signal recognition domains such as GAF, PAS, or receiver domains (11, 12). Conversely, c-di-GMP PDEs contain either a conserved EAL or a HD-GYP domain in their C terminus (13, 14). Bacteria typically encode many DGCs and PDEs within their genome; *V. cholerae* possesses 61 predicted c-di-GMP turnover enzymes (15). It is not well understood why *V. cholerae* encodes so many DGCs and PDEs, and the functions of the variable N-terminal domains for most of these enzymes have not been characterized. One hypothesis is that each DGC or PDE senses and responds to a specific environmental signal by altering c-di-GMP synthesis or degradation activity in the C-terminal domain. While a few environmental cues, including light, oxygen, zinc, arginine, quorum sensing (QS) autoinducers, and norspermidine, have been shown to directly regulate DGCs or PDEs in various bacteria (14, 16–24), the environmental signals recognized by the vast majority of DGCs and PDEs remain unidentified.

Upon entering the human host, it is imperative that *V. cholerae* recognize environmental signals to mediate the transition from an aquatic bacterium to a human pathogen. One signal prevalent in the small intestine is bile. Bile is an antimicrobial substance secreted from the liver into the proximal small intestine that aids in digestion by emulsifying lipids. The composition of secreted bile is heterogeneous and includes inorganic salts, cholesterol, phospholipids, pigments, and bile acids (25). Bile acids are derived from cholesterol in the liver and are processed by hepatic cells to produce a mixture dominated by taurine and glycine conjugates (25, 26). Bile acids have detergent properties that enable the interaction between bile and digestive lipids. For a complete review on the properties of bile, refer to the work of Begley et al. (27).

The interplay between enteric bacteria and bile is extensive. In bacteria, there is evidence that bile causes oxidative stress and

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DNA damage and perturbs the cell membrane (28–30). To counteract this stress, *V. cholerae* upregulates the porin protein *ompU* in a *toxR*-dependent manner to increase bile resistance (31). *V. cholerae* also employs six resistance-nodulation-division family efflux pumps that have been implicated in bile resistance (32–34). Additionally, *V. cholerae* increases biofilm formation in a *vpsR*-dependent manner in the presence of bile acids, presumably to increase resistance to the deleterious effects of bile (35). It is clear that the sensing of bile by *V. cholerae* leads to distinct physiological changes, but a role for c-di-GMP in this process has not been described.

We hypothesized that bile is an environmental signal sensed by *V. cholerae* to recognize and adapt to growth in the human environment by modulating c-di-GMP signaling pathways. Based on current models postulating that *V. cholerae* reduces intracellular c-di-GMP in the human host, we predicted that bile acids would reduce global c-di-GMP concentrations. Surprisingly, we discovered that bile acids increase intracellular c-di-GMP. A screen of the activity of all 61 *V. cholerae* c-di-GMP turnover enzymes in the presence and absence of bile acids identified three DGCs that showed increased c-di-GMP synthesis in the presence of bile acids. Furthermore, a screen on the expression of all 61 *V. cholerae* c-di-GMP turnover enzymes revealed that bile acids inhibited the expression of one PDE. Deletion of these four enzymes abolished the induction of c-di-GMP by bile and negated the ability of *V. cholerae* to form biofilms in the presence of bile. Bicarbonate, a biological pH buffer secreted by intestinal epithelial cells in the small intestine, suppressed the bile-induced increases in intracellular c-di-GMP. We propose that bile and bicarbonate inversely control c-di-GMP levels in *V. cholerae*, allowing this bacterium to sense and adapt to local environmental niches within the small intestine.

MATERIALS AND METHODS

Growth conditions and molecular methods. The *V. cholerae* El Tor biotype strain C6706str2 was used for all experiments (36), and *Escherichia coli* strains DH10B (Invitrogen) and S17-λpir (37) were used to harbor and conjugate plasmid DNA into *V. cholerae*. The construction of the Δ*vpsL*, Δ*luxO*, and Δ*hapR* strains has been described elsewhere (38–40). For all experiments, unless otherwise specified, cultures were grown in Luria-Bertani (LB) medium at 35°C with shaking at 220 rpm. When necessary, medium was supplemented with kanamycin (Sigma) at 100 μg/ml or chloramphenicol (Sigma) at 10 μg/ml. The inducer isopropyl-β-D-thiogalactopyranoside (IPTG) was added at 0.1 mM when required.

Synthetic human bile (SHB) is a mixture of six purified conjugated bile acids added to LB medium at physiologically relevant concentrations to mimic the human small intestine (25, 26, 41, 42). All bile acids were purchased from Sigma. The conjugated bile acids added were taurocholate (0.46 mM), glycocholate (0.93 mM), taurochenodeoxycholate (0.46 mM), glycochenodeoxycholate (0.93 mM), taurodeoxycholate (0.32 mM), and glycodeoxycholate (0.64 mM). Bovine bile (BV; Sigma) was added as a supplement at 0.4% (wt/vol). 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) was added as a supplement at 0.4% (wt/vol), and sodium dodecyl sulfate (SDS) was added as a supplement at 0.01% (wt/vol) due to its potent bactericidal activity. Taurine and glycine were each added as a supplement at 4 mM. Sodium bicarbonate (BiC) was added as a supplement when indicated at 0.3% (wt/vol) (49.2 mM), consistent with cholera toxin-inducing conditions (43), while Tris was added as a supplement when indicated at 0.6% (wt/vol) (49.5 mM).

All of the protocols used in this study for DNA manipulation and plasmid construction were performed as previously described (44), and all strains, plasmids, and primers are listed in Table S1 in the supplemental material. The DNA polymerase Phusion (Thermo Scientific) was used for all PCRs. The expression plasmids for the DGCs and PDEs were constructed as described elsewhere (45). Briefly, these plasmids allow controlled expression of each DGC encoded by *V. cholerae* via induction of the Ptac promoter with IPTG. To construct the HD-GYP expression plasmids, each HD-GYP gene was amplified from the *V. cholerae* chromosome and then inserted into the pEVS143 vector using the EcoRI and BamHI insertion sites, as previously described (38). The identification of the c-di-GMP reporter plasmid 6:C9-*lux* is described elsewhere (46). The DGC mutant allele plasmids were generated using the Lightning site-directed mutagenesis kit (Agilent) with the DGC expression plasmid as the template using the ASM primers listed in Table S1. The VC2497-*lux* reporter was constructed by amplifying the promoter of VC2497 from the *V. cholerae* chromosome by PCR and then inserting it into the pBBRlux vector using the SacI and BamHI insertion sites.

To generate the DGC and PDE deletion strains, natural transformation and homologous recombination were used. A PCR product was generated that contained a chloramphenicol resistance cassette (*cat*) bordered by FLP recombination target (FRT) sites from the plasmid pKD3 (47), flanked by 500 bp upstream and downstream of the targeted gene using the primers KO1/KO2 and KO3/KO4. The PCR products generated with these promoters were fused to the *cat* gene using zipper PCR. Natural competence was induced by ectopically expressing *tfoX* (VC1153) (48) from the Ptac promoter using the plasmid pANDA2. pANDA2 was constructed by amplification of VC1153 with the primers CMW464 and CMW465 and insertion of this product into the EcoRI/BamHI sites of the plasmid pEVS143. Homologous recombination events were selected by growing the culture on LB medium supplemented with chloramphenicol at 1 μg/ml. The *cat* gene was then removed by ectopically expressing a FLP recombinase on the vector pTL17 (49).

Quantifying the intracellular concentration of c-di-GMP. All c-di-GMP quantifications were analyzed using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Unless otherwise specified, cultures were grown to an optical density at 600 nm (OD_{600}) between 0.6 and 0.9, and a 1.5-ml aliquot of this culture was removed and centrifuged for 30 s at maximum speed. The supernatant was immediately removed, and the pellet was resuspended in 100 μl of cold extraction buffer (40% acetonitrile, 40% methanol, 0.1 N formic acid) and incubated for 20 min at −20°C. The insoluble fraction was pelleted in a benchtop centrifuge at 4°C for 5 min, and the supernatant was collected and stored at −80°C. Prior to mass spectrometry, the extraction buffer was evaporated using a vacuum manifold. The pellet was then resuspended in 100 μl water. Ten microliters of each sample was then analyzed on a Quattro Premier XE mass spectrometer (Waters) coupled with an Acquity Ultra Performance LC system (Waters) as previously described (50). The intracellular concentration of c-di-GMP was calculated by dividing the intracellular c-di-GMP of the sample by the total volume of the extracted bacteria, which was estimated by multiplying the number of bacterial cells in the extract by the average volume of the bacterial cell. The average cellular volume was determined for each strain analyzed under each growth condition by measuring individual cell dimensions using differential image contrast microscopy and assuming the cells to be cylindrical.

Systematic screen of DGC and PDE activity. To determine the *in vivo* activity of each *V. cholerae* DGC, each of the 40 DGC expression vectors was conjugated into a Δ*vpsL* strain of *V. cholerae* harboring a separate reporter vector encoding a luciferase-transcriptional fusion of a c-di-GMP-inducible promoter located within the open reading frame (ORF) of VC1673 (6:C9-*lux* [46]). These strains were then grown in solid white 96-well clear-bottom plates (Costar 3903) in 150 μl of LB medium or LB medium with SHB in the presence of IPTG inoculated as a 1/100 dilution from an overnight culture. Luminescence and OD_{600} values were recorded in a SpectraMax M5 plate reader (Molecular Devices) after 8 h of growth and were reported as relative luminescence units (RLU). The screen for PDE activity was performed in the same manner as the DGC

assay with the addition of a third vector, pBRP02, which carries the *qrgB* allele of DGC under Ptac control. pBRP02 has the *Vibrio harveyi qrgB* allele cloned into the vector pMMB67eh (51), allowing it to coexist with the PDE expression vectors and the 6:C9-*lux* reporter plasmid.

Systematic screen of DGC and PDE expression. DGC-*gfp* (GGDEF domain proteins) and PDE-*gfp* (GGDEF + EAL and EAL domain proteins) transcriptional fusion plasmids were constructed as previously described (38). Strains containing the plasmids were grown in triplicate overnight and then inoculated 1/100 in 150 μl LB medium or LB medium with SHB in a Costar black, clear-bottom 96-well plate (catalog no. 3904). Cultures were grown for 8 h, and then fluorescence was quantified (excitation, 475 nm; emission, 510 nm) using an M5 SpectraMax plate reader (Molecular Devices). Relative fluorescence was quantified by dividing the fluorescence by the OD_{600} reading of the culture. PDE-*lux* transcriptional fusion plasmids were constructed as previously described (52). Strains containing the plasmids were grown in triplicate overnight and then inoculated 1/100 in 150 μl LB medium or LB medium with SHB in a Costar white, clear-bottom 96-well plate (catalog no. 3903). Cultures were grown for 6 h, and then luminescence was quantified using an Envision plate reader (PerkinElmer). Relative luminescence was quantified by dividing the luminescence by the OD_{600} reading of the culture.

Biofilm quantification. Static biofilm formation was determined using a protocol modified from that in reference 35. Overnight planktonic cultures of *V. cholerae* and *V. cholerae* DGC mutants were grown in LB medium, and cultures were diluted 1:1,000 into 1 ml LB medium or LB medium with BV as specified in 17- by 100-mm polystyrene test tubes (BD Falcon). These tubes were incubated at 35°C for 24 h without shaking. The supernatant was then removed, an absorbance reading of the supernatant was taken (OD_{600}), and the biofilm was gently washed with approximately 2 ml of phosphate-buffered saline (PBS). The biofilm was then stained with 0.41% crystal violet in 12% ethanol for 3 min, followed by three washes with PBS, elution of the crystal violet in 10 ml 95% ethanol, and OD_{570} measurement for each biofilm. Each OD_{570} biofilm measurement was normalized to the OD_{600} measurement of the planktonic culture to account for differences in growth.

RESULTS

Bile increases the intracellular concentration of c-di-GMP in *V. cholerae*. It has been proposed that *V. cholerae* has a high intracellular c-di-GMP concentration in environmental reservoirs where it predominantly exists as surface-associated biofilm communities and reduces c-di-GMP upon entrance into the human host, leading to the activation of virulence factor expression (53). As bile is a prevalent constituent of the human small intestine, we hypothesized that *V. cholerae* would reduce c-di-GMP levels in response to bile. To test this hypothesis, we grew wild-type (WT) *V. cholerae* in the presence of a crude bovine bile extract (BV) or synthetic human bile (SHB) and quantified the intracellular c-di-GMP concentration using LC-MS/MS. BV was added as a supplement to the medium at 0.4% (wt/vol), a concentration which is physiologically relevant to that found in the small intestine (27, 54). SHB is a mixture of six purified bile acids that replicate the physiological concentrations of bile acids found in the human small intestine (25, 26, 41, 42). To our surprise, growth in both BV- and SHB-containing media significantly increased the intracellular concentration of c-di-GMP, 4.4- and 3.6-fold, respectively (Fig. 1).

To determine if the increase in intracellular c-di-GMP was due to the detergent activity of the bile acids, we grew *V. cholerae* in the presence of either the anionic detergent SDS (0.01%, wt/vol) or the zwitterionic detergent CHAPS (0.4%, wt/vol). We observed no significant difference in intracellular c-di-GMP upon addition of either detergent, suggesting that this increase in intracellular c-di-GMP is specific to bile acids. As the SHB mixture of bile acids contains bile acids conjugated to either taurine or a glycine, it was possible that these moieties were causing the increase in intracellular c-di-GMP. However, there was no significant difference in the intracellular c-di-GMP concentration of *V. cholerae* grown with the addition of either taurine or glycine at equivalent concentrations (Fig. 1). Therefore, we conclude that bile acids increase the intracellular concentration of c-di-GMP in *V. cholerae*.

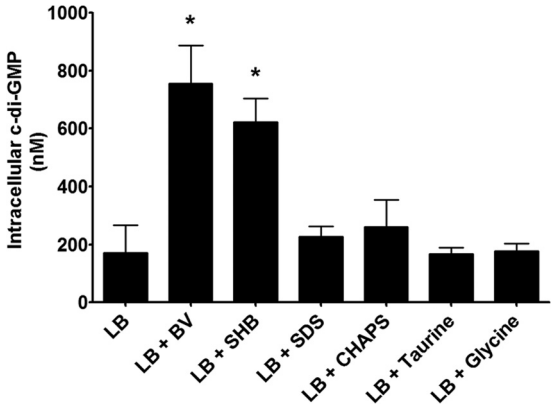

FIG 1 *V. cholerae* was grown in LB medium and LB medium with bovine bile (BV), synthetic human bile (SHB), the detergents SDS and CHAPS, and the amino acids taurine and glycine. Intracellular c-di-GMP was measured using LC-MS/MS. The reported values indicate the means. Error bars indicate standard deviations, and asterisks indicate statistical significance compared to LB medium as determined by a Student two-tailed *t* test ($n = 3$, $P < 0.05$).

The bile-mediated increase of intracellular c-di-GMP is growth phase dependent. The experiments described thus far analyzed cultures grown to late exponential growth. We therefore examined the temporal dynamics of c-di-GMP induction by bile at various points during bacterial growth. When the bacterium is grown under standard culture conditions with shaking, there is no significant difference in the doubling time of *V. cholerae* in the presence or absence of SHB (LB medium, 22.3 ± 0.3 min; LB medium with SHB, 21.6 ± 0.6 min). We measured intracellular c-di-GMP in three growth phases in the presence and absence of SHB: early exponential growth (OD_{600}, 0.2 to 0.3), late exponential growth (OD_{600}, 0.6 to 0.8), and stationary phase (OD_{600}, 1.0 to 1.2). When *V. cholerae* was grown in LB medium alone, we observed elevated c-di-GMP in early exponential growth, and the concentrations of c-di-GMP decreased as the cell density increased. These observations are consistent with previous studies showing that c-di-GMP is elevated in the low-cell-density quorum sensing (QS) state (38, 52). We observed significant increases of the intracellular c-di-GMP concentration in the presence of bile in early exponential and late exponential growth phases (Fig. 2). Alternatively, no significant induction of c-di-GMP was observed in stationary phase. These results indicate that bile induction of c-di-GMP is growth phase dependent. We speculated that QS might be responsible for density-dependent bile induction of c-di-GMP. However, bile induction of c-di-GMP was unchanged in *V. cholerae* mutants locked in either the low- or high-cell-density QS state, showing that QS does not drive growth-phase-dependent bile induction of c-di-GMP in *V. cholerae* (data not shown).

Three DGCs are more active in the presence of bile. As SHB increases intracellular c-di-GMP, we hypothesized that specific DGCs in *V. cholerae* would increase c-di-GMP synthesis in the presence of SHB. To identify these DGCs, we developed a high-

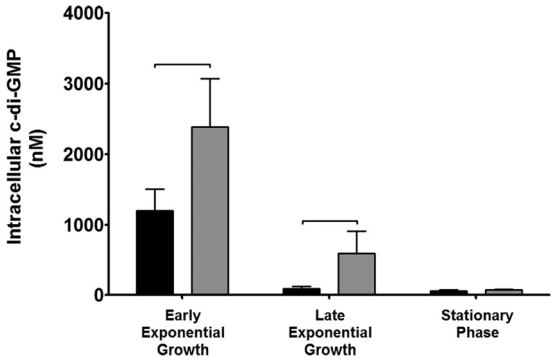

FIG 2 The intracellular c-di-GMP concentration of wild-type *V. cholerae* was quantified over the course of growth. All intracellular c-di-GMP concentrations were determined using LC-MS/MS. Black bars indicate strains grown in LB medium, and gray bars indicate strains grown in LB medium with SHB. The reported values indicate the means, and the error bars indicate the standard deviations. Brackets indicate statistical significance as determined by a one-tailed Student *t* test ($n = 3$, $P > 0.05$).

throughput screen where the *in vivo* activity of each of the 40 *V. cholerae* DGCs could be determined in the presence and absence of SHB. This screen utilized a series of expression vectors carrying every *V. cholerae* DGC under the control of the IPTG-inducible Ptac promoter and a common ribosome binding site (45). Therefore, we infer in this system that differences in c-di-GMP synthesis activity are not due to changes in gene expression. In addition to the DGC expression vector, each strain contained a separate reporter plasmid that carried a transcriptional fusion of a c-di-GMP-inducible promoter located within the ORF of VC1673 with the luciferase operon (named 6:C9-*lux* [46]). These vectors were introduced into a Δ*vpsL* strain of *V. cholerae* which abrogates biofilm formation and eliminates interference of aggregate formation in the analysis of reporter gene expression. In the absence of additional c-di-GMP generated by exogenous expression of DGCs, the reporter plasmid does not produce significant luminescence in the presence or absence of SHB (Fig. 3, vector control). c-di-GMP production by active DGCs leads to increased luciferase production, as can be seen by induction of luminescence by expression of the constitutively active DGC *qrgB* from *V. harveyi* (Fig. 3, QrgB). Induction of this transcriptional reporter is dependent on c-di-GMP synthesis as expression of *qrgB**, an allele of *qrgB* harboring a mutation in the active site of the protein, does not induce luminescence (Fig. 3, *qrgB**).

6:C9-*lux* expression was determined upon induction of each DGC in the presence of LB medium and LB medium with SHB. Seven DGCs showed reduced luminescence in the presence of SHB, although the extent of this reduction was not large (<3.5-fold). It is not currently known if this reduction of expression is related to bile inhibition of the DGC activity of these proteins, unrelated transcriptional regulation of 6:C9-*lux*, or a nonspecific effect of SHB on luminescence. Due to the large number of DGCs that are negatively affected (seven, $P < 0.01$), the relatively low reduction, and the fact that bile increases the total intracellular c-di-GMP concentration, we favor the latter two possibilities. Nevertheless, three DGCs demonstrated more than a 5-fold increase in luminescence in the presence of SHB ($P < 0.01$; Fig. 3); these DGCs are VC1067 (7.0-fold increase), VC1372 (47.6-fold increase), and VC1376 (6.7-fold increase).

The activity of PDEs in *V. cholerae* is not altered by bile. The hydrolysis of c-di-GMP is driven by c-di-GMP-specific PDE enzymes, which contain either a C-terminal EAL or an HD-GYP domain. Like DGC enzymes, these proteins are also modular, and the variable N-terminal domain is thought to respond to environmental stimuli. We hypothesized that an increase in intracellular c-di-GMP in response to bile acids could be caused by a repression of PDE activity. To examine this possibility, we developed a second high-throughput screen to analyze the enzymatic activity of

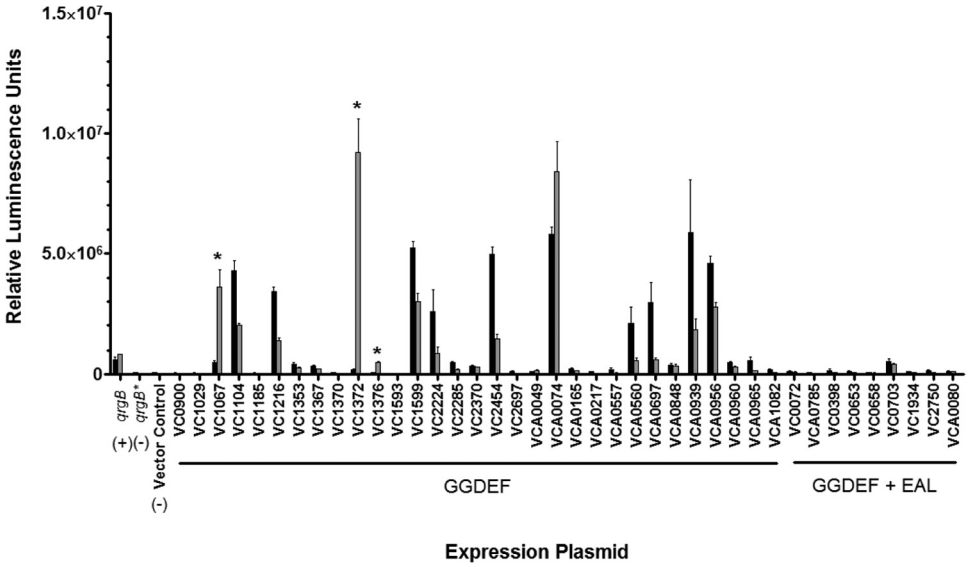

FIG 3 *V. cholerae* strains containing a DGC expression plasmid and the 6:C9-*lux* reporter plasmid were measured for luminescence in LB medium (black bars) and LB medium with SHB (gray bars). Expression plasmids carrying *qrgB* and its mutant allele counterpart, *qrgB**, were included as positive and negative controls, respectively. The vector control indicates expression of 6:C9-*lux* in the absence of protein induction. The error bars indicate standard deviations. Statistical significance (*) was determined for cultures exhibiting a positive fold change (LB medium + SHB/LB medium) greater than 2 as determined by a Student one-tailed *t* test ($n = 3$, $P < 0.01$).

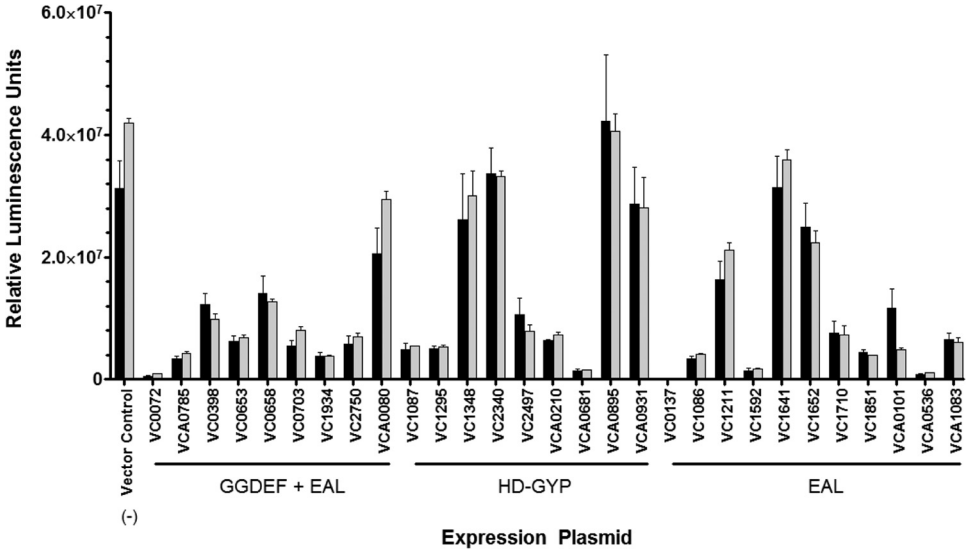

FIG 4 *V. cholerae* strains containing a PDE expression plasmid, the 6:C9-*lux* reporter plasmid, and the *qrgB* expression plasmid were measured for luminescence in LB medium (black bars) and LB medium with SHB (gray bars). The vector control indicates expression of 6:C9-*lux* with *qrgB* induction and without PDE expression. The error bars indicate standard deviations ($n = 3$).

the 29 predicted PDEs of *V. cholerae*. The sequence of one EAL protein, VC0515, was highly divergent from the sequenced N16961 strain upon amplification from the genome and was thus excluded from analysis; this is consistent with other studies reporting sequence variance (38). Similar to the screen above, each *V. cholerae* PDE is expressed from an inducible expression vector that has been introduced into a Δ*vpsL* strain of *V. cholerae* containing the 6:C9-*lux* reporter vector. A third vector (pBRP02) containing the constitutively active DGC *qrgB* was introduced, effectively increasing the baseline c-di-GMP concentration and subsequently the baseline luminescence values. Robust reporter gene expression can be seen in a strain expressing *qrgB* with a vector control that has no exogenous PDE expression (Fig. 4, vector control).

Strains of *V. cholerae* expressing each PDE alongside the DGC *qrgB* were grown in LB medium and LB medium with SHB, and the expression of 6:C9-*lux* was quantified. Twenty-two of the 29 PDEs showed appreciable decreases (>2-fold, $P < 0.01$) in luminescence when grown in LB medium compared to the vector control (Fig. 4), indicating that these PDEs are actively hydrolyzing c-di-GMP. However, none of the PDEs demonstrated any appreciable difference when grown in LB medium with SHB compared to LB medium alone (Fig. 4). Thus, we conclude that SHB does not affect the c-di-GMP hydrolysis of PDEs under the conditions that we examined here.

The three bile-inducible DGCs are inner membrane proteins. We analyzed the predicted domain structure and subcellular localization of the three bile-inducible DGCs to search for commonalities. An analysis of the relative hydrophobicity of the amino acid sequence of these three DGCs using the program toppred predicts that all contain transmembrane spanning domains (Fig. 5) (55–57). The DGC VC1067 (GI 15641080), which has also been referred to as *cdgH* (58), is predicted to contain two sequential periplasmic substrate-binding domains (PBPb) in the N terminus while VC1376 (GI 15641388), which has also been referred to as *cdgM* (59), is predicted to contain a conserved CHASE domain. VC1372 (GI 15641384) does not contain any conserved protein domains in the N terminus; however, analysis of the relative hydrophobicity of the amino acid sequence using toppred predicts 6 sequential intramembrane spanning domains.

Three DGCs increase c-di-GMP synthesis in the presence of bile acids. To directly examine if SHB increases the DGC activity of these three enzymes, we quantified the intracellular c-di-GMP concentration of strains ectopically expressing VC1067, VC1372, or VC1376 grown in the presence and absence of SHB. We similarly analyzed expression of active site mutant alleles of these genes (GG[D/E]EF→AA[D/E]EF) that render them incapable of c-di-GMP synthesis. As addition of SHB increases the intracellular c-di-GMP concentration of *V. cholerae* (Fig. 1), we hypothesized that the strains expressing each AA[D/E]EF mutant would show increased intracellular c-di-GMP in the presence of bile, similar to that of the wild-type (WT) strain. Moreover, induction of the bile-activated DGCs would lead to more c-di-GMP synthesis in the presence of bile than in the presence of LB medium alone.

When grown in LB medium, there was no notable difference between strains expressing any of the AA[D/E]EF alleles and the vector control in LB medium. As expected, growth of the vector control and that of DGC mutants in bile showed similar increases in c-di-GMP. Induction of the DGCs VC1067 and VC1376 produced significantly increased amounts of c-di-GMP in LB medium alone, leading to intracellular c-di-GMP concentrations of 48.4 ± 7.0 μM and 19.3 ± 10.0 μM, respectively (Fig. 6). Alternatively, the intracellular c-di-GMP of the VC1372 expression strain in the absence of bile (116.9 ± 17.4 nM) was not significantly altered compared to that of the vector control (263.7 ± 119.7 nM), indicating that this DGC does not produce c-di-GMP in LB medium alone. Importantly, the intracellular concentration of c-di-GMP was increased significantly upon ectopic expression of VC1067, VC1372, and VC1376 in the presence of LB medium with SHB over that in the presence of LB medium alone, resulting in intracellular concentrations of 89.2 ± 31.5 μM, 4.3 ± 0.4 μM,

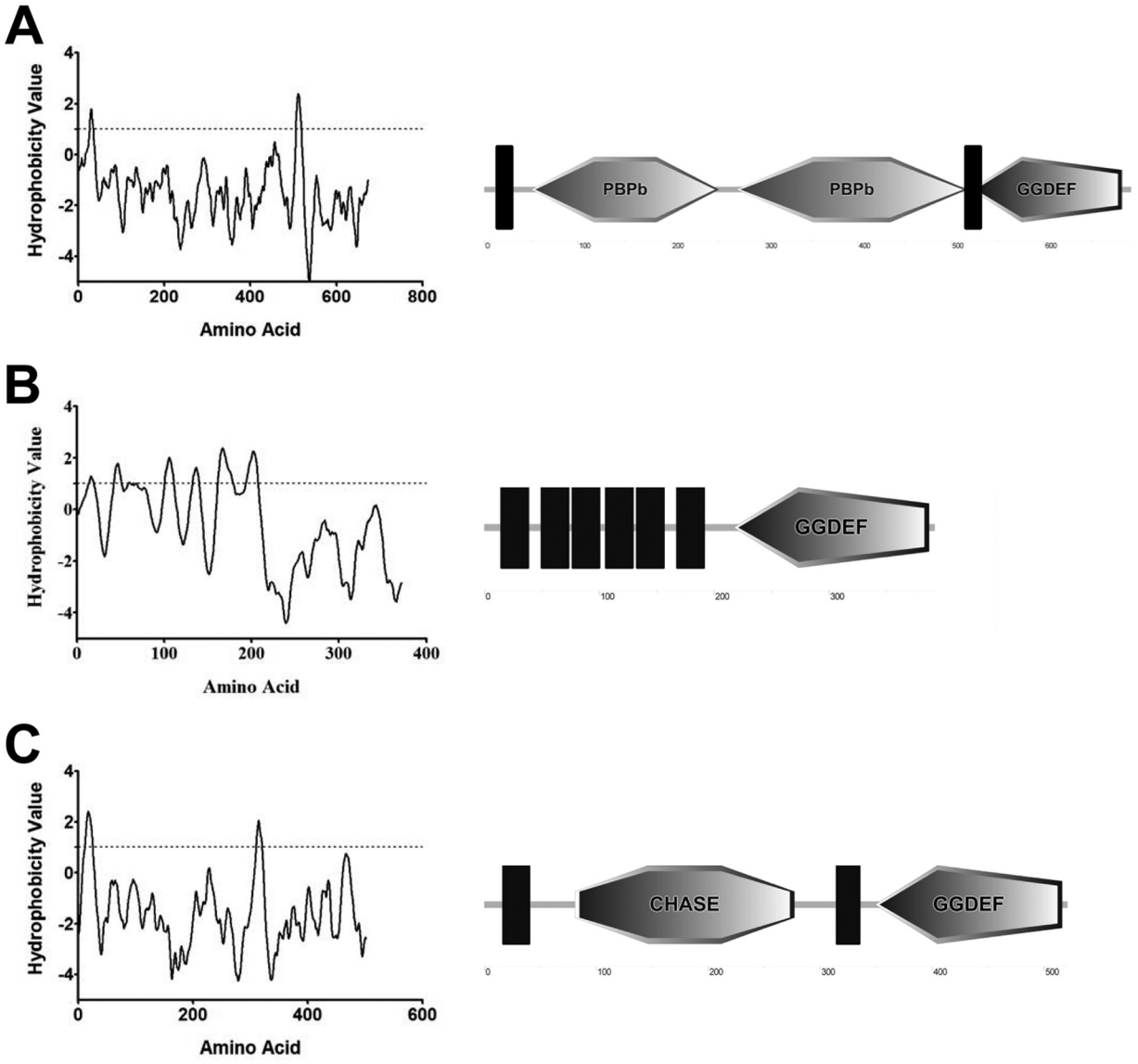

FIG 5 The predicted hydrophobicity of the amino acid sequence of VC1067 (A), VC1372 (B), and VC1376 (C) was used to predict transmembrane domains using the toppred program (left). The dotted line indicates the cutoff value for a potential transmembrane domain. A depiction of the potential N-terminal signaling domains and transmembrane domains (black bars) and the C-terminal GGDEF domain of each DGC is shown (right). The images were created using the SMART database (57).

and 67.4 ± 20.1 μM c-di-GMP, respectively (Fig. 6). These data together indicate that VC1067, VC1372, and VC1376 synthesize more c-di-GMP in the presence of bile acids.

Transcription of VC1295 is inhibited by bile acids. We hypothesized that the presence of bile could also regulate the transcription of DGCs or PDEs to control intracellular c-di-GMP concentrations. Specifically, bile could increase the expression of DGCs or decrease the expression of PDEs to increase intracellular c-di-GMP. To examine this possibility, we measured the relative transcription of 51 GGDEF, GGDEF + EAL, and EAL proteins in LB medium and LB medium with SHB by growing strains of Δ*vpsL V. cholerae* containing transcriptional fusions of approximately 500 bp of DNA upstream of each gene to *gfp*. After 8 h of growth, we found that there was less than a 2-fold difference in fluorescence between all GGDEF, GGDEF + EAL, and EAL strains grown in the presence and absence of bile (see Fig. S1 in the supplemental material), suggesting that under the conditions examined here, regulation of GGDEF and EAL gene transcription by bile does not play a significant role in the bile-mediated increase of intracellular c-di-GMP.

We also examined the relative transcription of the 8 HD-GYP proteins in LB medium and LB medium with SHB using transcriptional fusions of each promoter, defined as approximately 500 bp upstream of each HD-GYP gene, to the *lux* operon. The HD-GYP gene VC1087 is carried in a putative operon with the EAL gene VC1086, and thus, the expression is presumed to be synonymous with VC1086 (see Fig. S1 in the supplemental material). The luminescence of each reporter strain was determined after 6 h of growth in either LB medium or LB medium with SHB. The expression of six of the genes encoding HD-GYP proteins was

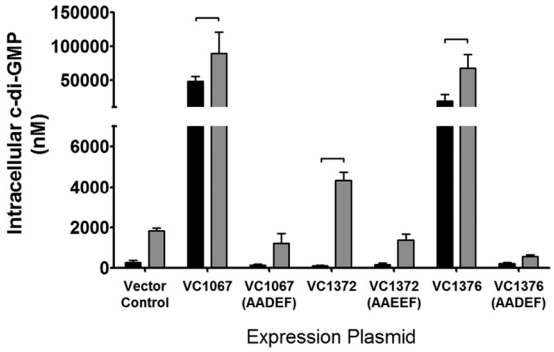

FIG 6 Intracellular levels of c-di-GMP in *V. cholerae* expressing an empty vector or DGC VC1067, VC1372, or VC1376 quantified with LC-MS/MS. The intracellular c-di-GMP levels of strains expressing alleles containing mutations in the active site motif of each DGC were also quantified. The black bars indicate strains grown in LB medium, while the gray bars indicate strains grown in LB medium with SHB. Error bars indicate standard deviations. Brackets indicate statistical significance, which was determined using a Student one-tailed *t* test ($n = 3$, $P < 0.05$).

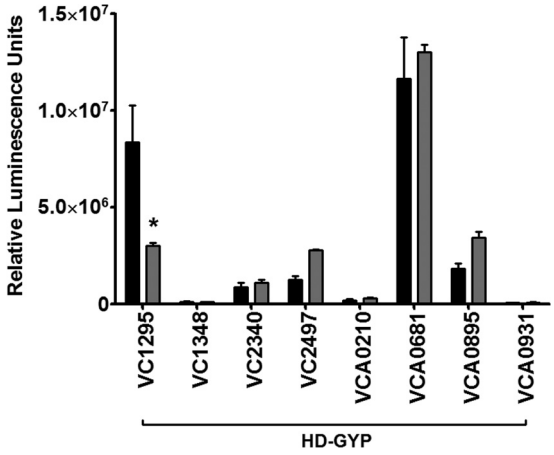

FIG 7 *V. cholerae* strains containing a transcriptional fusion of each HD-GYP promoter to luciferase were grown in LB medium (black) or LB medium with SHB (gray). Luminescence was quantified after 6 h of growth ($n = 4$) under each environmental condition. Each culture was normalized to an OD_{600} reading. Error bars indicate standard deviations. The asterisk indicates a statistically significant differences from the LB medium condition, determined by a one-tailed Student *t* test ($P < 0.05$).

not significantly changed in LB medium with SHB from that in LB medium alone (>2-fold), whereas the expression of VC2497 was modestly increased in the presence of SHB (2.2-fold, $P < 0.05$). Importantly, the expression of VC1295 (GI 15641308) was decreased 2.8-fold when grown in the presence of SHB (Fig. 7; $P < 0.05$). VC1295 appears to be an active PDE when ectopically expressed in *V. cholerae* (Fig. 4). This result indicates that bile acids decrease the expression of VC1295, possibly resulting in decreased c-di-GMP hydrolysis contributing to increased intracellular c-di-GMP.

Three DGCs and one HD-GYP account for bile-mediated c-di-GMP induction. To determine if the DGCs VC1067, VC1372, and VC1376 contribute to the bile-mediated increase of intracellular c-di-GMP in *V. cholerae*, we constructed unmarked *V. cholerae* DGC deletion mutants and examined SHB-mediated induction of c-di-GMP (Fig. 8). Similar to our previous findings, the intracellular c-di-GMP concentration of *V. cholerae* increased 4.1-fold in the presence of SHB in the WT strain. Both the ΔVC1067 and ΔVC1376 single mutants showed a modest but significant reduction of c-di-GMP when grown in bile compared to the WT strain, having 29.6% and 24.9% less c-di-GMP, respectively. The ΔVC1372 single mutant and ΔVC1372 Δ1376 double mutant were not statistically different from the WT strain. Importantly, the ΔVC1067 ΔVC1372 ΔVC1376 triple mutant strain exhibited the greatest reduction of intracellular c-di-GMP in the presence of bile, losing 46.8% of the intracellular c-di-GMP compared to the WT strain in LB medium with SHB ($P < 0.05$). These results suggest that VC1067, VC1372, and VC1376 function redundantly in the bile-mediated c-di-GMP induction.

As the expression of the HD-GYP VC1295 was inhibited by bile acids, we constructed an unmarked *V. cholerae* VC1295 deletion mutant and quantified intracellular c-di-GMP in the presence and absence of SHB. In LB medium alone, the intracellular c-di-GMP was modestly increased 1.8-fold in the ΔVC1295 mutant compared to the WT (Fig. 8; $P < 0.05$). This result is expected, as deletion of an active PDE will increase intracellular c-di-GMP. However, the intracellular c-di-GMP concentrations of the ΔVC1295 strain grown in the presence of SHB were indistinguishable from those of the WT. We hypothesized that both activation of DGC activity and transcriptional regulation of VC1295 contribute to bile induction of c-di-GMP. To test this, we created a quadruple ΔVC1295 ΔVC1067 ΔVC1372 ΔVC1376 mutant and measured intracellular c-di-GMP in the presence and absence of SHB. Similar to the ΔVC1295 single mutant, when grown in LB medium alone, the quadruple mutant had elevated intracellular c-di-GMP compared to the WT strain (2.4-fold, $P < 0.05$). Importantly, this strain showed no change in intracellular c-di-GMP in the presence of SHB. This indicates that these four proteins are responsible for the bile-mediated changes in intracellular c-di-GMP.

Deletion of the bile-responsive DGCs and PDE reduces bile induction of *V. cholerae* biofilm formation. It has been previously reported that BV (i.e., bovine bile) increases biofilm formation of *V. cholerae*, and this induction is dependent on the transcriptional regulator *vpsR* (35). VpsR binds c-di-GMP to regulate the transcription of biofilm genes (46). We wondered if the levels of c-di-GMP measured in the various DGC and PDE mutant strains with and without bile would correlate with biofilm formation. To test this, we performed a static biofilm assay where the wild-type, Δ*vpsL* mutant, and the DGC and PDE *V. cholerae* mutant strains were grown in polystyrene test tubes containing LB medium or LB medium with BV without shaking followed by crystal violet staining of the resulting attached biofilm. The Δ*vpsL* mutant cannot produce exopolysaccharide and thus does not form biofilms. BV was used to induce biofilm formation to remain consistent with prior studies (35, 60) and because it induced more robust biofilm formation in this assay than did SHB (data not shown).

We observed that all cultures of *V. cholerae* grew to a significantly lower optical density, as measured by OD_{600}, after static growth in the presence of BV. To account for these growth differences, the biofilm formation of each culture was normalized to the OD_{600} of the planktonic culture. Consistent with previous reports (35), BV increased biofilm formation in the WT strain 2.7-fold, and this response was eliminated in the Δ*vpsL* strain (Fig. 9).

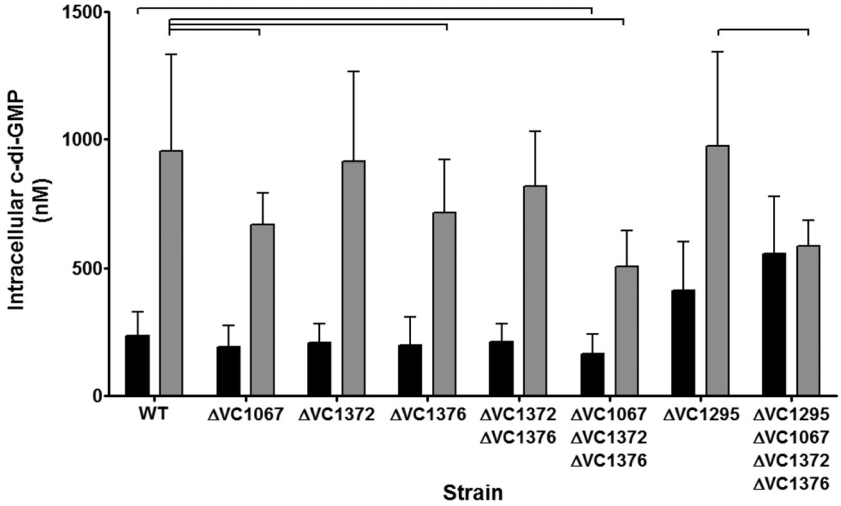

FIG 8 The intracellular c-di-GMP concentrations of wild-type *V. cholerae* and the DGC and PDE mutant strains were quantified after growth in LB medium (black bars) or LB medium with SHB (gray bars) using LC-MS/MS. The reported values indicate the means, and the error bars indicate standard deviations. Brackets indicate statistical significance as determined by a one-tailed Student *t* test ($n = 9$ to 10, $P < 0.05$).

While the ΔVC1067, ΔVC1376, and ΔVC1372 ΔVC1376 mutants all showed modest to no loss of bile induced c-di-GMP (Fig. 8), these mutants no longer exhibited substantial bile-induced biofilm formation. Only the ΔVC1372 mutant induced biofilm formation in response to bile similar to that of the WT, indicating that this DGC contributes less to biofilm formation. As expected, the triple DGC mutant showed the lowest level of biofilm formation and was not responsive to bile addition. Although the ΔVC1295 strain had modestly elevated c-di-GMP in LB medium, deletion of VC1295 had no noticeable impact on biofilm formation. Like the triple mutant, the DGC/PDE quadruple mutant exhibited low biofilm formation that was unresponsive to bile addition. These results demonstrate that the bile-responsive DGCs are required for c-di-GMP-dependent biofilm formation in response to bile acids. However, the HD-GYP VC1295 did not contribute to biofilm formation in this assay.

Bicarbonate inhibits the bile-mediated increase of c-di-GMP in *V. cholerae*. As c-di-GMP has been shown to repress virulence gene expression in *V. cholerae*, it remained puzzling why bile would increase c-di-GMP (53). We hypothesized that additional signals in the small intestine could override this induction. Another major component of the human small intestine is bicarbonate, which is secreted by the pancreas as well as the small intestinal epithelial cells. Bicarbonate acts as a pH buffer to neutralize acids secreted by the stomach (61, 62). Bicarbonate has also been implicated in virulence gene regulation in *V. cholerae* (63). As bicarbonate is abundant in the small intestine, we hypothesized that it may contribute to the regulation of intracellular c-di-GMP in *V. chol-*

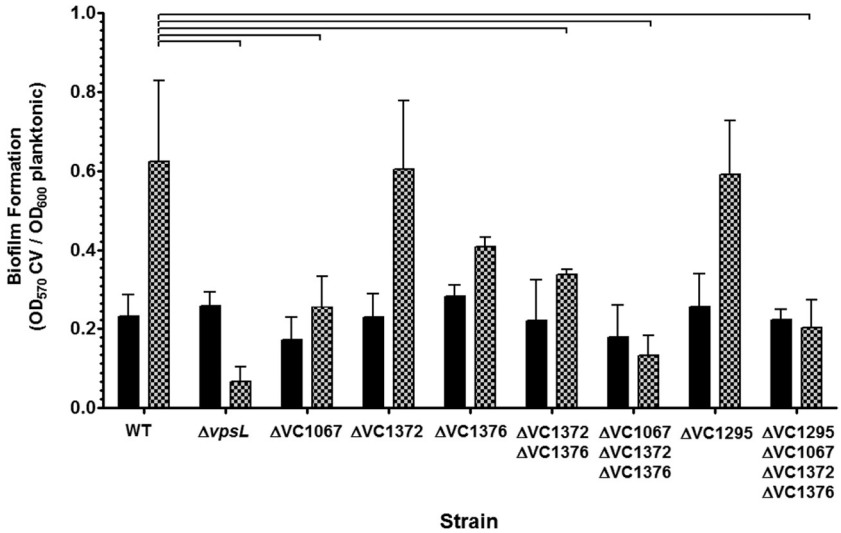

FIG 9 The biofilm formation of wild-type *V. cholerae* and *V. cholerae* DGC and PDE mutants was quantified in test tubes using crystal violet (CV). A strain containing a mutation in the *vpsL* gene was included as a negative control. The CV value was normalized by the OD_{600} value of the planktonic culture to account for differences in growth. The reported values indicate the means, and error bars indicate standard deviations from the means. Brackets indicate statistical significance as determined by a Student one-tailed *t* test ($n = 3$, $P < 0.05$).

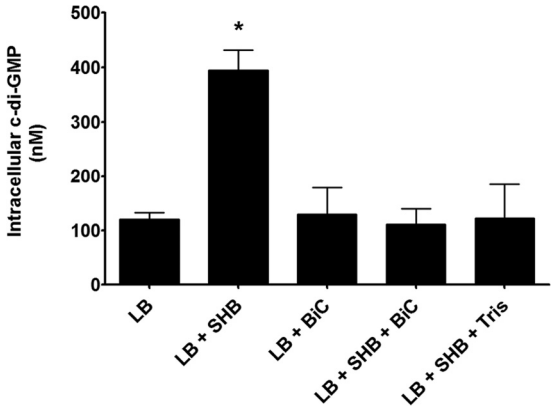

FIG 10 The intracellular c-di-GMP concentration of wild-type *V. cholerae* was quantified in LB medium, LB medium with bicarbonate (BiC), or LB medium with SHB and different supplements using LC-MS/MS. The reported values indicate the means, and the error bars indicate standard deviations. The asterisk indicates statistical significance compared to LB medium as determined by a one-tailed Student *t* test ($n = 3$, $P > 0.05$).

erae. To examine if bicarbonate impacted bile induction of c-di-GMP, we measured intracellular c-di-GMP of *V. cholerae* when grown in the presence and absence of SHB and bicarbonate. Bicarbonate was added as a supplement at 0.3% (wt/vol), consistent with toxin-inducing conditions (43). We observed no significant difference in intracellular c-di-GMP of *V. cholerae* in the presence of bicarbonate alone (Fig. 10). However, growth of *V. cholerae* in the presence of both SHB and bicarbonate completely abolished bile induction of c-di-GMP (Fig. 10).

As bicarbonate acts as a buffer in the small intestine, we hypothesized that bicarbonate suppression of the bile induction of c-di-GMP was due to pH changes. We tested this idea by growing *V. cholerae* in the presence of SHB with the pH buffer Tris. The pHs of LB medium and LB medium with SHB were approximately the same and did not change significantly over the course of growth (pH of LB medium, 7.3 pregrowth, 7.0 postgrowth; pH of LB medium with SHB, 7.3 pregrowth, 7.1 postgrowth). Upon addition of bicarbonate, the pH increased substantially (pH of LB medium with SHB and BiC, 8.1 pregrowth, 8.7 postgrowth). The addition of Tris caused a similar increase in pH (pH of LB medium with SHB and Tris, 8.7 pregrowth, 8.6 postgrowth). Analogously to bicarbonate, the addition of Tris to LB medium with SHB inhibited the normal induction of c-di-GMP by bile (Fig. 10), indicating that this inhibition of bile-induced c-di-GMP is pH dependent.

DISCUSSION

Bile is an abundant component of the human small intestine and thus a probable physiological cue for *V. cholerae* to recognize upon entry into this environment. A number of lines of evidence suggest that bile is an important signal in the transition of *V. cholerae* between environmental and infectious lifestyles. Bile acids increase the expression of *ompU* in a *toxR*-dependent manner to increase bile resistance, indicating that the classical biotype of *V. cholerae* is capable of sensing the presence of bile (64). There are conflicting reports regarding bile control of virulence factor expression. Bile acids were reported to negatively regulate the expression of the toxin-coregulated pilus (TCP) and cholera toxin (CT) in a *toxT*-dependent manner in a classical biotype, as well as positively regulating motility, demonstrating that there is a link between bile and virulence (65, 66). Specifically, it has been shown that unsaturated fatty acids in bile inhibit the expression of virulence factors (67). Contrary to these findings, bile acids were reported to induce CT and TCP in a *toxR*-dependent manner in a classical *V. cholerae* biotype and a *tcpP*-dependent manner in the El Tor biotype used in this study (60, 68). From these studies, it is clear that *V. cholerae* responds to bile to induce a number of physiological changes. In this work, we explore the connections between bile and c-di-GMP.

Based on the prevailing *V. cholerae* disease model hypothesizing that c-di-GMP levels are decreased upon infection, we predicted that bile acids would decrease intracellular c-di-GMP concentrations. Contrary to this prediction, we found that bile acids increase the intracellular c-di-GMP concentration of *V. cholerae* (Fig. 1). This finding suggests that the dynamics of the c-di-GMP signaling system in the human host are more complex than previously appreciated. Furthermore, we have found that the difference in the bile-mediated change of c-di-GMP is largest during exponential growth and that at stationary phase this difference was negated. Consistent with these findings, it has been shown that intracellular c-di-GMP is depleted at high cell density and that these changes are due in part to quorum sensing (38, 52, 69). Our results indicate that this regulation is dominant over the increase in intracellular c-di-GMP caused by bile.

To begin to understand how *V. cholerae* modulates its intracellular c-di-GMP in response to bile, we determined if the activity of any DGCs or PDEs was affected by bile using a novel high-throughput *in vivo* assay. This assay is easily adaptable to examine the response of DGCs and PDEs to any environmental cue, and it can be modified to explore other bacteria if a suitable *in vivo* reporter of c-di-GMP levels is available. Three DGCs (VC1067, VC1372, and VC1376) exhibited increased c-di-GMP synthesis activity in the presence of SHB (Fig. 3 and 6). The DGC activity of VC1372 appeared to be absolutely dependent on bile whereas bile modulated the basal activities of VC1067 and VC1376. All of these DGCs are important for the bile-induced increase of intracellular c-di-GMP and biofilm formation of *V. cholerae* (Fig. 8 and 9).

The mechanisms by which these three DGCs respond to bile acids are currently unknown. All three DGCs are predicted to be associated with the inner membrane. VC1067 has been implicated in biofilm formation as it induces rugosity-associated phenotypes in *V. cholerae* (58). Furthermore, it has been shown that both VC1067 and VC1376 actively produce c-di-GMP and stimulate biofilm formation (45, 58), and a VC1376 mutant strain also demonstrates lower *vpsL* expression (59). VC1376 has also been implicated in the indole-induced increase in biofilm formation (70). Furthermore, all three DGCs also have been shown to repress motility when ectopically expressed in *V. cholerae*; this is particularly interesting as the levels of motility repression of the VC1372 and VC1376 expression strains do not seem to correlate with the intracellular c-di-GMP levels reported here (Fig. 6) (71). Analysis by BLAST revealed that sequences homologous to VC1372 are found only in a few *Vibrio* species, dominated by strains of *V. cholerae*. A previous study has also noted that VC1372 is unique to *V. cholerae* among members of the genus *Vibrio* (72).

The phylogenetic link of VC1372 with an enteric human pathogen, the domain structure of VC1372, and our observation

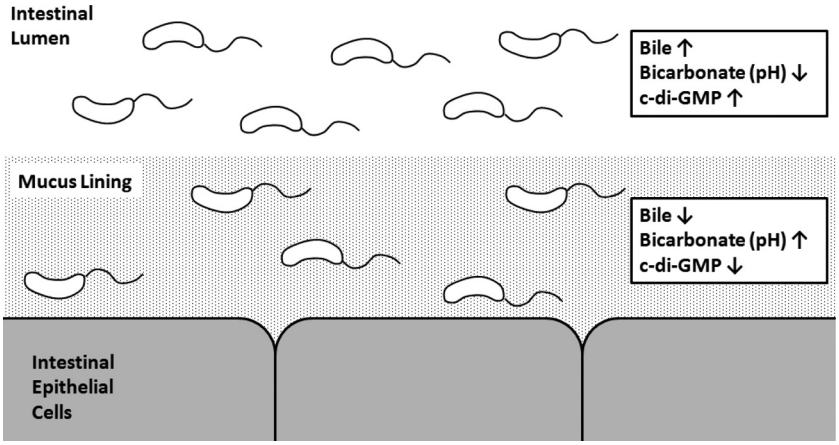

FIG 11 Proposed model of *V. cholerae* c-di-GMP regulation in the human small intestine. c-di-GMP is elevated in the lumen, where the concentration of bile is elevated and the concentrations of bicarbonate and pH are low. Upon entry into the mucosal layer, where bile is low and the bicarbonate concentration and pH are elevated, c-di-GMP is repressed.

that the activity of VC1372 is absolutely dependent on the presence of bile suggest that the physiological cue which controls VC1372 is bile. Bile acids are known to interact with the cell membrane due to their detergent activity (29). Moreover, we observed bile-mediated activation of VC1372 in *E. coli* (data not shown), an orthologous system with no clear homolog to VC1372. Alternatively, as both VC1067 and VC1376 maintain robust activity upon exogenous expression even in the absence of bile, we postulate that these DGCs might be controlled indirectly by bile through sensing perturbations in the membrane.

Additionally, the expression of the PDE VC1295 was inhibited by bile acids (Fig. 7). This HD-GYP actively hydrolyzes c-di-GMP in both LB medium and LB medium with SHB, but bile acids do not affect this activity (Fig. 4). VC1295 is predicted to be composed of 492 amino acids and contains 6 predicted N-terminal transmembrane domains preceding a HAMP domain linked to the HD-GYP domain (55–57). The mechanism governing this transcriptional regulation of VC1295 by bile remains unknown. Analysis of the VC1295 promoter region reveals motifs resembling the ToxR binding site (−127 bp, TCAAA-N_{11}-TTAAA [73]). While this gene is not listed among the known genes regulated by ToxR (74), there is evidence that the activity of ToxR is altered by bile (60), suggesting that the ToxR regulon could be altered when bile acids are present. We are currently investigating the connection between the transcriptional regulation of VC1295, bile, and ToxR. Although VC1295 contributed to bile-induced c-di-GMP, we did not observe any effect of VC1295 on biofilm formation. We speculate that this result is due to the distinct experimental growth conditions under which c-di-GMP and biofilm formation were measured.

Another important host-derived cue is bicarbonate, a biological pH buffer that is highly abundant in the human small intestine (62). Bicarbonate has been shown to be important for virulence, as bicarbonate is critical for *in vitro* production of CT (43). Bicarbonate is capable of activating *V. cholerae* virulence gene expression via the transcriptional regulator *toxT* (63). We have shown that bicarbonate is able to suppress the bile-mediated induction of c-di-GMP in *V. cholerae*. Furthermore, this regulation is driven by changes in pH, as the bile-mediated induction is similarly repressed by Tris (Fig. 10). It is possible that the change in pH alters the structure of bile so that it no longer triggers the c-di-GMP synthesis activity of the DGCs. Alternatively, the bicarbonate could directly interact with DGCs or PDEs to competitively alter intracellular c-di-GMP.

As bile has strong antimicrobial properties (75), it may be physiologically advantageous for *V. cholerae* to increase c-di-GMP to promote biofilm formation in order to grant elevated tolerance to bile acids and other stresses associated with the intestinal environment. Other studies have indicated that biofilm formation is important for increased acid shock tolerance and protection from bile acids (35, 76), and biofilms increase infectivity and intestinal colonization in a mouse infection model, which has implications for transmission (77). Indeed, we confirmed that BV induces biofilm formation in *V. cholerae* and showed that all three bile-responsive DGCs were required for bile induction of biofilm formation.

We propose that bile stimulates a high intracellular c-di-GMP concentration in the intestinal lumen (Fig. 11). Upon penetration of the mucosal layer where the bicarbonate concentration and thus the pH are elevated (78), the response to bile is abrogated, leading to a corresponding decrease in intracellular c-di-GMP. The physiological consequences of spatial alteration of c-di-GMP within the small intestine remain to be determined, although we speculate that c-di-GMP could be modulating biofilm formation, motility, and virulence gene expression. Our model predicting high c-di-GMP concentrations in the lumen and decreased c-di-GMP proximal to the intestinal epithelial cells is consistent with previously described virulence gene regulatory models for *V. cholerae* and *Salmonella enterica* (63, 79, 80).

Our findings indicate that both bile and bicarbonate are environmental cues that modulate c-di-GMP signaling in *V. cholerae* and facilitate the transition from aquatic environments to the human host. They suggest that modulation of c-di-GMP levels by *V. cholerae* upon entry into the human host is more complex than previously appreciated and that both bile and bicarbonate act together to inversely regulate the intracellular concentration of c-di-GMP to presumably enable the bacteria to adapt and thrive in the diverse intestinal environment.

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