



# Cyclic di-GMP Positively Regulates DNA Repair in *Vibrio cholerae*

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**ABSTRACT** In *Vibrio cholerae*, high intracellular cyclic di-GMP (c-di-GMP) concentrations are associated with a biofilm lifestyle, while low intracellular c-di-GMP concentrations are associated with a motile lifestyle. c-di-GMP also regulates other behaviors, such as acetoin production and type II secretion; however, the extent of phenotypes regulated by c-di-GMP is not fully understood. We recently determined that the sequence upstream of the DNA repair gene encoding 3-methyladenine glycosylase (*tag*) was positively induced by c-di-GMP, suggesting that this signaling system might impact DNA repair pathways. We identified a DNA region upstream of *tag* that is required for transcriptional induction by c-di-GMP. We further showed that c-di-GMP induction of *tag* expression was dependent on the c-di-GMP-dependent biofilm regulators VpsT and VpsR. *In vitro* binding assays and heterologous host expression studies show that VpsT acts directly at the *tag* promoter in response to c-di-GMP to induce *tag* expression. Last, we determined that strains with high c-di-GMP concentrations are more tolerant of the DNA-damaging agent methyl methanesulfonate. Our results indicate that the regulatory network of c-di-GMP in *V. cholerae* extends beyond biofilm formation and motility to regulate DNA repair through the VpsR/VpsT c-di-GMP-dependent cascade.

**IMPORTANCE** *Vibrio cholerae* is a prominent human pathogen that is currently causing a pandemic outbreak in Haiti, Yemen, and Ethiopia. The second messenger molecule cyclic di-GMP (c-di-GMP) mediates the transitions in *V. cholerae* between a sessile biofilm-forming state and a motile lifestyle, both of which are important during *V. cholerae* environmental persistence and human infections. Here, we report that in *V. cholerae* c-di-GMP also controls DNA repair. We elucidate the regulatory pathway by which c-di-GMP increases DNA repair, allowing this bacterium to tolerate high concentrations of mutagens at high intracellular levels of c-di-GMP. Our work suggests that DNA repair and biofilm formation may be linked in *V. cholerae*.

**KEYWORDS** DNA repair, c-di-GMP, VpsT, *Vibrio cholerae*, biofilm, cyclic di-GMP

Many bacterial species use the second messenger signaling molecule cyclic dimeric GMP (c-di-GMP) to control cellular behavior. Since its discovery in 1987, research in c-di-GMP signaling has uncovered a largely invariable paradigm, that high intracellular c-di-GMP stimulates an adherent static lifestyle known as a biofilm, while decreasing c-di-GMP results in a motile lifestyle (1). Two classes of enzymes are responsible for changes in intracellular c-di-GMP. Diguanylate cyclase enzymes (DGCs), containing GGDEF active-site motifs, catalyze the cyclization of two GTP molecules to form c-di-GMP. c-di-GMP is degraded into pGpG or 2 GMP molecules by phosphodiesterase enzymes (PDEs) containing EAL or HD-GYP active-site motifs, respectively (1–3). pGpG is further degraded to GMP by the Orn nuclease (4, 5). Furthermore, the enzymatic activities of DGCs and PDEs are thought to be controlled by environmental cues (3).

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Thus, c-di-GMP functions as one of the major global regulatory pathways that allows bacteria to respond and adapt to their environment.

c-di-GMP additionally regulates bacterial behaviors beyond biofilm formation and motility. For example, *Streptomyces* species undergo a morphological transition between vegetative and aerial mycelial growth that is controlled by the c-di-GMP-dependent transcription factor BldD (6). In *Caulobacter crescentus*, certain DGC and PDE enzymes localize to different cellular regions to control the transition from a swarmer to stalked cell phenotype (7, 8). c-di-GMP also regulates virulence and the type II secretion apparatus in *V. cholerae* (9, 10). However, this list of bacterial phenotypes controlled by c-di-GMP has not been fully elucidated. Further, whether these phenotypes act in a coordinated manner to promote adaptation to a sessile or motile lifestyle is not clear. Here, we report that DNA repair is a new phenotype controlled by c-di-GMP in *V. cholerae*.

The human pathogen *V. cholerae* causes the diarrheal disease cholera, ultimately resulting in death by dehydration if left untreated. Currently, the 7th *V. cholerae* pandemic caused by the El Tor biotype is responsible for numerous infections worldwide, including recent outbreaks in Haiti, Ethiopia, and Yemen (11–13). While infamously known for its epidemics throughout human history, *V. cholerae* resides harmlessly between disease outbreaks in marine environments, where it is thought to associate with chitinous surfaces as biofilms (14–16). Biofilm formation and motility also contribute to *V. cholerae* infections. Growth in biofilms induces a hyperinfectious state, while motility is necessary for colonization of the proximal small intestine (17–19). Thus, the transition between biofilm formation and motility is considered a key aspect of the pathogenesis of this bacterium.

The mechanism for c-di-GMP-dependent biofilm formation in *V. cholerae* has been well studied. In response to specific environmental cues, such as bile acids (20), intracellular c-di-GMP increases. c-di-GMP binds to and activates the c-di-GMP-dependent transcription factor VpsR (27). Activated VpsR then induces the expression of another c-di-GMP-dependent transcription factor, VpsT, which is also directly activated by c-di-GMP binding (21, 22). At high c-di-GMP concentrations, these two transcription factors induce the expression of genes needed for biofilm formation, in particular, the *Vibrio* polysaccharide (VPS) operons and the gene cluster *rbmBCDEF* encoding biofilm matrix proteins (23, 24). Concurrently, high c-di-GMP concentrations directly decrease the expression of the flagellar biosynthesis gene cluster through inhibition of the transcription factor FlrA (25). Thus, high c-di-GMP concentrations increase the expression of genes involved in biofilm formation and simultaneously decrease the expression of genes involved in flagellar motility. Moreover, the production of VPS itself inhibits motility (25).

In response to c-di-GMP, VpsT and VpsR regulate the gene expression of other processes in addition to biofilm formation. *aphA*, a gene involved in virulence regulation and acetoin biosynthesis, is induced by VpsR and c-di-GMP, but only acetoin utilization is significantly impacted (26, 27). VpsR, activated by c-di-GMP, induces transcription of the *eps* operon encoding proteins necessary for production of the type II secretion system. While not impacting protein secretion, the induction of *eps* leads to the production of a surface pseudopilus (10). VpsR and c-di-GMP induce transcription of the transcription factor gene *tfoY*, which impacts motility and type VI secretion (28, 29). In addition, transcriptomic analyses indicate that c-di-GMP signaling and VpsT regulate the expression of various genes; however, the physiological effect of these global gene responses is not well characterized (15, 22).

In the marine environment and human host, *V. cholerae* likely incurs DNA damage that must be repaired to maintain genome integrity and fitness. Indeed, mutations in key genes of the base excision repair (BER) pathway decrease infant mouse colonization and increase sensitivity to DNA-damaging chemicals (30). DNA glycosylases stimulate the repair of DNA damage through the BER pathway by specifically recognizing and removing lesioned bases (31). While the mechanistic details of the BER pathway have been well studied, only a few regulatory pathways controlling BER gene expression in

bacteria have been identified (31–33). Here, we report our findings that increased c-di-GMP induces transcription of the DNA glycosylase Tag (3-methyladenine glycosylase) gene in *V. cholerae*. We demonstrate that c-di-GMP positively regulates *tag* expression through the c-di-GMP-dependent transcription factors VpsT and VpsR but not FlrA, indicating that *tag* may play a role in homeostasis within a biofilm or during biofilm formation. We further demonstrate that VpsT directly regulates the expression of *tag* in a c-di-GMP-dependent manner. Last, we show that cells with high intracellular c-di-GMP concentrations have increased tolerance to the DNA-methylating agent methyl methanesulfonate (MMS).

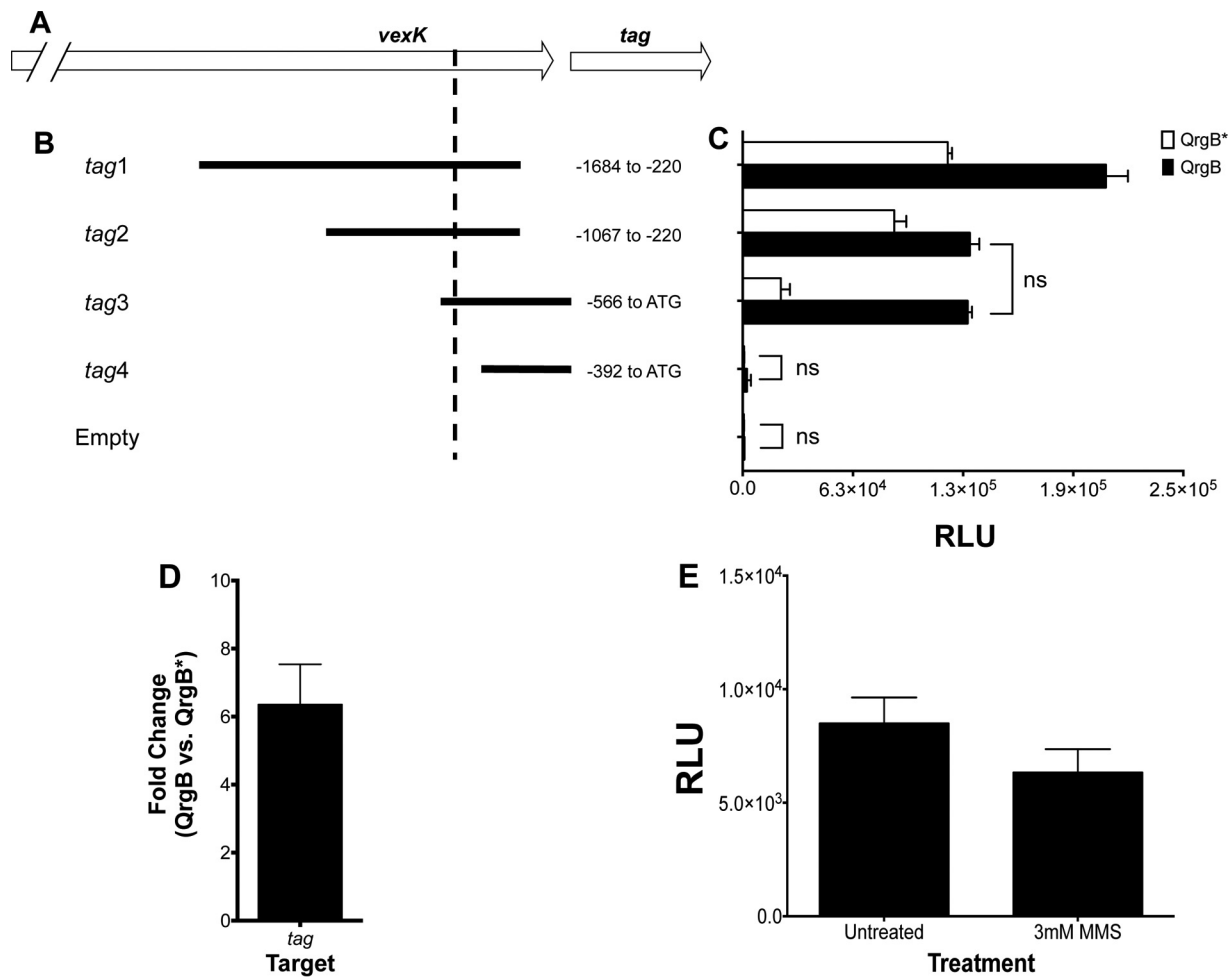
## RESULTS

**c-di-GMP induces expression of *tag*.** Bacteria use c-di-GMP to modulate disparate aspects of cell physiology ranging from biofilm formation to cell cycle progression. While some of these phenotypes have been characterized in *V. cholerae*, we hypothesized that there were more remaining to be discovered (34). Thus, we screened a library of transcriptional fusion reporters for promoters regulated by c-di-GMP, as previously reported (27). Of the seven promoters identified, one mapped upstream of the gene VC1672 (referred to as 6:C9 in our previous publication [27]), encoding the DNA glycosylase Tag. In *Escherichia coli*, Tag initiates the BER pathway by recognizing and removing adenines and guanines methylated at the 3-position in DNA (35, 36).

The original region of DNA isolated from the promoter screen, which we refer to as *tag1* (Fig. 1A), was ~1.4 kb long and extended to the upstream gene VC1673, which encodes the AcrB family transporter VexK. Based on the orientation of this DNA sequence, transcription could proceed into *tag*. To determine the region necessary for c-di-GMP-mediated reporter activity, we modified the lengths of the 5' and 3' ends of *tag1* and measured promoter activity under different c-di-GMP levels (Fig. 1B and C). To increase intracellular c-di-GMP levels, we overproduced the *Vibrio harveyi* DGC QrgB from a plasmid. We found that this system generates c-di-GMP at concentrations that are similar to those at growth at low cell density, a growth phase of *V. cholerae* that naturally has high intracellular c-di-GMP concentrations due to quorum sensing regulation (29, 37). As a negative control, the active-site mutant QrgB\*, which does not change c-di-GMP levels, was similarly induced. Induction of QrgB significantly increased the promoter activities of regions *tag1* through *tag3* relative to QrgB\* (Fig. 1B). Moreover, the DNA sequence from –1067 to –566 in both *tag1* and *tag2* increases basal expression at low c-di-GMP concentrations relative to *tag3* (Fig. 1C, white bars). *tag3*, which contains the sequence from –566 to the ATG start codon of *tag*, exhibited the greatest fold induction by c-di-GMP. Importantly, this reporter also demonstrates that transcription from these upstream regions proceeds into the *tag* open reading frame. The promoter activity of the DNA sequence immediately upstream of *tag* (*tag4*) was similar to the activity of the promoterless vector control, indicating that this region lacks an active promoter under our conditions (Fig. 1C). 5' rapid amplification of cDNA ends (RACE) experiments identified the transcriptional start site at bp –502 relative to the ATG start codon (Fig. 1A and B, dashed line). To confirm that *tag* was induced by c-di-GMP, we quantified *tag* mRNA by quantitative real-time PCR and observed an ~6-fold increase when QrgB is induced relative to QrgB\* (Fig. 1D).

Cellular stressors often act as signals to induce gene expression of the pathways that repair their damage, such as double-stranded breaks in DNA and the SOS response in *E. coli* (38). We were interested in whether *tag* expression was also modulated by the addition of the methylating agent methyl methanesulfonate (MMS), as this chemical induces damage that would be repaired by Tag. Therefore, we measured *tag3* expression in the presence and absence of 3 mM MMS. However, we observed no change in *tag* expression with the addition of MMS (Fig. 1E).

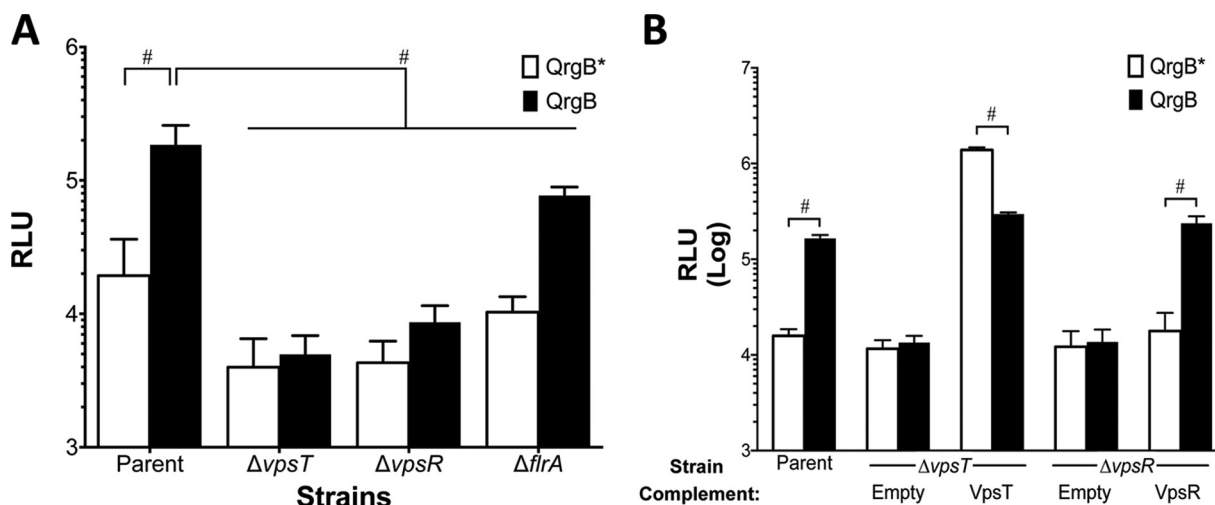
Together, these data indicate that c-di-GMP-regulated expression of *tag* is driven by a promoter 502 bp upstream of the ATG start codon and that the sequence from bp –566 to –392 is important for expression. Further, regions in the 5' direction of this



**FIG 1** Promoter characterization of *tag*. (A and B) Diagrams depicting sizes and locations of promoter truncations relative to the ATG start codon of *tag* utilized for luciferase reporter assays. The dashed line indicates the transcriptional start site (A, 502 bp upstream of ATG start codon). (C) Luciferase reporter assay of the promoter truncations in panel B. White bars are strains overproducing QrgB\*, while black bars are strains overproducing QrgB. The results are averages of the results from three independent experiments with 3 technical replicates each. Error bars indicate standard deviations. All comparisons are considered significant, except those with brackets and ns (nonsignificant;  $P > 0.05$ ), as determined by a two-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test. (D) qRT-PCR analysis of *tag* mRNA expression levels. The data are average fold changes between QrgB- and QrgB\*-expressing strains from 3 biological replicates, and the error bar is the standard deviation. (E) The parent strain ( $\Delta vpsL$  mutant) harboring *tag3* (luciferase reporter with *tag* promoter) was grown in the absence (left bar) or presence (right bar) of 3 mM MMS. The data are the averages of the results from at least two experiments, and the error bars indicate standard deviations.

promoter sequence increase basal expression. For the remainder of the experiments, the *tag3* reporter construct was used to assess *tag* transcription.

**VpsT and VpsR are necessary for *tag* expression.** *c*-di-GMP stimulates biofilm production by activating the transcription factors VpsT and VpsR (22, 27). It also inhibits motility by inactivation of the master regulator of flagellar biosynthesis, FlrA (25). Therefore, we reasoned that *c*-di-GMP induction of *tag* could depend on either of these three *c*-di-GMP-dependent transcription factors. To test this hypothesis, we conjugated the *tag* reporter plasmid into *V. cholerae* strains lacking either *vpsT*, *vpsR*, or *flrA*, and we assayed *tag* promoter activity at different intracellular *c*-di-GMP levels. In the parent and  $\Delta flrA$  isogenic mutant, *tag* expression increased approximately 10-fold with QrgB overproduction compared to the QrgB\* control. *tag* expression with QrgB overproduction was 2.4-fold lower in the  $\Delta flrA$  mutant strain than in the wild type (WT), but significant induction was observed (Fig. 2A). However, in the  $\Delta vpsT$  and  $\Delta vpsR$  mutant backgrounds, QrgB overproduction had no effect on *tag* expression compared to the QrgB\* control, indicating that VpsT and VpsR are necessary for *tag* expression (Fig. 2A).



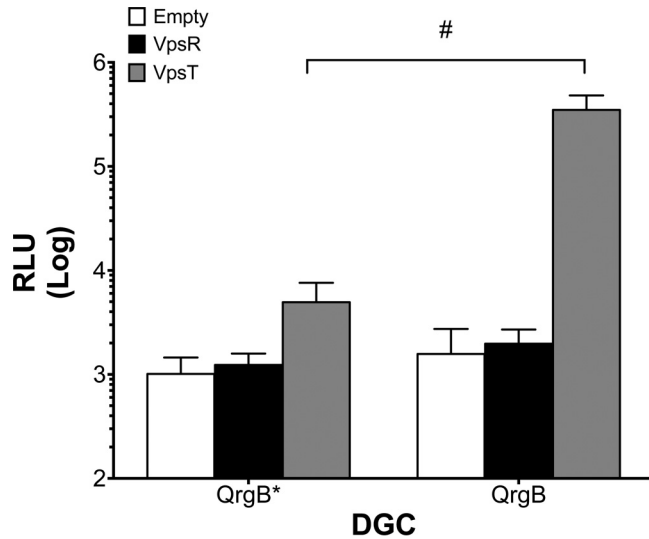
**FIG 2** VpsT and VpsR are required for *tag* induction by c-di-GMP. (A) Parent strain ( $\Delta vpsL$  mutant) and isogenic knockouts ( $\Delta vpsT$ ,  $\Delta vpsR$ , and  $\Delta flrA$  mutants) harboring either QrgB\* or QrgB and the *tag3* luciferase reporter construct were grown in the presence of 100  $\mu$ M IPTG to induce protein expression. The data are the averages from the results from at least three experiments, and the error bars indicate standard deviations. #, statistical significance ( $P < 0.05$ ) between strains, as determined by a two-way ANOVA followed by Tukey's multiple-comparison test. (B) *tag* expression in the  $\Delta vpsT$  and  $\Delta vpsR$  mutant backgrounds with vectors harboring IPTG-inducible VpsT and VpsR, respectively. All strains contain *tag3* and either QrgB\* or QrgB, as well as an expression vector for VpsT or VpsR or an empty vector negative control. Data represent averages of the results from at least three different experiments. Error bars indicate standard deviations. #, statistical significance ( $P < 0.05$ ) comparing QrgB\* and QrgB for that given strain/condition, as determined by a two-tailed Student *t* test.

We then complemented the  $\Delta vpsT$  and  $\Delta vpsR$  mutants by overexpressing VpsT and VpsR, respectively, from a plasmid. Complementing the  $\Delta vpsR$  mutant strain with VpsR restored c-di-GMP-mediated *tag* expression to a level similar to that of the parent strain (Fig. 2B). Interestingly, complementation of the  $\Delta vpsT$  mutant by overexpressing VpsT resulted in increased *tag* expression regardless of QrgB\* or QrgB expression (Fig. 2B). We did observe a 2-fold decrease in *tag* expression when VpsT and QrgB were overexpressed compared to VpsT and QrgB\*; however, this difference was relatively small compared with the 100-fold induction caused by VpsT in a comparison with the empty vector control, and thus, we do not consider it to be biologically meaningful (Fig. 2B). This experiment suggests that high concentrations of VpsT do not require elevated c-di-GMP to activate *tag*. Either VpsT can function in a c-di-GMP-independent manner or the basal concentration of c-di-GMP in *V. cholerae* under these conditions is high enough to activate the increased concentrations of VpsT. These results demonstrate that VpsT and VpsR are necessary for c-di-GMP-mediated *tag* expression.

#### VpsT activates *tag* transcription in *E. coli* and binds to the *tag* promoter *in vitro*.

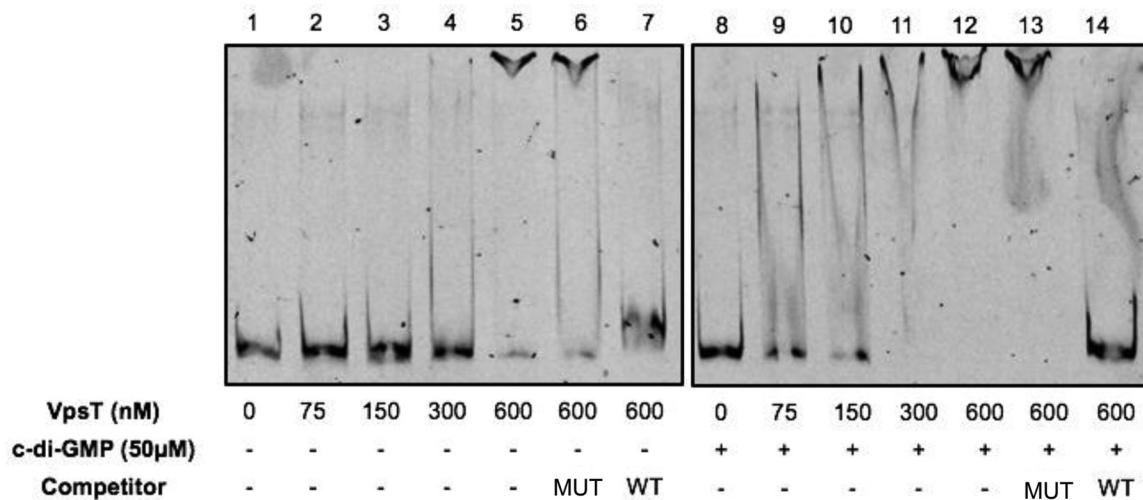
Since *vpsT* expression is activated by *vpsR* in a c-di-GMP-dependent manner (39), and *vpsT* was required for *tag* expression (Fig. 2A and B), we hypothesized that VpsT was the primary c-di-GMP-dependent activator of *tag*. To test this hypothesis, we measured *tag* expression in a heterologous host, *E. coli*. We reasoned that this *in vivo* system would allow us to isolate the impact of VpsT and VpsR on *tag* expression without pleiotropic effects due to the complex regulatory relationship between VpsT and VpsR (40). *tag* expression increased approximately 200-fold relative to the empty vector control when VpsT was coexpressed with QrgB to generate high c-di-GMP concentrations, while no induction was observed with VpsR coexpressed with QrgB (Fig. 3). *tag* expression was not induced when the inactive QrgB\* was coexpressed with VpsT or VpsR, indicating that c-di-GMP is required for this induction (Fig. 3). These data indicate that VpsT, but not VpsR, is sufficient to regulate *tag* expression in a c-di-GMP-dependent manner in *E. coli* and that no other *V. cholerae*-specific proteins are required for this induction.

To determine if VpsT bound to the *tag* promoter *in vitro*, we carried out electrophoretic mobility shift assays (EMSA). We generated 5'-6-carboxyfluorescein (5'-FAM)-labeled probes containing the *tag3* region (Fig. 1B, FAM-*tag3*) and incubated them with purified histidine-tagged VpsT for *in vitro* binding. VpsT partially bound to FAM-*tag3* at



**FIG 3** VpsT and c-di-GMP induce *tag* expression in *E. coli*. A three-plasmid system was established wherein the *tag* reporter, either QrgB\* or QrgB, and either VpsT, VpsR, or empty vector were transformed into DH10b *E. coli*. IPTG (100 μM) was added at subculture, and luciferase activity was measured at mid-exponential phase. The data are averages of the results from three experiments. Error bars indicate standard deviations. #, statistical significance ( $P < 0.05$ ) comparing QrgB\* and QrgB for each condition, as determined by a two-tailed Student *t* test.

a concentration of 300 nM and almost completely shifted the probe at 600 nM (Fig. 4, lanes 1 to 7). c-di-GMP enhances the *in vitro* DNA binding ability of VpsT at promoters of genes involved in biofilm formation (22). Thus, to determine if c-di-GMP enhanced DNA binding at the *tag* promoter, we included 50 μM c-di-GMP in the binding reaction mixture (Fig. 4, lanes 8 to 14). Partial binding occurred at 75 nM VpsT with near-complete shifting observed at 300 nM VpsT, indicating that c-di-GMP enhances VpsT DNA binding at the *tag* promoter *in vitro* in a manner similar to the *vpsL* promoter (41, 42). The U-shaped bands that we observed when c-di-GMP was added to the binding reaction mixture (Fig. 4, lanes 9 to 11) are likely the result of the dissociation between c-di-GMP and VpsT during electrophoresis, which would result in VpsT dissociating from the labeled probe, as previously described by Ayala et al. (42).



**FIG 4** c-di-GMP enhances VpsT affinity at the *tag* promoter *in vitro*. Lanes 1 to 7, increasing concentrations of purified VpsT-HIS were incubated with the fluorescently labeled *tag* promoter (FAM-*tag3*). When indicated, the mutant (MUT) or wild-type (WT) VpsT binding site competitor was added to the reaction at 100× excess relative to the probe concentration. Lanes 8 to 14, the same as lanes 1 to 7, except that 50 μM c-di-GMP was added to the reaction mixtures.

Recently, a 20- to 22-bp VpsT binding consensus site containing a palindromic sequence was described (41). We reasoned if the interaction between VpsT and the *tag* promoter was specific, the addition of a 22-bp oligonucleotide containing the VpsT binding site to the binding reaction (WT competitor) would compete away interactions between VpsT and FAM-*tag3*. Conversely, adding an oligonucleotide with a disrupted palindromic region (mutant [MUT] competitor) would have no effect on interactions between VpsT and FAM-*tag3*. Indeed, the addition of the WT competitor to the binding reaction without c-di-GMP resulted in a nearly complete reduction in the shifted probe, while the addition of the mutant competitor did not compete away VpsT binding from FAM-*tag3* (Fig. 4, lanes 6 and 7). In the presence of 50  $\mu$ M c-di-GMP, the WT competitor completely competed away binding of VpsT, while the mutant competitor had no effect (Fig. 4, lanes 13 and 14). These results indicate that VpsT specifically binds to the *tag3* region, and c-di-GMP increases the affinity of VpsT binding. Taken together, these data suggest that VpsT directly regulates *tag* expression and that VpsR regulates *tag* expression indirectly through the induction of VpsT (41).

**c-di-GMP increases tolerance of the alkylating agent MMS.** Tag is an enzyme in the BER pathway that recognizes and removes methylated adenines (3meA) and guanines (3meG) at the N3 position (35). 3meA is a lethal nonmutagenic lesion that blocks DNA replication, whereas 3meG is innocuous (36). Since c-di-GMP induces *tag* expression, we hypothesized that cells with high intracellular c-di-GMP concentrations would be more tolerant of MMS treatment, which generates 3meA, than cells with basal intracellular c-di-GMP concentrations. To test this, QrgB or QrgB\* was induced for 2 h in the parent background before treatment with 3 mM MMS, and growth was quantified every hour for 10 h by measuring the optical density at 600 nm (OD<sub>600</sub>). The parent strain used in these experiments is a *V. cholerae*  $\Delta$ vpsL mutant that is incapable of forming biofilms; thus, biofilm formation itself cannot be induced by c-di-GMP and is not responsible for the observed differences.

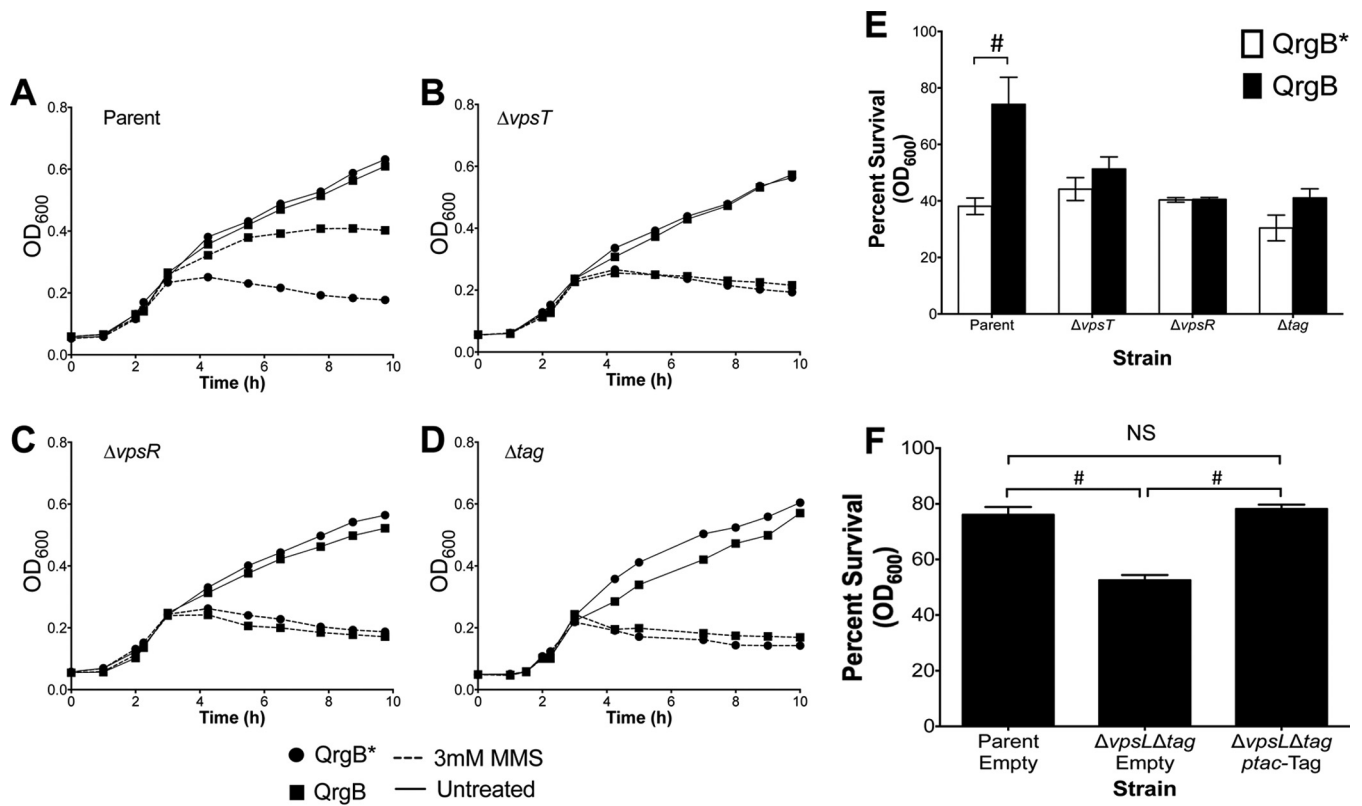
*V. cholerae* strains expressing QrgB\* or QrgB displayed similar growth curves for all strains examined in the absence of MMS treatment (Fig. 5A to D, solid lines), indicating that c-di-GMP has no deleterious effects on growth under these conditions. In the parent strain, the induction of c-di-GMP led to significantly more growth in the presence of MMS (Fig. 5A, squares). Quantification of optical density after 6 h of treatment revealed that increased c-di-GMP led to an approximate 2-fold increase in survival compared to the QrgB\* control (Fig. 5E).

As VpsT and VpsR were necessary for *tag* expression, we repeated the experiment in  $\Delta$ vpsT and  $\Delta$ vpsR mutant backgrounds and found that these strains lacked a c-di-GMP-mediated increase in MMS tolerance (Fig. 5B, C, and E). Similarly, high c-di-GMP concentrations did not significantly increase the survival of a *V. cholerae*  $\Delta$ tag mutant (Fig. 5D and E). Interestingly, the  $\Delta$ tag mutant was only slightly more sensitive to MMS than the parent strain (Fig. 5E), indicating that under basal c-di-GMP conditions, Tag plays a small role in MMS resistance. Complementing the  $\Delta$ tag mutant strain by overproducing Tag from a plasmid restored parent survival (Fig. 5F). Together, these data indicate that high c-di-GMP conditions promote the growth of *V. cholerae* in the presence of alkylation damage.

Induction of *tag* by c-di-GMP suggests that DNA repair might be important in *V. cholerae* biofilm formation. To test this hypothesis, we measured biofilm formation of the WT and the  $\Delta$ tag mutant exposed to 0, 3, and 6 mM MMS. The deletion of *tag* did not affect biofilm formation at either concentration, which we hypothesize is due to redundant mechanisms for dealing with MMS damage in a biofilm. Interestingly, the addition of MMS significantly increased biofilm formation in a dose-dependent manner in both the WT and  $\Delta$ tag mutant backgrounds (see Fig. S1 in the supplemental material).

## DISCUSSION

The dinucleotide second messenger c-di-GMP is best known for its role in transition from a motile state to a biofilm state in many Gram-negative bacteria, including *V.*



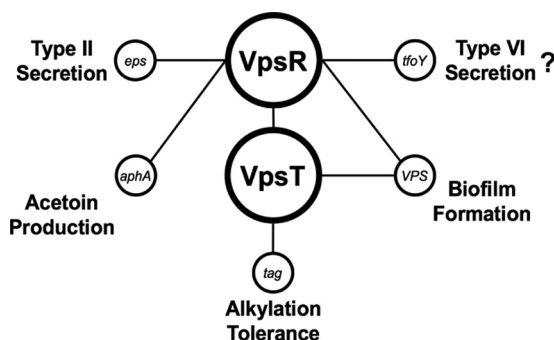
**FIG 5** High c-di-GMP increases tolerance to the methylating agent MMS. (A to D) Strains were cultured 1:1,000 and grown in the presence of 100  $\mu$ M IPTG to overproduce QrgB\* and QrgB. After 2 h, cells were left untreated (solid lines) or treated with 3 mM MMS (dashed lines), and the OD<sub>600</sub> was measured every hour for 10 h. Error bars, which are obscured by symbols, are standard deviations. (E) Quantification of survival (percentage of treated OD divided by untreated OD) for a given strain after 6 h of treatment. (F) Complementation of the  $\Delta vpsL \Delta tag$  mutant strain by expressing Tag in *trans*. Error bars are standard deviations. #, *P* value of <0.05, determined by a two-tailed Student *t* test; NS, comparison was not significant (*P* > 0.05).

*cholerae* (3). In many cases, genes involved in biofilm formation are turned on through changes in the activities of transcription factors (22, 25, 27, 43). Past studies indicate that there are potentially multiple genes or pathways regulated by c-di-GMP besides genes directly related to motility or biofilm formation (10, 22, 27, 44). In this study, we demonstrate that c-di-GMP regulates expression of the DNA repair gene *tag*. Strains lacking the biofilm regulators VpsT and VpsR were unable to induce *tag* expression at high c-di-GMP concentrations. Importantly, we demonstrate that VpsT induction of *tag* leads to increased tolerance to MMS when cells have high intracellular c-di-GMP concentrations independent of biofilm formation.

We determined that the region necessary for *tag* expression by c-di-GMP is between 566 bp and 392 bp upstream of the ATG translational start codon. Additionally, this region is in the coding region of the upstream gene *vexK* encoding the resistance-nodulation-division (RND) multidrug efflux pump VexK. VexK can efflux detergents and was upregulated in human volunteers of *V. cholerae* infection, while *tag* was not identified as an *in vivo*-expressed gene, suggesting that *tag* is not upregulated during human host infection and that these genes are under independent regulatory control (45, 46).

We determined that the transcriptional start of *tag* is located 502 bp upstream of the translation start site. This transcription start site was independently identified in a systematic RNA sequencing (RNA-seq) analysis to identify all the transcription start sites of *V. cholerae* (47). Thus, the 5' untranslated region (UTR) for *tag* mRNA is 502 bp long. The most common 5' UTR lengths in the Gram-negative organisms *E. coli* and *Klebsiella pneumoniae* range from 25 to 35 bp, with the longest reaching 700 bp (48). The relatively long 5' UTR of the *tag* transcript suggests that c-di-GMP may be only one of





**FIG 6** Model of the VpsR/VpsT c-di-GMP regulatory network in *V. cholerae*. In response to high c-di-GMP concentrations, VpsR becomes an active transcription factor and induces the expression of *vpsT*. VpsT is also a c-di-GMP-dependent transcription factor and together, VpsT and VpsR induce the expression of genes involved in biofilm formation. c-di-GMP-activated VpsR has been shown to directly regulate promoters other than those that regulate biofilm production, such as *tfoY*, which regulates motility and type VI secretion, the *eps* operon involved in type II secretion, and *aphA*, which regulates acetoin production. In this work, we uncovered another phenotype regulated by the central c-di-GMP-dependent transcription factors, which is alkylation tolerance through the induction of *tag* indirectly by VpsR and directly by VpsT.

the signals regulating *tag* expression in *V. cholerae*. It is possible that there are additional transcriptional start sites closer to the translation start site of *tag* that were missed in our analyses; however, a luciferase fusion to this region (*tag4*) showed no activity above the vector control level, and no such start sites were detected in the previously published RNA-seq analysis (47). The function of this long 5' UTR in DNA repair is an intriguing question that warrants further investigation. Interestingly, a systematic search for small RNAs (sRNAs) detected a putative sRNA in this 5' UTR from bases  $-227$  to  $-121$  relative to the ATG start codon located on the coding strand of the *tag* gene (49). Due to the lack of additional transcription start sites in this region, we hypothesize that this sRNA might be processed from the 5' UTR of *tag*. Whether this sRNA has any biological function remains to be determined.

In *V. cholerae*, three transcription factors act to sense and respond to c-di-GMP by altering transcription at key promoters of genes necessary for VPS or flagellar biosynthesis. VpsR and VpsT are c-di-GMP-responsive transcription factors that induce expression of the VPS operon when intracellular c-di-GMP concentrations are high (22, 27, 50). We demonstrate that both VpsR and VpsT are necessary for the induction of *tag* by c-di-GMP, but VpsT appears to directly regulate this gene (Fig. 6). Binding site sequences exist for VpsT in the promoters of *vpsL*, a component of the VPS operon, and the promoter for the stationary-phase sigma factor *rpoS*; however, we could not identify a canonical VpsT binding site in the *tag* promoter (41, 51). We are currently working to determine the VpsT binding sequence in the *tag* promoter. FlrA, the c-di-GMP-responsive transcription factor necessary for flagellar biosynthesis, was not needed for *tag* induction by c-di-GMP, suggesting that *tag* expression is not advantageous in a motile lifestyle.

A previous microarray analysis suggests that c-di-GMP can regulate other forms of DNA repair in addition to the BER pathway in *V. cholerae*. Overproduction of a DGC in the EI Tor biotype A1552 resulted in the upregulation of *mutL*, a gene involved in methyl-directed mismatch repair, while in the classical biotype O395, DGC overproduction led to an increase in the gene encoding photolyase (*phrB*), which is involved in light-dependent DNA repair after UV radiation damage (15). Although these regulatory pathways have not been studied, the multiple connections to DNA repair suggest that *tag* induction by c-di-GMP may just be one example of how *V. cholerae* uses c-di-GMP signaling to coordinate DNA repair.

Various stressors, including DNA-damaging agents, increase biofilm formation in *E. coli* and *Pseudomonas aeruginosa* (52, 53). Additionally, the SOS response pathway regulates biofilm formation in *P. aeruginosa*, demonstrating a link between DNA

damage and biofilm formation (54). In contrast, growth in a biofilm increases mutability in *P. aeruginosa* through the production of endogenous reactive oxygen species (55). These findings demonstrate that stress can induce biofilm formation, or, conversely, that biofilms can induce stress. We hypothesized that c-di-GMP-induced *tag* expression likely accompanies environmental biofilm formation in *V. cholerae*, based on the following pieces of evidence: (i) VpsT regulates *tag* expression under high-c-di-GMP conditions, (ii) *vpsT* expression is epistatic to *vpsR* (39), and (iii) high levels of VpsR activated by c-di-GMP result in biofilm formation (50). However, biofilm formation in the WT strain was similar to that in the  $\Delta tag$  mutant strain under untreated and MMS-treated conditions. We hypothesize that the fitness advantage of *tag* induction by c-di-GMP may be relevant under other environmental conditions not tested here.

We demonstrate a novel example of dinucleotide second messenger signaling regulating a core cellular process, DNA repair. It is becoming clear that the roles of the VpsR and VpsT pathways in *V. cholerae* expand beyond biofilm formation. Indeed, this c-di-GMP regulatory node controls a variety of phenotypes in response to changing c-di-GMP concentrations, including type II secretion, acetoin production, and *tfoY* transcription (Fig. 6). Consequently, these two transcription factors should not be viewed solely as biofilm regulators but rather as the central transcriptional regulators of c-di-GMP-regulated phenotypes in *V. cholerae*.

## MATERIALS AND METHODS

**Growth conditions and strain construction.** All strains, primers, and plasmids used in this study are listed in Tables S1 to S3 in the supplemental material. The *V. cholerae* strains used in this study were derived from the El Tor biotype strain C6706str2. Where indicated, a strain unable to form mature biofilms was used (CW2034,  $\Delta vpsL$  mutant [37]) to allow for accurate measurement of optical density and *lux* reporter gene expression. The  $\Delta vpsT$ ,  $\Delta vpsR$ ,  $\Delta vpsT \Delta vpsR$ , and  $\Delta tag$  unmarked mutant strains were constructed in the  $\Delta vpsL$  mutant parent strain using the pKAS32 suicide vector, as described previously (56). All *V. cholerae* strains were grown overnight in Luria-Bertani (LB) broth at 35°C with shaking at 220 rpm, unless stated otherwise. Concentrations of 100  $\mu$ g/ml ampicillin, 100  $\mu$ g/ml kanamycin, 10  $\mu$ g/ml chloramphenicol, and 100  $\mu$ M isopropyl- $\beta$ -D-1-thiogalactopyranoside (IPTG) were added when needed. *Escherichia coli* S17-*Apir* was used as the donor in biparental conjugation to mobilize plasmids into *V. cholerae* with selection against the donor using polymyxin B (10 U/ml).

**DNA manipulations.** Standard procedures were used for DNA manipulations (57). PCR was carried out with Phusion polymerase (NEB). Restriction enzymes were purchased from NEB. The primers used in this study are listed in Table S3. Reporter plasmids were constructed by Gibson Assembly (NEB) using PCR-generated inserts and pBBRLux linearized by BamHI and SpeI restriction digestion. Expression vectors were constructed by Gibson Assembly using PCR-amplified inserts (*vpsT*, *vpsR*, and *tag*) and pEV5143 linearized by EcoRI and BamHI restriction digestion. The vectors used for gene deletions were generated by Gibson Assembly using three fragments: 1 kb of sequence upstream of the gene of interest, 1 kb of sequence downstream of the gene of interest, and MfeI- and BglII-linearized pKAS32. pET28B (Novagen) was used for protein purification. PCR-amplified *vpsT* (*vca0952*) was cloned into XhoI- and NcoI-linearized pET28b using Gibson Assembly.

**Reporter gene expression.** Overnight cultures of *V. cholerae* or *E. coli* harboring reporter plasmids (pBBRLux derivatives) were diluted to a starting OD<sub>600</sub> of 0.04 in 1 ml LB supplemented with appropriate concentrations of antibiotics and IPTG. Two hundred microliters of dilutions was added to black 96-well plates (Cellstar) in triplicate and grown at 35°C while shaking at 220 rpm. Luminescence readings were taken at mid-exponential phase using an EnVision multilabel plate reader (PerkinElmer) and were normalized for relative light units (RLU) by dividing the luminescence reading by the OD<sub>600</sub>.

**RNA isolation, quantitative real-time PCR, and 5' RACE.** Overnight cultures of *V. cholerae* harboring QrgB or QrgB\* were diluted 1:1,000 in 10 ml LB in triplicate, supplemented with appropriate concentrations of antibiotics and IPTG. Cultures were grown for 3 h (OD<sub>600</sub> ~1.0) at 35°C with shaking at 220 rpm. Cells were pelleted, and RNA was isolated using the TRIzol reagent (Thermo Fisher Scientific), according to the manufacturer's instructions. Five nanograms of total RNA was treated with Turbo DNase (Thermo Fisher Scientific), according to the instructions in the manual. DNA-free RNA was converted into cDNA using GoScript reverse transcriptase (Promega). Quantitative real-time PCRs (qRT-PCRs) were carried out using SYBR green PCR master mix (Thermo Fisher Scientific) and the StepOnePlus real-time PCR system (Thermo Fisher Scientific), with final primer concentrations of 250 nM. Data were analyzed via the  $\Delta\Delta C_T$  method using *gyrA* as a reference gene and cDNA from QrgB\*-overproducing strains as the calibrator.

5' RACE was carried out using the 5' RACE assay (Invitrogen), according to the manufacturer's instructions. RNA was extracted from *V. cholerae* cells harboring the *tag1* pBBRLux reporter plasmid and converted to cDNA. Primers for nested PCR amplification can be found in Table S3.

**Protein expression and purification.** The VpsT-pET28b expression vector was transformed into *E. coli* BL21(DE3). Sequence-verified clones were cultured into 250 ml LB supplemented with kanamycin and grown at 35°C shaking at 220 rpm to an OD<sub>600</sub> of 0.7. Protein expression was induced by adding a

1 mM final concentration of IPTG. The growth temperature was switched to 16°C and the shaking speed was lowered to 160 rpm, and cells were incubated overnight (approximately 16 h). Cells were pelleted and resuspended in buffer A (25 mM Tris-HCl [pH 7.5], 550 mM NaCl, 20 mM imidazole, cOmplete mini EDTA-free Roche protease inhibitor tablets [2 per 50 ml], 5 mM  $\beta$ -mercaptoethanol) and lysed by homogenization.

Nickel nitrilotriacetic acid (Ni-NTA) resin (Qiagen) was incubated in buffer A for 20 min at 4°C. Cell lysates were centrifuged for 25 min at  $10,000 \times g$  and 4°C to pellet insoluble material. The soluble lysate was incubated with the Ni-NTA resin for 30 min at 4°C. The column was washed with 20 column volumes of buffer A and eluted by step elution with buffer A supplemented with 75, 125, 250, and 500 mM imidazole. Elution fractions were analyzed by SDS-PAGE, and those containing protein were pooled and dialyzed against dialysis buffer (25 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM  $\beta$ -mercaptoethanol [BME], 10% glycerol) overnight at 16°C. Protein concentrations were estimated using Bradford reagent (Bio-Rad) with purified bovine serum albumin (Sigma) to generate a standard curve.

**Electrophoretic mobility shift assay.** EMSA reactions were carried out by incubating purified VpsT-HIS with 5'-FAM-labeled probes (FAM-*tag3*). Twenty-microliter reaction mixtures consisted of 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM BME, and 10% glycerol. Poly(dI-dC) (Sigma) was added to all reaction mixtures at a concentration of 1  $\mu\text{g}/\mu\text{l}$ . When indicated, c-di-GMP was added at a final concentration of 50  $\mu\text{M}$ ; otherwise, an equal volume of water was added. The 22-bp wild-type or 20-bp mutant VpsT binding site single-stranded oligomers were annealed to generate double-stranded oligomers by incubating equimolar ratios of complementary oligonucleotides (Table S3) at 94°C for 2 min and were cooled at room temperature overnight. A 100 $\times$  molar excess competitor was added when indicated. All components except the labeled probe were mixed at room temperature and incubated for 10 min. FAM-*tag3* probe was then added at a final concentration of 2.5 nM, and the reaction mixtures were incubated for 30 min at 30°C. Ten microliters of the reaction mixture was loaded onto a prerun 5% polyacrylamide Tris-borate-EDTA (TBE) gel, and electrophoresis was carried out for 90 min at 90 V at 4°C. Fluorescent band migration was visualized on Typhoon FLA 9000 imager (GE Healthcare Life Sciences).

**Growth assays.** Overnight cultures were diluted to a starting OD<sub>600</sub> of 0.004 in 1 ml LB supplemented with ampicillin and IPTG. Two hundred microliters of the culture was moved into wells of a 96-well plate (Cellstar) in triplicate and incubated at 35°C with shaking at 220 rpm. After 2 h of growth, phosphate-buffered saline (PBS) or MMS (Santa Cruz Biotechnology) was added at the indicated concentrations, and growth was monitored by measuring the OD<sub>600</sub> every hour for 10 h using an EnVision multilabel plate reader (PerkinElmer). For calculating the percent survival, the quotient of the OD<sub>600</sub> of MMS-treated wells divided by the OD<sub>600</sub> of untreated wells was multiplied by 100 at a given time point.

**Statistical analysis.** Data are represented as the mean  $\pm$  standard deviation (SD). Statistical analyses (details in figure legends) were calculated with Prism version 6 (GraphPad, San Diego, CA). A *P* value of <0.05 was considered significant.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00005-18>.

**SUPPLEMENTAL FILE 1**, PDF file, 0.3 MB.

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