Cyclic di-GMP Increases Catalase Production and Hydrogen Peroxide Tolerance in *Vibrio cholerae*

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ABSTRACT *Vibrio cholerae* is a Gram-negative bacterial pathogen that causes the disease cholera, which affects nearly 1 million people each year. In between outbreaks, *V. cholerae* resides in fresh and salt water environments, where it is able to persist through changes in temperature, oxygen, and salinity. One key characteristic that promotes environmental persistence of *V. cholerae* is the ability to form multicellular communities, called biofilms, that often adhere to biotic and abiotic sources. Biofilm formation in *V. cholerae* is positively regulated by the dinucleotide second messenger cyclic dimeric GMP (c-di-GMP). While most research on the c-di-GMP regulon has focused on biofilm formation or motility, we hypothesized that the c-di-GMP signaling network encompassed a larger set of effector functions than reported. We found that high intracellular c-di-GMP increased catalase activity ~4-fold relative to strains with unaltered c-di-GMP. Genetic studies demonstrated that c-di-GMP mediated catalase activity was due to increased expression of the catalase-encoding gene *katB*. Moreover, c-di-GMP mediated regulation of catalase activity and *katB* expression required the c-di-GMP dependent transcription factors VpsT and VpsR. Lastly, we found that high c-di-GMP increased survival after H₂O₂ challenge in a *katB*-, *vpsR*-, and *vpsT*-dependent manner. Our results indicate that antioxidant production is regulated by c-di-GMP uncovering a new node in the growing VpsT and VpsR c-di-GMP signaling network of *V. cholerae*.

IMPORTANCE As a result of infection with *V. cholerae*, patients become dehydrated, leading to death if not properly treated. The aquatic environment is the natural reservoir for *V. cholerae*, where it can survive alterations in temperature, salinity, and oxygen. The second messenger molecule c-di-GMP is an important signal regulating host and aquatic environmental persistence because it controls whether *V. cholerae* will form a biofilm or disperse through flagellar motility. In this work, we demonstrate another function of c-di-GMP in *V. cholerae* biology: promoting tolerance to the reactive oxygen species H₂O₂ through the differential regulation of catalase expression. Our results suggest a mechanism where c-di-GMP simultaneously controls biofilm formation and antioxidant production, which could promote persistence in human and marine environments.

KEYWORDS *Vibrio cholerae*, VpsT, catalase, cyclic di-GMP, reactive oxygen species

The Gram-negative bacterium *Vibrio cholerae* is the human pathogen that causes the diarrheal disease cholera. The most common route to infection is consumption of contaminated food or water, after which *V. cholerae* traverses the stomach and colonizes the small intestines. Cholera patients lose liters of fluid and dissolved ions through toxin-mediated changes to the host intestinal tract, allowing *V. cholerae* to exit the host, reenter a water source, and perpetuate its infectious cycle. In addition to the harsh conditions of the human gastrointestinal tract, *V. cholerae* must adapt to numerous stresses in the aquatic environment. These environmental stresses include temperature

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fluctuations, eukaryotic predation, and exposure to chemical insults such as reactive oxygen species (ROS) (1).

As an aquatic organism, *V. cholerae* is exposed to various concentrations of dissolved oxygen and ROS produced abiotically through photochemical reactions between sunlight and dissolved organic matter in the ocean (2, 3). ROS can also be produced biotically through metabolic processes in aerobic environments by phytoplankton, another potential reservoir of *V. cholerae* (4). In response to the multiple routes of exposure to ROS, it is not surprising that *V. cholerae* has multiple ROS defense systems, including two paralogues of the oxidative stress responsive transcription factor OxyR, two catalases, and multiple peroxidases (5–7). Another mechanism to increase tolerance to ROS is the production of surface-adhered communities encased in an exopolysaccharide matrix also known as biofilms.

Many bacterial species, including *V. cholerae*, have increased tolerance to ROS such as hydrogen peroxide (H_2O_2) when grown in biofilms compared to planktonic counterparts (8–10). Biofilm formation in *V. cholerae* is regulated by the bacterial second messenger molecule cyclic dimeric GMP (c-di-GMP), which is produced by diguanylate cyclase (DGC) enzymes. c-di-GMP alters bacterial physiology by modulating transcription, translation, and/or protein function (11). In *V. cholerae*, a common mechanism of c-di-GMP signaling is the modulation of gene expression by three c-di-GMP-dependent transcription factors: VpsR, VpsT, and FlrA (12–14). c-di-GMP activates the transcription factors VpsR and VpsT, resulting in increased transcription of genes involved in synthesis of the biofilm matrix component *Vibrio* polysaccharide (VPS) (12, 13, 15). In contrast, c-di-GMP acts as an anti-activator of FlrA, which causes decreased expression of genes necessary for flagellar biosynthesis (14). c-di-GMP can also bind to two riboswitches, Vc1 and Vc2. Binding of c-di-GMP to Vc1 functions as an ON-switch to induce production of the adhesin GbpA, while binding of c-di-GMP to Vc2 functions as an OFF-switch to inhibit production of the transcription factor TfoY (16, 17). As cells receive signals to disperse from the biofilm, phosphodiesterase (PDE) enzymes, which degrade c-di-GMP, become activated. These PDEs then deplete the intracellular concentration of c-di-GMP, promoting a switch from a sessile biofilm lifestyle to a motile one (reviewed in reference 11).

While VpsR and VpsT were initially discovered as regulators of biofilm production, other c-di-GMP-dependent functions have emerged. For example, c-di-GMP and VpsR transcriptionally regulate genes in the type II secretion operon, as well as *tfoY*, a gene involved in driving dispersive motility and regulating type VI secretion (17–19). VpsT negatively regulates the expression of genes involved in flagellar biosynthesis; however, the mechanism is not known (13). In addition, Wang and coworkers demonstrated VpsT negatively regulates the transcription of the stationary-phase sigma factor RpoS (20). Recently, we found that c-di-GMP and VpsT induced expression of the DNA repair gene *tag* to promote survival after alkylation stress (21). These studies demonstrate that c-di-GMP regulation extends beyond biofilm formation and motility in *V. cholerae*.

In this study, we uncovered an additional role for c-di-GMP: positively regulating catalase activity by increasing transcription of the catalase-encoding gene *katB* via a VpsR- and VpsT-dependent mechanism. We further show that c-di-GMP-dependent catalase activity was necessary for survival after exposure to the ROS H_2O_2 . Our results expand the regulatory network of c-di-GMP to include antioxidant production, demonstrating that elevated c-di-GMP enhances the oxidative stress response in *V. cholerae*.

RESULTS

c-di-GMP positively regulates catalase activity. We have shown that c-di-GMP regulates genes involved in DNA repair and that this regulation increased tolerance to the methylating agent methyl methanesulfonate (21). Thus, we hypothesized c-di-GMP had a role in mitigating other forms of cellular stress besides DNA methylation damage. We chose to test whether c-di-GMP increased H_2O_2 tolerance because it is a common ROS produced by aerobic microorganisms as a by-product of cellular respiration, and H_2O_2 is found in high concentrations in aquatic environments (reviewed in reference

FIG 1 Cyclic di-GMP increases catalase activity. Catalase activity was measured in parent and mutant strains overproducing QrgB^{Mut} (open bars) or QrgB (solid bars). Brackets and asterisks (*) indicate differences with a *P* value of <0.05 determined by two-way analysis of variance (ANOVA), followed by Tukey's multiple-comparison test. The data are the average of three biological replicates with error bars indicating the standard deviations. ##, activity below the limit of detection.

22). To test the effects of c-di-GMP on H₂O₂ tolerance in *V. cholerae*, we expressed the *Vibrio harveyi* DGC QrgB in a strain unable to form mature biofilms ($\Delta vpsL$ mutant, designated the parent strain here) which would introduce a confounding variable in our experiments (23). In addition, we chose a heterologous DGC from *V. harveyi*, as opposed to *V. cholerae*, to minimize effects associated with potential protein-protein complexes within the *V. cholerae* c-di-GMP signaling network. As a control, we expressed an inactive allele of QrgB (QrgB^{Mut}) that is unable to synthesize c-di-GMP. Immediately after the addition of H₂O₂, both cultures began to produce gas bubbles, which is a phenomenon indicative of catalase activity as H₂O₂ is degraded into water and gaseous oxygen. Interestingly, the culture with a higher intracellular c-di-GMP exhibited a larger amount of gas production despite the cultures having similar numbers of bacteria, suggesting that c-di-GMP increased catalase activity. Quantification of catalase activity revealed an approximate 5-fold increase when comparing strains expressing QrgB to QrgB^{Mut} (Fig. 1), indicating c-di-GMP increased catalase activity. Importantly, we have previously demonstrated that the concentration of c-di-GMP generated by QrgB overexpression is similar to that seen naturally in the low-cell-density quorum-sensing state, demonstrating that these results are physiologically relevant (17).

c-di-GMP can directly modulate protein activity or change gene expression through allosteric interactions with c-di-GMP-dependent transcription factors or riboswitches (13–15). The c-di-GMP-dependent transcription factors VpsT and VpsR induce transcription of genes involved in biofilm formation, protein secretion, and DNA repair under high c-di-GMP conditions (12, 13, 17, 18, 21). Therefore, we hypothesized that these transcription factors control c-di-GMP-regulated catalase activity. To test this hypothesis, we repeated the catalase assay using $\Delta vpsT$, $\Delta vpsR$, and $\Delta vpsT \Delta vpsR$ *V. cholerae* mutants and observed a loss of c-di-GMP-mediated induction of catalase activity, suggesting the increased catalase activity was part of the VpsR/VpsT/c-di-GMP regulatory network (Fig. 1).

V. cholerae has two catalase-encoding genes: *katG* (VC1560), a bifunctional enzyme with both catalase and peroxidase functions, and *katB* (VC1585), which only exhibits catalase activity (7, 24). Mutants of these enzymes render *V. cholerae* more susceptible to H₂O₂ treatment; however, regulation of either *katB* or *katG* by c-di-GMP has not been described (24). We therefore measured c-di-GMP induction of catalase activity in the $\Delta katB$, $\Delta katG$, or $\Delta katB \Delta katG$ mutants. We observed that the $\Delta katB$ background did not display c-di-GMP-regulated catalase activity but did possess basal level catalase activity similar to the parent strain expressing QrgB^{Mut} (Fig. 1). Expression of *katB* from an

FIG 2 Characterization of *katB* promoter. (A) *katB* genetic locus and promoter truncations and reporter activity. The 5' promoter truncations were constructed and cloned upstream of the luciferase operon. Lines and numbers on the y axis indicate the length of the promoter relative to the ATG start codon for each promoter construct. Normalized luminescence is light production normalized to the OD₆₀₀ to control for cell number. For each promoter construct, luminescence was measured while overproducing QrgB^{Mut} (open bars) or QrgB (solid bars). An empty pBBRlux vector control was included in each experiment. Brackets and asterisks (*) indicate differences with a *P* value of <0.05 determined by two-way ANOVA, followed by Tukey's multiple-comparison test. The data are the average of three biological replicates with error bars indicating the standard deviations. (B) *katG* expression is unresponsive to changes in c-di-GMP. *katG* expression was measured in the parent strain while overproducing QrgB^{Mut} (open bars) or QrgB (solid bars). Bars represent averages of three biological replicates, with error bars indicating the standard deviations.

arabinose-inducible, multicopy plasmid in the $\Delta katB$ background complemented catalase activity regardless of the levels of c-di-GMP (see Fig. S2 in the supplemental material). Strains lacking *katG* were still able to induce catalase activity by c-di-GMP; however, the level of catalase activity induced by c-di-GMP was approximately 20% lower than that of the parent strain (Fig. 1). In addition, the catalase activity in the $\Delta katG$ strain expressing QrgB^{Mut} was ~2-fold lower than that of the parent strain under the same conditions (Fig. 1). Strains lacking both *katG* and *katB* did not have measurable catalase activity under these conditions, which is expected because these are the only annotated genes encoding catalase activity in the El Tor *V. cholerae* reference genome N16961 (7) (Fig. 1).

Characterization of the *katB* promoter and regulation of *katB* expression. Our results suggest *katB*, but not *katG*, is positively regulated by c-di-GMP at the transcriptional level. We addressed this hypothesis by measuring *katB* mRNA from cultures inducing QrgB and QrgB^{Mut} using quantitative real-time PCR (qRT-PCR). We found that *katB* expression increased ~12-fold in QrgB-expressing strains relative to QrgB^{Mut}-expressing strains (see Fig. S1 in the supplemental material).

We next were interested in the promoter architecture of the region upstream of *katB* driving expression and hypothesized that specific regions in the *katB* promoter were necessary for c-di-GMP-mediated transcriptional control. We tested this hypothesis by measuring luciferase activity from a series of *katB* transcriptional reporters that have been truncated at the 5' end in the presence of QrgB or QrgB^{Mut} expression (Fig. 2A). In the full-length promoter construct *katB1*, expression of QrgB induced *katB* expression 5-fold compared to strains overproducing QrgB^{Mut} (Fig. 2A). Inclusion of 212 bp upstream of *katB* was sufficient to maintain c-di-GMP induction (Fig. 2A, *katB2* and *katB3*). However, if the promoter was truncated to include 172 bp upstream of *katB* (*katB4*), c-di-GMP-dependent induction of *katB* was abrogated. This result suggested the necessary *cis*-acting sequences for c-di-GMP-mediated activation of *katB* expression are found between -172 and -212 bp upstream of the *katB* relative to the ATG start codon (Fig. 2A). Deleting 30 bp from *katB4* resulted in expression levels similar to that of the promoterless vector control regardless of the c-di-GMP concentrations, suggesting components necessary for the basal expression of *katB* are between -172 and -142 relative to the ATG start codon (Fig. 2A). We also measured the effect of c-di-GMP

FIG 3 *vpsT* and *vpsR* are Necessary for c-di-GMP mediated expression of *katB* but dispensable for H₂O₂ induction. (A) *katB* expression was measured in parent, Δ*vpsT*, Δ*vpsR*, and Δ*vpsT* Δ*vpsR* backgrounds while overproducing QrgB^{Mut} (white bars) or QrgB (black bars). Brackets and asterisks (*) indicate differences with a *P* value of <0.05 determined by two-way ANOVA, followed by Tukey's multiple-comparison test. The data are the average of three biological replicates with error bars indicating the standard deviations. (B) *katB* expression was measured in parent and Δ*vpsT* Δ*vpsR* backgrounds 30 min after the addition of 50 μM H₂O₂ or PBS control (untreated). The data are averages of three biological replicates, and error bars indicate the standard deviations.

on a *katG*-luciferase transcriptional fusion and did not observe significant c-di-GMP-dependent changes in expression, in agreement with our initial hypothesis (Fig. 2B).

VpsT and VpsR are necessary for the c-di-GMP-dependent induction of catalase activity, suggesting that these transcription factors activate transcription of *katB* at high intracellular concentrations of c-di-GMP (Fig. 1). Thus, we hypothesized that c-di-GMP-mediated induction of *katB* would be lost in strains lacking *vpsT*, *vpsR*, or both *vpsT* and *vpsR*. We tested this hypothesis by measuring *katB2* reporter activity under different c-di-GMP conditions in the parent, single-knockout, and double-knockout strains. We found that *katB2* expression increased 5-fold in the parent background. *katB* expression increased 2.5- to 3-fold in the Δ*vpsT* and Δ*vpsR* backgrounds, but the differences in expression between QrgB^{Mut} and QrgB were not statistically significant (Fig. 3A). It was only in the double-mutant Δ*vpsT* Δ*vpsR* background that c-di-GMP-mediated *katB* expression was completely lost (Fig. 3A). These data suggest that both VpsR and VpsT are needed for full induction by c-di-GMP and that individually they may be able to change expression of the *katB* reporter. However, if induction of *katB* occurs in the single Δ*vpsT* and Δ*vpsR* mutants, it is not sufficient to alter catalase activity (Fig. 1).

katB expression is upregulated in response to hydrogen peroxide in *V. cholerae* through the transcriptional activator OxyR (6, 24). To determine whether the same region necessary for c-di-GMP-mediated regulation of *katB* was necessary for H₂O₂ induction of *katB*, we measured the ability of H₂O₂ to induce *katB4*, the promoter region that no longer responded to c-di-GMP (Fig. 2A). We found that 50 μM H₂O₂ induced *katB4* expression ~6-fold in the parent background (Fig. 3B). Next, to determine whether VpsT and VpsR contributed to the H₂O₂ inducible response of the *katB4* promoter, we repeated the assay in a Δ*vpsT* Δ*vpsR* background. In this background, H₂O₂ increased *katB4* expression to the same extent as the parent; however, the basal and induced expression level was 1.5-fold lower compared to the parent (Fig. 3B).

In *Mycobacterium smegmatis*, H₂O₂ can act as a first messenger to promote c-di-GMP synthesis (25). Whether H₂O₂ acts as a first messenger to modulate c-di-GMP in *V. cholerae* has not been demonstrated. To test the hypothesis that H₂O₂ acts as a first messenger, we measured intracellular c-di-GMP in control and H₂O₂-treated cultures and found no differences in intracellular c-di-GMP (see Fig. S3 in the supplemental material). Together, these results indicate that *katB* transcription is induced by c-di-GMP and the oxidative stress response through distinct regulatory mechanisms and demonstrate that H₂O₂ does not alter global levels of intracellular c-di-GMP.

VpsT induces *katB* expression in a heterologous host and binds to the *katB* promoter *in vitro*. To test whether VpsT or VpsR directly regulates *katB*, we used

FIG 4 VpsT and c-di-GMP induce *katB* expression in a heterologous host. QrgB^{Mut} (open bars) or QrgB (solid bars) was overproduced, along with either an empty vector, VpsR, or VpsT in DH10b *E. coli* harboring the *katB3* promoter fused upstream of the luciferase operon. The data are average luminescences between strains overproducing QrgB and QrgB^{Mut} from three biological replicates, and error bars indicate the standard deviations. Brackets and asterisks (*) indicate statistically significant differences determined by two-way ANOVA, followed by Tukey's multiple-comparison testing.

Escherichia coli as a heterologous host to measure *katB* expression at high versus low concentrations of c-di-GMP in the presence of either VpsR or VpsT. We reasoned that the genetic dissimilarity between *V. cholerae* and *E. coli* would allow us to isolate the effects of VpsR and VpsT directly on the *katB* promoter without the regulatory feedback for these transcription factors (23, 26, 27). Expression of QrgB with an empty vector increased *katB* expression in *E. coli* ~2.5-fold compared to expression of QrgB^{Mut} through an unknown mechanism (Fig. 4). Similarly, coexpression of VpsR with QrgB resulted in approximately the same fold change (2-fold) as the empty vector, indicating that VpsR is not sufficient to induce *katB* expression when expressed in *E. coli* along with increased c-di-GMP (Fig. 4). Interestingly, expression of VpsT with QrgB^{Mut} increased *katB3* expression 20-fold compared to the empty vector control. Although this comparison had a *P* value of 0.8, the large difference between averages suggests VpsT can contribute to modulating gene expression under low-c-di-GMP conditions (Fig. 4). The largest difference in *katB3* expression was observed when VpsT was expressed with QrgB, resulting in a 344-fold induction compared to the empty vector (Fig. 4). Together, these data suggest VpsT is a key regulator of *katB* expression and that transcriptional activation is increased when c-di-GMP is present.

As a transcription factor, VpsT binds to DNA in the presence of c-di-GMP to modulate gene expression (13, 20, 21, 27). Since VpsT was necessary for c-di-GMP-mediated *katB* expression and was able to induce *katB* expression when expressed in a heterologous host, we hypothesized VpsT directly interacted with the *katB2* promoter in a c-di-GMP-dependent manner. Thus, we purified C-terminal His-tagged VpsT and measured its ability to bind to the *katB2* promoter *in vitro*. VpsT only partially shifted the *katB2* probe at the highest concentration tested (600 nM) in the absence of c-di-GMP (Fig. 5, lane 5). However, with the addition of 50 μ M c-di-GMP, VpsT was able to decrease the intensity of the unshifted band at 150 nM and completely shift the probe at 300 nM (Fig. 5, lanes 10 to 12). The addition of an unlabeled, 20-bp oligonucleotide composed of a VpsT binding site found in the *vpsL* promoter at a 100-fold molar excess was able to outcompete VpsT binding to the labeled *katB2* probe in the presence or absence of c-di-GMP (Fig. 5, lanes 7 and 14) (27). When the unlabeled competitor had transversion mutations introduced into the palindromic region, it was no longer able to abrogate the VpsT-*katB2* band migration (Fig. 5, lanes 6 and 13) (27). Together, the *in vivo* and *in vitro* data suggest that VpsT directly interacts with the *katB* promoter to induce expression under high-c-di-GMP conditions.

c-di-GMP-mediated HOOH survival is dependent on catalase. Since c-di-GMP increased *katB* expression and catalase activity, we hypothesized that high levels of

FIG 5 c-di-GMP enhances VpsT interaction with the *katB* promoter *in vitro*. Increasing concentrations of VpsT-HIS were incubated with a FAM-labeled probe corresponding to the *katB4* promoter (lanes 1 to 5). Unlabeled mutant (MT) or wild-type (WT) VpsT binding site competitor was added at a 100× molar excess relative to the labeled probe in reactions with 600 nM VpsT-His (lanes 6 and 7). Lanes 8 to 14 represent the same reaction conditions as lanes 1 to 7 except that 50 μM c-di-GMP was added to the binding reactions (indicated by the “+” sign).

intracellular c-di-GMP would provide a survival advantage during H₂O₂ stress. We tested this hypothesis by measuring survival after H₂O₂ treatment in *V. cholerae* backgrounds (Δ *vpsL*) unable to make mature biofilms to specifically test whether the transcription regulation of *katB* by c-di-GMP was responsible for any observed protection as opposed to matrix production or the formation of multicellular biofilms (8, 10, 28, 29). *V. cholerae* strains expressing QrgB^{Mut} or QrgB were challenged with H₂O₂, and the gross culture viability (i.e., the optical density at 600 nm [OD₆₀₀]) was monitored for 3 h. We found that in the parent strain the expression of QrgB, but not QrgB^{Mut}, led to significant protection from H₂O₂ stress (Fig. 6A). To test whether this production was dependent on c-di-GMP-mediated catalase activity, we measured H₂O₂ survival in the Δ *katB*, Δ *katG*, and Δ *katB* Δ *katG* mutants. Consistent with our catalase activity results, deletion of *katB* in either the Δ *katB* and Δ *katB* Δ *katG* mutants resulted in no c-di-GMP-mediated H₂O₂ survival (Fig. 6E and G), whereas the survival of the Δ *katG* mutant during H₂O₂ treatment resembled that of the parent strain (Fig. 6F).

Since *vpsT* and *vpsR* were necessary for c-di-GMP-dependent induction of *katB* expression and catalase activity (Fig. 1 and 4), we hypothesized that deletion mutations of these transcription factors would decouple c-di-GMP signaling from survival during H₂O₂ treatment. Indeed, strains lacking *vpsT*, *vpsR*, or both *vpsT* and *vpsR* lost c-di-GMP-mediated survival during H₂O₂ treatment (Fig. 6B to D). Taken together, these data suggest that c-di-GMP increases *katB* expression and KatB catalase activity through the c-di-GMP-dependent transcription factors *vpsT* and *vpsR*, resulting in increased survival during H₂O₂ treatment (Fig. 7).

DISCUSSION

We sought here to determine whether providing resistance to ROS is a c-di-GMP-regulated phenotype in *V. cholerae*. Using a plasmid-based system to modulate intracellular c-di-GMP, we observed increased gas production after the addition of H₂O₂, suggesting that c-di-GMP positively influenced catalase activity. We determined that *katB* was responsible for c-di-GMP-regulated catalase activity and found that *katB* transcription was increased 5-fold in the parent background but was lost in strains lacking the c-di-GMP-dependent transcription factors VpsT and VpsR. Measuring *katB*

FIG 6 c-di-GMP enhances survival after H₂O₂ treatment. QrgB^{mut} (open squares) or QrgB (solid squares) were induced with 100 μ M IPTG in parent (A), $\Delta vpsT$ (B), $\Delta vpsR$ (C), $\Delta vpsT \Delta vpsR$ (D), $\Delta katB$ (E), $\Delta katG$ (F), and $\Delta katB \Delta katG$ (G) strains until reaching an OD₆₀₀ of 0.3. H₂O₂ was added to cultures at a final concentration of 12.5 mM. Cell death was monitored by measuring OD₆₀₀ every hour after H₂O₂ addition (time 0). *, $P < 0.05$ compared to the untreated control at that time point as determined by two-way ANOVA, followed by Tukey's multiple-comparison test. Bars indicate averages from three biological replicates, and error bars indicate the standard deviations.

transcription in a heterologous host revealed that VpsT was sufficient to induce *katB* expression under high-c-di-GMP conditions, and *in vitro* DNA binding assays demonstrated that VpsT specifically bound to the *katB* promoter in a c-di-GMP-dependent fashion. Lastly, we showed that c-di-GMP-mediated survival after H₂O₂ treatment was dependent on *vpsT*, *vpsR*, and *katB*.

FIG 7 Model for the cyclic di-GMP regulatory network controlling biofilm formation and H₂O₂ tolerance. In response to high intracellular levels of cyclic di-GMP, VpsR becomes activated and induces the expression of *vpsT*. VpsT, in turn, is activated by cyclic di-GMP, and both VpsR and VpsT activate genes in the *vps* operons to promote biofilm formation. Our data indicate that cyclic di-GMP-activated VpsT also induces the expression of *katB*, which increases the catalase activity of the population. The catalase degrades H₂O₂ and promotes survival after H₂O₂ treatment. Our data also demonstrate that the induction of *katB* by H₂O₂ occurs independently of the cyclic di-GMP signaling network and is likely controlled by OxyR, a transcription factor involved in a broader antioxidant production program.

In agreement with our findings, other groups have observed an increase in *katB* expression under certain biofilm-inducing conditions that modulate intracellular c-di-GMP, such as incubation with norspermidine (30). In addition, rugose variants of *V. cholerae* were shown to be resistant to H₂O₂ in a VPS-independent manner and were later found to have increased expression of *katB* (31, 32). Transcriptomic data from experiments where c-di-GMP or VpsT was artificially induced in *V. cholerae* suggested that *katB* expression was positively regulated by c-di-GMP (13, 33).

Interestingly, c-di-GMP and VpsT were shown to downregulate the production of the stationary-phase sigma factor RpoS, in turn decreasing survival to various stressors, including H₂O₂, which is in contrast to our results (Fig. 5A) (20). These experiments were done with the El Tor biotype strain C7258, which is part of the serogroup Ogawa, whereas the strain used in the present study (C6076 Str2) is part of the serogroup Inaba. Although there are no studies describing differences in transcriptional regulation between the two serogroups, we hypothesize there may be serogroup-dependent differences in c-di-GMP signaling, which would explain the contrasting results.

Unlike the $\Delta vpsT \Delta vpsR$ double mutant, c-di-GMP was able to induce the expression of the *katB* reporter in the single *vpsT* and *vpsR* deletion backgrounds, although the difference was not statistically significant. To further clarify which transcription factor was the direct effector, we reconstructed the system in *E. coli* and found that only production of VpsT and c-di-GMP, but not that of VpsR and c-di-GMP, increased the expression of the *katB* promoter in *E. coli* (Fig. 4). c-di-GMP-mediated catalase activity and survival in H₂O₂ were also lost in both $\Delta vpsT$ and $\Delta vpsR$ backgrounds. Thus, our evidence suggests that phenotypes are primarily controlled by VpsT (Fig. 7). VpsR is required for c-di-GMP-mediated induction of *vpsT* in *V. cholerae*; therefore, we conclude that it has an indirect effect on *katB* transcription (26) (Fig. 7).

Interestingly, we observed that VpsT was able to induce *katB* expression 20-fold under unaltered c-di-GMP conditions in *E. coli* (Fig. 4). In addition, VpsT was also able to bind to the *katB* promoter without exogenously added c-di-GMP, albeit at high protein concentrations (Fig. 5). It is possible these results are a consequence of relatively high VpsT levels driven by the expression plasmid; however, it is also possible that VpsT may have some c-di-GMP-independent effect on gene expression. Indeed, allele variants of VpsT that are unable to bind to c-di-GMP are still able to modulate gene expression (13). If and how VpsT functions in the absence of c-di-GMP is an area that requires more research.

A predicted VpsT binding site ~150 bp upstream of the ATG start codon of *katB* was previously reported (34). Our results indicate that even when the VpsT binding site was present in the *katB4* transcriptional fusion, induction by c-di-GMP was lost. We note that the predicted VpsT sequence was found in the template strand, while predicted and validated VpsT binding sites in the *vpsL* and *vpsA* promoters were found in the coding strand (27, 34). As a result of this difference, the binding site would position VpsT in different directionalities, i.e., positioned toward or away from the open reading frame. Whether this difference in directionality alters VpsT transcriptional regulation warrants further investigation.

Although our work focuses on *V. cholerae*, the association between c-di-GMP and protection against ROS has been shown in other bacteria. In *Listeria monocytogenes*, deletion of genes encoding proteins with PDE domains resulted in elevated biofilm production and H₂O₂ tolerance (9). However, it is not known whether the H₂O₂ tolerance was caused by increased EPS production, antioxidant production, or both. In *Mycobacterium smegmatis*, a relative of the human pathogen *M. tuberculosis*, H₂O₂ stimulates the production of intracellular c-di-GMP, which inactivates the transcriptional repressor HpoR. As a result, ROS defense genes are upregulated and increase H₂O₂ tolerance (25). This differs from our work in two ways: (i) H₂O₂ does not act as a first messenger that stimulates c-di-GMP activity in *V. cholerae* (see Fig. S3) and (ii) VpsT and c-di-GMP regulate a wide array of genes, whereas HpoR-c-di-GMP has been shown to only regulate expression of the *hpoR* operon. Despite the differences in regulation,

TABLE 1 Plasmids used in this study

Plasmid	Description ^a	Copy no.	Source or reference
pBBRlux	<i>luxABCDE</i> containing promoterless plasmid; Cam ^r	5–10	Lab collection
pEVS143	<i>pTac</i> overexpression vector; Kan ^r	10	40
pKAS32	λ pir-dependent allele exchange vector; Amp ^r	15–20	41
pBRP333	Control expression vector; Kan ^r	10	17
pBRP1	<i>qrgB</i> * (inactive DGC) cloned into expression vector pMMB67Eh; Amp ^r	10–12	17
pBRP2	<i>qrgB</i> (active DGC) cloned into expression vector, pMMB67Eh; Amp ^r	10–12	17
pNF031	<i>katB1</i> promoter in pBBRlux; Cam ^r	5–10	This study
pNF032	<i>katB2</i> promoter in pBBRlux; Cam ^r	5–10	This study
pNF033	<i>katB3</i> promoter in pBBRlux; Cam ^r	5–10	This study
pNF036	<i>katB4</i> promoter in pBBRlux; Cam ^r	5–10	This study
pNF038	<i>katB5</i> promoter in pBBRlux; Cam ^r	5–10	This study
pNF030	<i>katG</i> promoter in pBBRlux; Cam ^r	5–10	This study
pCMW131	<i>vpsR</i> in pEVS143; Kan ^r	10	23
pCMW132	<i>vpsT</i> in pEVS143; Kan ^r	10	23
pNF011	<i>vpsT</i> in pET28b (C-terminal His tag); Kan ^r	15–20	21
pNF057	pHERD20T μ BAD expression vector, with Cam ^r replacing Amp ^r	10–15*	This study
pNF067	<i>katB</i> in pNF057; Cam ^r	10–15*	This study
pNF068	<i>katG</i> in pNF057; Cam ^r	10–15*	This study
pNF045	Deletion construct for <i>katB</i> in pKAS32; Amp ^r	15–20	This study
pNF048	Deletion construct for <i>katG</i> in pKAS32; Amp ^r	15–20	This study

^aCam^r, chloramphenicol resistance; Kan^r, kanamycin resistance; Amp^r, ampicillin resistance. *, the copy number of the pRO1600 origin is host dependent.

the connection between c-di-GMP and ROS tolerance is evident in bacteria from diverse phylogenetic backgrounds.

MATERIALS AND METHODS

DNA manipulations and growth conditions. *V. cholerae* C6706 Str2 was used as the wild type, and the low-biofilm-forming Δ vpsL mutant derivative was used as the parent strain (23, 35). All vectors were constructed by Gibson Assembly (NEB). Chromosomal deletion strains were constructed using the allele exchange vector pKAS32 digested with KpnI and SacI (NEB, High Fidelity). Luciferase reporters were constructed using the luciferase reporter vector pBBRlux digested with BamHI and SpeI (NEB) (Table 1). Expression vectors for VpsT and VpsR were constructed by removing the ribosome-binding site, green fluorescent protein, and chloramphenicol acetyltransferase from pEVS143 with BamHI and EcoRI digests (NEB) (23). The VpsT purification vector was constructed elsewhere (21). Expression vectors for *katB* and *katG* were constructed as follows. pHERD20T was amplified with primers flanking the ampicillin resistance gene using inverse PCR, resulting in a linear PCR fragment lacking the ampicillin resistance gene (36). The chloramphenicol resistance gene from pBBRlux was amplified by PCR, and the two linear fragments were circularized by Gibson Assembly. Plasmids were introduced into *V. cholerae* through biparental conjugation using *Escherichia coli* S17- λ pir as the donor strain. *V. cholerae* harboring the plasmid of interest was selected for using polymyxin B (10 U/ml) with the relevant antibiotic. Antibiotics and reagents were used at the following concentrations unless stated otherwise: ampicillin (100 μ g/ml), kanamycin (100 μ g/ml), chloramphenicol (10 μ g/ml), and 100 μ M IPTG (isopropyl- β -D-thiogalactopyranoside). Cultures were grown in lysogeny broth (LB; Acumedia) at 35°C and 220 rpm unless otherwise stated (Table 2 shows strains used in this study).

TABLE 2 Strains used in this study

Strain	Description	Source or reference
<i>E. coli</i>		
DH10b	F [−] <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80/ <i>lacZ</i> Δ M15 Δ <i>lacX74</i> <i>recA1</i> <i>endA1</i> <i>araD139</i> Δ (<i>ara-leu</i>)7697 <i>galU</i> <i>galK</i> λ [−] <i>rpsL</i> <i>nupG</i>	Thermo Fisher Scientific
S17- λ pir	Tp ^r Sm ^r <i>recA</i> <i>thi</i> <i>pro</i> <i>hsdR17</i> (r _K [−] m _K ⁺) <i>RP4::2-Tc::Mu</i> Km Tn7 λ pir	Lab stock
BL21(DE3)	F [−] <i>ompT</i> <i>hsdSB</i> (r _B [−] m _B ⁺) <i>gal</i> <i>dcm</i> (DE3)	Invitrogen
<i>V. cholerae</i>		
C6706 Str2	Wild-type El Tor (serogroup Inaba)	35
CW2034	C6706 Str2 Δ vpsL	23
NF03	CW2034 Δ katB	This study
NF04	CW2034 Δ katG	This study
NF05	CW2034 Δ katB Δ katG	This study
JC1195	CW2034 Δ vpsT	Lab Stock
WN310	CW2034 Δ vpsR	Lab Stock
JC1220	CW2034 Δ vpsT Δ vpsR	Lab Stock

TABLE 3 Oligonucleotides used for qRT-PCR and EMSA experiments

Expt and description	Sequence (5'–3')	T_m^a (qRT-PCR only)
qRT-PCR		
<i>gyrA</i> -For	AAATTCATCTTCATGGCAAC	58
<i>gyrA</i> -Rev	GCGATGTTTTCTTCACAG	58
<i>katB</i> -For	CGTATGCCGATACACAGCTTTA	64
<i>katB</i> -Rev	ACGACTGGGTGGTAGTTAATG	64
EMSA		
pBBRLux-FAM-For	FAM-ATTTTGGCGCCGCAACTAGA	
pBBRLux-FAM-Rev	FAM-CCGCGGTGGCGCCGCTCTA	
WT-VpsT binding site-For	AAAGTAACTAAAGTTTATTTT	
WT-VpsT binding site-Rev	AAAATAAACTTTAGTTTACTTT	
MT-VpsT binding site-For	AAAAGGTCTAAACTAGGTT	
MT-VpsT binding site-Rev	AACCTAGTTTTAGACCTTTT	

^a T_m , melting temperature.

Catalase assay. Measurement of catalase activity was adapted from an earlier study (37) with the following changes. Overnight cultures were diluted 1:100, the OD₆₀₀ was measured, and the resulting value was multiplied by the dilution factor of 100 to obtain the actual optical density of the solution. The cell solutions were diluted to a starting OD₆₀₀ of 0.040 in 5 ml of LB in 18- by 150-mm borosilicate test tubes supplemented with the necessary antibiotics and IPTG. Cultures were grown at 35°C with shaking at 220 rpm until the OD₆₀₀ reached ~2.0, moved to 15-ml Falcon tubes (Corning), and pelleted by centrifugation (4,000 × *g* for 3 min). Pellets were resuspended in 100 μl of sterile 1× phosphate-buffered saline (PBS) to create a viscous cell solution that was adjusted to an OD₆₀₀ of 150 in a 100-μl final volume in Pyrex test tubes (13 by 100 mm, borosilicate). Then, 200 μl of catalase reaction buffer (1% Triton X-100 and 15% hydrogen peroxide in 1× PBS) was added to the test tubes, and the solution was mixed using disposable 10-μl loops (BD Difco). Tubes were incubated at room temperature until gas production subsided (approximately 5 to 10 min). A standard curve was generated by mixing purified bovine catalase (Sigma, 570 U/μl) diluted in 1× PBS with 200 μl of catalase reaction buffer. At 10 min of incubation, images of the tubes were taken with an iPad Air (iOS 12.1.4), and the height from the bottom of the tube to the top of the foam was measured in both the standards and the samples using ImageJ software and an internal 1-inch reference mark for each picture. GraphPad Prism was used to generate the standard curve and interpolate the sample catalase activity using linear regression. The data are presented as catalase activity (units) normalized to the cell number (OD₆₀₀).

RNA isolation and qRT-PCR. Three biological replicate overnight cultures were diluted to a starting OD₆₀₀ of 0.040 in 2 ml of LB supplemented with ampicillin and IPTG and grown until reaching an OD₆₀₀ of ~1.0 at 35°C and 220 rpm. Next, 1 ml of each replicate was pelleted, and RNA was extracted using the TRIzol reagent following the directions in the manual (Thermo Fisher Scientific). Purified DNA was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific). Then, 5 μg of purified RNA was treated with DNase (Turbo DNase; Thermo Fisher Scientific). cDNA synthesis was carried out using a GoScript reverse transcription kit (Promega). cDNA was diluted 1:30 into molecular-biology-grade water and used as the template in qRT-PCR analyses with SYBR green (Applied Biosystems) as the method of detection. Reactions consisted of 5 μl of 2.5 μM primer 1, 5 μl of 2.5 μM primer 2, 5 μl of diluted cDNA template, and 15 μl of 2× SYBR green consisting of deoxynucleoside triphosphates and AmpliTaq Gold DNA polymerase (see Tables 3 and 4 for the primer sequences). Each plate had technical duplicates and biological triplicate samples, as well as no-reverse transcriptase controls, to check for genomic DNA contamination. A StepOnePlus real-time PCR system was used for qRT-PCR with the following thermocycling conditions: 95°C for 20 s, followed by 40 cycles of 95°C for 2 s and 60°C for 30 s. Melting curves were included to ensure that PCR products had single amplicons and that primer dimers were absent. Data were analyzed by the $\Delta\Delta C_T$ method using *gyrA* as a housekeeping or reference target.

Luciferase reporter assays. (i) *V. cholerae* reporter assays. Overnight cultures of *V. cholerae* harboring *katB* transcriptional fusions to luciferase in pBBRLux were diluted 1:100 in 1 ml of LB supplemented with ampicillin, chloramphenicol, and IPTG in 1.5-ml microcentrifuge tubes. Then, 200-μl portions of cell solution were aliquoted into the wells of black 96-well plates (Costar). The plates were incubated at 35°C with shaking at 220 rpm until the OD₆₀₀ reached ~0.250, and the luciferase activity was measured using an Envision plate reader (Perkin-Elmer). The luciferase activity (in relative light units [RLU]) was normalized for the cell number by dividing the RLU by the OD₆₀₀ at the time of the reading (normalized luminescence). For experiments where H₂O₂ were added to the cultures, overnight cultures of *V. cholerae* were diluted as described above except that H₂O₂ was added to the cultures at a final concentration of 50 μM when the OD₆₀₀ values reached ~0.225, followed by shaking at 35°C for an additional 30 min before measuring the luciferase and the OD₆₀₀.

(ii) *E. coli* DH10b luciferase assays. Overnight cultures of *E. coli* DH10b harboring vectors to modulate transcription factor, c-di-GMP production, and the luciferase reporter were diluted 1:100 as described above and grown at 35°C and 220 rpm until the OD₆₀₀ reached 0.450. The luciferase activity was measured and normalized to the OD₆₀₀ to yield the normalized luminescence.

Protein purification and EMSAs. C-terminal His-tagged VpsT purification and electrophoretic mobility shift assay (EMSA) experiments using the FAM-labeled *katB* promoter region from *katB2* were

TABLE 4 Oligonucleotides used for vector construction

Construct ^a	Orientation ^b	Sequence (5'–3')	
		5' overhang	3' gene specific
pNF031	Forward	CGGTGGCGGCCGCTCTAGAA	TTCACATTTGCTGGTTCAAAGG
pNF032	Forward	CGGTGGCGGCCGCTCTAGAA	AAACGCCCCGAGAATAGAGA
pNF033	Forward	CGGTGGCGGCCGCTCTAGAA	TTGGCTTAAATAATGACGGTTG
pNF036	Forward	CGGTGGCGGCCGCTCTAGAA	AAGTCATCTAAACTTTTCGTTTAAATCGATTTTC
pNF038	Forward	CGGTGGCGGCCGCTCTAGAA	TTTCTCTATTAAATTCATCGTTATTTCCCATTTAG
pNF031,032,033,036,038	Reverse	CCATTTTGC GGCCGCAACTAGAG	GGTTGCTCTCCAATGCGAC
pNF030	Forward	CGGTGGCGGCCGCTCTAGAA	CAAACAGCGCGATATCTTCTT
	Reverse	CCATTTTGC GGCCGCAACTAGAG	TTTAGCGCTTTAGGCCACC
pNF057	Forward_1*		CACATTTCCCCGAAAAGTG
	Reverse_1*		CTGTCAGACCAAGTTTACTC
	Forward_2#	GCACTTTTCGGGGAAATGTG	TTAATGAATCGGCCAACG
	Reverse_2#	GAGTAAACTTGGTCTGACAG	TGATCGGCACGTAAGAGG
pNF067	Forward	ATCTGATAAGAATTCGAGCT	ATGCATATGTCAAAAAGCTTT
	Reverse	TGCATGCCTGCAGGTCGACT	TTACATCGCGGCCAGTTTTG
pNF068	Forward	ATCTGATAAGAATTCGAGCT	ATGGAGCACAATAAAGC
	Reverse	TGCATGCCTGCAGGTCGACT	TTACACCAGATCAAATCG
pNF045 (UI)	Forward	AGCTATAGTTCTAGAGGTAC	GGTGGTGGTGAAGACAATTC
	Reverse	GTGGAATTCCTGGGAGAGCT	ATTTTACTTATCAACCGTCATTATTTTAAG
pNF045 (DI)	Forward	AGCTATAGTTCTAGAGGTAC	CCGATAAAAATTTGCCCC
	Reverse	GTGGAATTCCTGGGAGAGCT	GATCACCCAAGTGATTGTC
pNF048 (UI)	Forward	AGCTATAGTTCTAGAGGTAC	ACCTGAGTTGCAATAACCAAAG
	Reverse	GTGGAATTCCTGGGAGAGCT	TGATTACTCCTTGCTTCGCAT
pNF048 (DI)	Forward	AGCTATAGTTCTAGAGGTAC	TCCATCTCACCATCCCTTTCA
	Reverse	GTGGAATTCCTGGGAGAGCT	GATGAGATGGTCGCCCCC

^aUI, upstream insert; DI, downstream insert.

^b*, Forward_1 and Reverse_1, primers used for inverse PCR to amplify pHERD20T backbone without the ampicillin resistance gene; #, Forward_2 and Reverse_2, primers used to amplify the chloramphenicol acetyltransferase from pBBRLux.

carried out as previously described (21). For purification, an overnight culture of *E. coli* BL21 harboring the pET28b-VpsT expression construct was diluted 1:100 into 250 ml of LB supplemented with kanamycin in a 1-liter flask. The culture was grown to an OD₆₀₀ of ~0.7, at which point 1 mM IPTG was added, and the culture conditions were shifted to 16°C with shaking at 160 rpm for 16 h to induce protein production. Protein purification was carried out by standard Ni-NTA resin purification protocols. For EMSAs, various concentrations of purified His-tagged VpsT (0 to 600 nM) were incubated with 2.5 nM FAM-labeled *katB* probe, along with the nonspecific DNA competitor poly(dI-dC) at 1 µg/µl in VpsT buffer (25 mM Tris-Cl, 150 mM NaCl, 5 mM β-mercaptoethanol [pH 7.5]) at 30°C for 30 min. The binding reaction was loaded into prerun 5% nondenaturing Tris-borate-EDTA gels, and gel electrophoresis was performed by applying 90 V for 90 min at 4°C. Images of the gels were taken using a Typhoon FLA 9000 imager using settings for FAM label detection and the requisite software (GE Healthcare Life Sciences).

Hydrogen peroxide survival assay. Overnight cultures were diluted to a starting OD₆₀₀ of 0.040 in 1 ml of LB supplemented with the necessary antibiotics and IPTG in 1.5-ml microcentrifuge tubes. Then, 140-µl aliquots were added to a 96-well plate (Costar) and grown to an OD₆₀₀ of 0.300. H₂O₂ solutions were made from fresh H₂O₂ stocks in light-impermeable microcentrifuge tubes and sterile 1× PBS. At time zero, 10 µl of H₂O₂ was added to the cell solution, and growth was monitored over time by measuring the OD₆₀₀.

Measurement of intracellular c-di-GMP. Overnight cultures of Δ*vpsL* mutant harboring pBRP1 (QrgB^{Mut}) were diluted 1:100 in 2 ml of LB ampicillin in 18-by-150-mm borosilicate test tubes and grown to an OD₆₀₀ of 1.0. The cultures were split into two 1-ml aliquots in microcentrifuge tubes, and H₂O₂ was added to one aliquot at a final concentration of 500 µM. An equal volume of water was added to the other aliquot as the untreated control. Cultures were incubated statically at room temperature for 30 min and then collected for total protein quantification and nucleotide extraction. Briefly, 100 µl of culture was removed from each tube to quantify total protein, pelleted by centrifugation at full speed (15,000 × *g*) for 1 min, resuspended in 100 µl 1× PBS with 10% sodium dodecyl sulfate, and boiled at 95°C for 10 min. Lysed cell solutions were centrifuged at 15,000 × *g* for 1 min, and the supernatant was removed and placed in new tubes. Total protein was quantified by using a DC protein assay (Bio-Rad) following the instructions of the manufacturer. Protein standards consisting of bovine serum albumin (provided with the DC protein assay) were used to generate a standard curve to interpolate sample concentrations. Nucleotide extractions were carried out following the protocol as described in reference 38 with the following changes. Portions (900 µl) of the remaining culture were pelleted at 15,000 × *g* for 1 min in a benchtop microcentrifuge. The supernatants were removed, and the remaining pellets were resuspended in 100 µl of nucleotide extraction buffer (methanol-acetonitrile-water [40:40:20] with 0.1 N formic acid). The extraction solution was incubated at 20°C for 20 min and pelleted for 10 min at 15,000 × *g*. The supernatants were placed into new microcentrifuge tubes, and the solutions were dried overnight using a heated, vacuum centrifuge (SpeedVac concentrator; Savant). The resulting dried pellets

were resuspended in 100 μ l of high-pressure liquid chromatography-grade water and subjected to mass spectrometry analysis to quantify the c-di-GMP (39). The data are represented as picomoles of c-di-GMP normalized by total cellular protein (mg).

Statistical analysis. Data are represented as means $\pm$ the standard deviations. Statistical analyses (see details in the figure legends) were calculated using Prism v6 (GraphPad, San Diego, CA). A *P* value of <0.05 was considered statistically significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.01043-19>.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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