The Proline Variant of the W[F/L/M][T/S]R Cyclic Di-GMP Binding Motif Suppresses Dependence on Signal Association for Regulator Function

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ABSTRACT *Vibrio vulnificus* is an estuarine bacterium and potent opportunistic human pathogen. It enters the food chain by asymptotically colonizing a variety of marine organisms, most notably oysters. Expression of the *brp*-encoded extracellular polysaccharide, which enhances cell-surface adherence, is regulated by cyclic di-GMP (c-di-GMP) and the activator BrpT. The *Vibrio cholerae* and *Vibrio parahaemolyticus* homologs VpsT and CpsQ, directly bind c-di-GMP via a novel W[F/L/M][T/S]R motif, and c-di-GMP binding is absolutely required for activity. Notably, BrpT belongs to a distinct subclass of VpsT-like regulators that harbor a proline in the third position of the c-di-GMP binding motif (WLPR), and the impact of this change on activity is unknown. We show that the *brp* locus is organized as two linked operons with BrpT specifically binding to promoters upstream of *brpA* and *brpH*. Expression data and structural modeling suggested that BrpT might be less dependent on c-di-GMP binding for activity than VpsT or CpsQ. We show that the affinity of BrpT for c-di-GMP is low and that signal binding is not a requisite for BrpT function. Furthermore, a BrpT mutant engineered to carry a canonical WLTR motif (BrpT^{P124T}) bound c-di-GMP with high affinity and its activity was now c-di-GMP dependent. Conversely, introduction of the WLPR motif into VpsT suppressed its dependence on c-di-GMP for activity. This is the first demonstration of reduced dependence on signal association for regulator function within this motif family. Thus, BrpT defines a new class of VpsT-like transcriptional regulators, and the WLPR motif variant may similarly liberate the activity of other subclass members.

IMPORTANCE A *Vibrio* genome may encode nearly 100 proteins that make, break, and bind c-di-GMP, underscoring its central role in the physiology of these bacteria. The activity of the biofilm regulators VpsT of *V. cholerae* and CpsQ of *V. parahaemolyticus* is regulated by the direct binding of c-di-GMP via a novel W[F/L/M][T/S]R motif. The *V. vulnificus* homolog, BrpT, bears an unusual WLPR variant and remains active at low intracellular c-di-GMP levels. This suggests that the WLPR motif may also liberate the activity of other members of this subclass. A single point mutation at the 3rd position of the motif was sufficient to moderate dependence on c-di-GMP binding for activator function, highlighting the simplicity with which complex bacterial signaling networks can be rewired.

KEYWORDS biofilms, c-di-GMP, foodborne pathogens, regulation of gene expression

In nature, the majority of microorganisms live as surface-associated communities (biofilms) that are sheathed in a hydrated matrix of DNA, proteins, and polysaccharides produced by the resident microorganisms (1). Compared with their planktonic

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counterparts, biofilm consortia are highly resilient, allowing bacteria to withstand environmental stress, the onslaught of antimicrobials, and immune responses of the host.

The second messenger cyclic di-GMP (c-di-GMP) is a key signaling molecule used by bacteria to regulate biofilm development (2–6). Once triggered, the signaling pathway directs diguanylate cyclases (DGC) and phosphodiesterases (PDE), which synthesize and degrade, respectively, c-di-GMP to alter the internal c-di-GMP levels (7, 8). Downstream effector proteins sense this change and transduce the signal (9). The domains and motifs within effector proteins that bind c-di-GMP include the RXXD motif, the PilZ, EAL, and HD-GYP domains, and the novel binding sites identified within MshE and CuxR (9–12). The circuit culminates with a phenotypic response driven by modified protein activity and/or differential expression of target genes. In general, increases in intracellular c-di-GMP concentrations enhance the expression of adhesion factors and biofilm formation, while repressing motility and virulence gene expression.

Vibrio vulnificus is a potent opportunistic human pathogen. It is unique among *Vibrio* species in that it is best known for causing septicemia (13). Infection typically occurs through open wounds or via the food chain, in which *V. vulnificus* is known to colonize bivalves such as oysters. Disease progression is rapid, and the fatality rate of septicemic patients is greater than 50%, the highest death rate of any foodborne disease agent (14). Biofilm formation is important for the ecology and transmission of *V. vulnificus*; however, our understanding of its regulation is lacking. Greater insight could lead to the development of approaches to decrease the *Vibrio* load in filter feeders and lower the incidence of invasive disease.

We previously demonstrated that increased intracellular c-di-GMP levels induced biofilm formation in *V. vulnificus* (15). Among the plethora of genes with increased transcription were four that clustered to one region of the chromosome that was later designated the *brp* (biofilm and rugose polysaccharide) locus (16). The locus contained nine genes (*brpABCDHFHIJK*), and disruption of *brpF* and *brpI* prevented c-di-GMP-induced biofilm, aggregate, and rugose colony formation. Screening of a transposon mutant library for clones that failed to form a biofilm in response to elevated c-di-GMP levels led to the identification of two regulators of *brp* expression, designated *brpR* and *brpT*. Both BrpR and BrpT contained a helix-turn-helix (HTH) DNA binding domain and shared 79% and 33% amino acid identity with the transcriptional regulators VpsR and VpsT of *Vibrio cholerae* (17). VpsR binds c-di-GMP by an unknown mechanism and drives *vpsT* expression (18). VpsT in turn binds c-di-GMP (19) and activates transcription of two linked operons, *vpsI* and *vpsII*, which are part of the *Vibrio* polysaccharide synthesis (*vps*) locus (20).

The crystal structure of VpsT revealed that it interacts with c-di-GMP via a novel W[F/L/M][T/S]R motif. T¹³³ and R¹³⁴ of the WLTR motif participate in H bonding with the c-di-GMP dimer, and signal binding is required for activity. The *Vibrio parahaemolyticus* homolog CspQ, which activates capsular polysaccharide (*cps*) expression, harbors a WLSR motif and binds c-di-GMP. A CspQ^{R134A} (WLSA) mutant failed to bind c-di-GMP and consequently could not activate *cps* expression (21). BrpT belongs to a distinct subclass of VpsT-like regulators that deviate from the canonical c-di-GMP binding sites of VpsT and CspQ in that a proline, rather than a serine or threonine, is found in the third position of the motif (WLPR). Not only is proline conformationally rigid, but the lack of an amide hydrogen precludes it from donating to H bonding. As a consequence, proline is not commonly found in the binding or active sites of proteins (22). As such, its presence in the putative BrpT c-di-GMP binding site is unusual and its effect on signal binding by and subsequent activity of BrpT is unknown.

Here, we show that the *brp* locus is organized as two linked operons that are separately transcribed. Expression of the locus is BrpT dependent, and purified BrpT specifically bound to promoters upstream of *brpA* and *brpH*. Expression data and structural modeling suggested that BrpT could potentially activate transcription without binding c-di-GMP. We show that, in contrast to VpsT and CspQ, purified BrpT was able to bind target promoters and drive *brp* expression under low-c-di-GMP conditions.

FIG 1 Transcription of *brpA* and *brpT* is regulated by c-di-GMP. (A) Relative P_{brpA} -*gfp* expression in the WT, $\Delta brpT$, and complemented ($\Delta brpT$ -C) strains is shown in relative fluorescence units (RFU). (B) Relative P_{brpT} -*gfp* expression in the WT and $\Delta brpR$ strains. Plots show the means, and error bars represent the standard deviations. Significance: ns, not significant ($P > 0.05$); *, $P < 0.001$.

By engineering a canonical WLTR motif into BrpT and the proline variant into VpsT via single point mutations, we were able to alter the degree to which signal binding was required for activator function.

RESULTS

The activities of DcpA and CdgJ alter the intracellular c-di-GMP concentrations.

To begin examining the mechanism by which elevated c-di-GMP levels induce *brp* expression, we sought to controllably manipulate the intracellular c-di-GMP concentration. We previously identified a putative *V. vulnificus* DGC (DcpA) that, when over-expressed, increased *brp* expression and biofilm formation. To demonstrate that DcpA expression resulted in elevated intracellular c-di-GMP concentrations, we expressed it in *V. vulnificus* and measured the change in the level of the second messenger relative to a control strain. Following DcpA induction, c-di-GMP levels increased 14-fold, while expression of the *V. cholerae* PDE, CdgJ, decreased c-di-GMP levels 15-fold (see Fig. S1, top, in the supplemental material). Similar effects were observed in *Escherichia coli*, in which DcpA expression boosted the intracellular c-di-GMP concentration nearly 114-fold, while CdgJ expression decreased levels 3-fold. We also tested the phenotypic effect of DcpA and CdgJ expression on *V. vulnificus* morphology (Fig. S1, bottom). Wild-type cells were flat and featureless on solid medium and remained so following expression of CdgJ alone. DcpA expression induced a rugose-colony development that was punctuated by a dramatic wrinkled phenotype, while simultaneous expression of DcpA and CdgJ reverted the rugose phenotype. These results confirmed that we could use DcpA and CdgJ to manipulate intracellular c-di-GMP concentrations from the millimolar (high) to low nanomolar (low) range.

c-di-GMP regulates *brp* locus and *brpT* expression. Our previous studies suggested that elevated intracellular c-di-GMP levels induced biofilm formation in *V. vulnificus* and that the enhanced biofilm phenotype was dependent on BrpT (15, 16). We monitored the expression of P_{brpA} in *V. vulnificus* using an ectopically integrated transcriptional reporter (Fig. 1A). P_{brpA} -*gfp* expression was observed in wild-type (WT) cells only when intracellular c-di-GMP levels were increased. No P_{brpA} -*gfp* expression was observed in the *brpT*-null strain under unaltered or elevated c-di-GMP conditions. Moreover, ectopic expression of *brpT* in the null mutant ($\Delta brpT$ -C) restored P_{brpA} -*gfp* expression when intracellular c-di-GMP levels were elevated. This confirmed that *brp* expression was regulated by c-di-GMP and dependent on BrpT. To determine if the expression of *brpT* was also regulated by c-di-GMP and/or BrpR, we measured the activity of a P_{brpT} -*gfp* reporter in WT and $\Delta brpR$ strains under unaltered and elevated intracellular c-di-GMP conditions (Fig. 1B). P_{brpT} -*gfp* expression was low in WT cells but increased 4.5-fold when intracellular c-di-GMP was elevated. However, only a 1.5-fold increase in P_{brpT} -*gfp* expression was observed in the $\Delta brpR$ strain. This suggested that *brpT* expression was regulated by BrpR and c-di-GMP and BrpT in turn regulated *brp* expression.

FIG 2 Architecture of the *brp* locus and DNA binding by BrpT to the *brpA* and *brpH* promoter regions. (A) Reads per kilobase per million mapped sequence reads (RPKM) to the *brp* locus. The top, middle, and bottom panels show the traces for the WT and $\Delta brpT$ strains under unaltered (top panel) and elevated (middle and bottom panels) intracellular c-di-GMP conditions (i.e., expressing DcpA). Yellow arrows denote the respective *brp* genes. Green arrows, putative promoters upstream of *brpA* and *brpH*. The genes *brpF* and *brpH* are separated by 217 bp (gray region). (B) BrpT was purified and incubated with labeled DNA fragments spanning the *brpA* promoter (P_{brpA}) or the *brpH* promoter (P_{brpH}) regions. Unlabeled P_{brpA} was used as a cold competitor (cc) for DNA binding. The *flpA* promoter (P_{flpA}) was used as a control for specific binding.

BrpT directly regulates *brp* expression. We previously showed that increased c-di-GMP levels triggered the expression of four of the nine *brp* genes. However, it was still unclear if all of the genes were expressed or how their expression was organized. We used transcriptome sequencing (RNA-seq) to analyze c-di-GMP-dependent *brp* expression to better define its transcriptional architecture. Few transcripts mapping to the locus were detected in wild-type cells carrying the empty expression vector, but transcripts were readily detected in cells following DcpA expression (Fig. 2A, top and middle panels). Expression of all nine *brp* genes ceased in a $\Delta brpT$ strain, even when DcpA was expressed (Fig. 2A, bottom panel).

The *brpF* and *brpH* genes are separated by 217 bp, and we did not identify any transcripts that mapped to this intergenic region. This raised the possibility of the *brp* locus being expressed as two separate transcripts, the first initiating upstream of *brpA* and the second initiating upstream of *brpH*. To determine if BrpT specifically bound to promoter regions upstream of *brpA* and *brpH*, we purified it (see Fig. S2A in the supplemental material) and used it in gel mobility shift assays (Fig. 2B). BrpT was able to bind and shift a labeled 325-bp DNA fragment corresponding to the *brpA* promoter (P_{brpA}), and binding was prevented when excess unlabeled P_{brpA} (cold competitor) was added to the reaction mixture. Conversely, BrpT was unable to bind to a labeled 100-bp DNA fragment spanning the promoter region of *flpA* (P_{flpA}), which codes for a type IVb pilin of *V. vulnificus*. BrpT was also capable of shifting a 325-bp DNA fragment mapping to the *brpH* promoter region (P_{brpH}). These results suggested that BrpT specifically binds to target promoters upstream of *brpA* and *brpH* to regulate *brp* expression and that the *brp* locus consists of two operons, *brpABCD* and *brpHIJK*, that are separately transcribed.

***brp* expression is temperature dependent.** We previously demonstrated that the rugose phenotype in *V. vulnificus* was dependent on expression of the *brp* locus (15, 16).

FIG 3 P_{brpA} expression is temperature dependent. Colony morphology (top) and biofilm formation (bottom) (A) and RT-PCR analysis (B, top and bottom) of rugose *V. vulnificus* grown at 37°C or 25°C. Representative colony morphology and agarose gel images are shown. The transcript levels for *wzc* and *brpA* were normalized relative to the level of *rplT* (ribosomal L20) transcript in the same sample. Assays were performed in triplicate on three biological samples. Plots show the means, and error bars represent the standard deviations. White and gray bars indicate transcript levels at 37°C and 25°C, respectively. Significance: ns, not significant ($P > 0.05$); *, $P < 0.001$.

During the course of those studies, we noticed that rugosity was striking at 25°C and almost imperceptible at 37°C. This led us to speculate that *brp* expression might be temperature dependent. To investigate this, we monitored the expression of *brpA* and *wzc* (required for capsular polysaccharide [CPS] transport to the bacterial surface [23]) as a function of temperature in a *V. vulnificus* rugose isolate. There was a clear correlation between the level of rugosity, *brpA* expression, and temperature. Rugosity was more pronounced and biofilm formation was 4.6-fold greater at 25°C than 37°C (Fig. 3A), and while the expression of *wzc* was unaffected by the growth temperature, the expression of *brpA* was 4.9-fold higher at 25°C than 37°C (Fig. 3B). These results suggested that the loss of rugosity at 37°C was due to decreased *brp* expression and that *brp* expression was temperature dependent.

BrpT activates transcription at low intracellular c-di-GMP concentrations. VpsT and CpsQ harbor c-di-GMP binding motifs (WLTR and WLSR, respectively) that conform to the W[F/L/M][T/S]R consensus. BrpT is different; a proline occupies the third position of the motif (WLPR), and the impact of this change on BrpT function is not known. We monitored P_{brpA} -*gfp* expression and biofilm formation of the same cells under low, unaltered, and elevated c-di-GMP levels (Fig. 4). Wild-type cells harboring empty vector or expressing CdgJ exhibited very low P_{brpA} -*gfp* expression (Fig. 4, bars 1 and 3) and biofilm formation (Fig. 4, inset, bars 1 and 3). P_{brpA} -*gfp* levels increased 18-fold following DcpA expression (Fig. 4, bar 2), and biofilm formation increased 11-fold (inset, bar 2). P_{brpA} -*gfp* expression was entirely dependent on BrpT, as DcpA was unable to induce expression in a $\Delta brpT$ mutant (bars 4 to 6). Expression was restored when *brpT* was ectopically expressed from its native promoter (bars 7 to 9).

To minimize the potential influence of altered c-di-GMP levels on *brpT* transcription from its native promoter, we expressed *brpT* from an IPTG (isopropyl- β -D-thiogalactopyranoside)-inducible promoter. While the expression of DcpA alone increased biofilm formation in WT cells (Fig. 4, inset, compare bars 1 and 2), the expression of BrpT alone did not (inset, compare bars 1 and 4). This suggested that in

FIG 4 BrpT mediates the effect of c-di-GMP on *brp* locus expression and biofilm formation. Wild type (WT), $\Delta brpT$, and complemented ($\Delta brpT-C$) *V. vulnificus* strains expressing either *dcpA* or *cdgJ* from an arabinose-inducible promoter (P_{ara}) and/or *brpT* from an IPTG-inducible promoter (P_{IPTG}) were grown in LB under inducing conditions. Vector control strains contained the empty expression vectors. The relative level of $P_{brpA-gfp}$ expression is shown in relative fluorescence units (RFU). Inset, biofilm formation by the indicated WT strains. BrpT mediates the effect of c-di-GMP on *brp* expression and biofilm formation. Plots show the means, and error bars represent the standard deviations. Significance: ns, not significant ($P > 0.05$); *, $P < 0.001$. Refer to the text for bar numbering.

addition to stimulating *brpT* and ultimately *brp* expression, c-di-GMP may also regulate the function of additional factors required for biofilm development that cannot be overcome by simply overexpressing *brpT*. However, IPTG-induced expression of BrpT increased $P_{brpA-gfp}$ levels in each background (Fig. 4, bars 10 to 18), including when coexpressed with CdgJ (bars 12, 15, and 18). In fact, only a slight decrease in reporter expression was observed in strains coexpressing CdgJ relative to the corresponding vector controls (Fig. 4, bars 10, 13, and 16). This hinted at BrpT being capable of driving $P_{brpA-gfp}$ expression at low intracellular c-di-GMP concentrations.

To confirm that BrpT could activate gene expression under limiting c-di-GMP conditions, $P_{brpA-gfp}$ levels were monitored in *E. coli* cells expressing BrpT under unaltered (4 nM), low (1 nM), and high (400 nM) intracellular c-di-GMP concentrations. Under low conditions, the intracellular c-di-GMP levels were approaching the limit of our detection capability (~ 1 nM) (Fig. S1). We did not detect $P_{brpA-gfp}$ expression under any condition in the absence of BrpT (Fig. 5, bars 1 to 3). This is likely due to the lower overall intracellular c-di-GMP levels in *E. coli* than in *V. vulnificus* (Fig. S1) and the dependence of $P_{brpA-gfp}$ expression on BrpT. Induction of BrpT resulted in a similar increase (>230 -fold) in $P_{brpA-gfp}$ expression under low, unaltered, and high intracellular c-di-GMP concentrations, with a 1.5-fold difference at most among the three strains (Fig. 5, bars 4 to 6). This suggested that BrpT can specifically bind to target promoters and activate gene expression over a wide range of intracellular c-di-GMP concentrations (nanomolar to micromolar).

The BrpT WLPR motif suppresses the requirement for c-di-GMP binding for activation. To delve further into the impact of the proline of the WLPR motif on BrpT activity, we mutated it to a T (BrpT^{P124T}) as found in the canonical VpsT c-di-GMP binding site. Structural modeling of the BrpT^{P124T} motif (WLTR) suggested that it would mirror the arrangement of the c-di-GMP binding pocket of VpsT (see Fig. S3 in the supplemental material). The R in the 4th position of the motif is essential for c-di-GMP binding by, and function of, VpsT and CpsQ, so the analogous change was introduced into BrpT (BrpT^{R125A}). However, the BrpT^{R125A} motif (WLPA) maintains the 3rd-position proline, so a BrpT^{P124T/R125A} double mutant was also generated. Each mutant was

FIG 5 BrpT^{WT}-driven P_{brpA} expression in *E. coli*. Plots show the means, and error bars represent the standard deviations. Significance: ns, not significant ($P > 0.05$). RFU, relative fluorescence units. Refer to the text for bar numbering.

assayed for its ability to drive P_{brpA} expression in a $\Delta brpT$ background under low (CdgJ), unaltered (empty vector), or high (DcpA) intracellular c-di-GMP concentrations.

Reporter expression in control cells was 2-fold higher with BrpT^{P124T} than observed with BrpT^{WT} (Fig. 6A, bar 1; compare with Fig. 4, bar 13). Although a similar increase (2.5-fold) was observed when BrpT^{P124T} was coexpressed with DcpA (Fig. 6A, bars 1 and 2; compare with Fig. 4, bars 13 and 14), overall $P_{brpA-gfp}$ expression was twice the level observed with BrpT^{WT}. Importantly, $P_{brpA-gfp}$ expression was 4-fold lower when CdgJ was coexpressed with BrpT^{P124T} than with BrpT^{WT} (Fig. 6A, bar 3; compare with Fig. 4, bar 15). This suggested that BrpT^{P124T} was more active and more sensitive to changes in the intracellular c-di-GMP concentrations than BrpT^{WT}. The BrpT^{R125A} mutant, which bears a proline in the 3rd position of the motif, could still activate $P_{brpA-gfp}$ expression, and its activity was largely insensitive to the intracellular c-di-GMP level (Fig. 6A, bars 4 to 6). Conversely, only a low level of promoter activity was detected with the BrpT^{P124T/R125A} double mutant under each condition (Fig. 6A, bars 7 to 9). This decrease suggested that the activity of BrpT^{P124T} was now dependent on R^{125A} (Fig. 6A, compare bars 1 to 3 with bars 7 to 9).

The same overall trend was observed when the reporter assays were conducted in *E. coli* (Fig. 6B). BrpT^{P124T}-driven $P_{brpA-gfp}$ expression was highly dependent on the intracellular c-di-GMP concentration (bars 1 to 3). While only a 1.5-fold change in $P_{brpA-gfp}$ expression was observed between high and low intracellular c-di-GMP concentrations for BrpT^{WT} (Fig. 5, bars 5 and 6), a 17-fold drop in $P_{brpA-gfp}$ expression occurred for BrpT^{P124T} under the same conditions (Fig. 6B, bars 2 and 3). This again suggested that BrpT^{P124T} was more active but more sensitive than BrpT^{WT} to changes

FIG 6 P_{brpA} expression driven by BrpT WLPR motif mutants. $P_{brpA-gfp}$ expression profiles in a *V. vulnificus* $\Delta brpT$ strain (A) and *E. coli* (B). RFU, relative fluorescence units. Plots show the means, and error bars represent the standard deviations. Significance: ns, not significant ($P > 0.05$); *, $P < 0.001$. Refer to the text for bar numbering.

FIG 7 c-di-GMP dependent binding of BrpT^{P124T} to the *brpA* promoter. BrpT^{P124T} was incubated with a labeled 325-bp fragment spanning the *brpA* promoter (P_{brpA}) or a 100-bp fragment of the *flpA* promoter (P_{flpA}). c-di-GMP was added where indicated.

in the intracellular c-di-GMP concentrations. Although P_{brpA} -*gfp* expression driven by BrpT^{R125A} (Fig. 6B, bars 4 to 6) was 2- to 3-fold lower than that driven by BrpT^{WT}, the overall expression pattern appeared to be largely independent of the intracellular c-di-GMP concentration. Finally, the BrpT^{P124T/R125A} double mutant could not support P_{brpA} -*gfp* expression under any condition tested (Fig. 6B, bars 7 to 9). Since both BrpT^{WT} and BrpT^{R125A} bear a proline in the third position of their respective WLP[R/A] motif (and each variant was stably expressed to comparable levels *in vivo* [Fig. S2]) and both still drove P_{brpA} -*gfp* expression when coexpressed with CdgJ, this suggested that a proline at the third position of the BrpT motif was sufficient to support BrpT activity at low intracellular c-di-GMP levels. Mutating the proline to a threonine rendered BrpT more active than BrpT^{WT} at high intracellular c-di-GMP levels but less active at unaltered and low intracellular c-di-GMP levels, and this activity was now dependent on the arginine in the 4th position of the motif.

BrpT has a low affinity for c-di-GMP. BrpT could specifically bind to promoters upstream of *brpA* and *brpH*. Since only nanomolar amounts of c-di-GMP were detected in our purified BrpT preparations, this suggested that DNA binding could occur at low intracellular c-di-GMP concentrations. Structural modeling of the WLPR motif of BrpT suggested that the proline would likely affect the H bond at that position and potentially at the neighboring arginine (R¹²⁵) as well (Fig. S3). Empirically, the ability of BrpT to stably bind c-di-GMP seemed suspect. Isothermal titration calorimetry (ITC) was used to measure the c-di-GMP binding potential of purified BrpT (see Fig. S4 in the supplemental material). We were unable to saturate BrpT^{WT} with c-di-GMP at any molar ratio (from 1:4 to 18:1). The data indicated minimal protein-molecule association, and the affinity of BrpT for c-di-GMP (apparent dissociation constant [K_d] = 135.4 μ M with $n = 1$, $\Delta S = 408.2$ J/mol/K) was >40 times weaker than reported for VpsT ($K_d = 3.2$ μ M). This result suggested that BrpT interacted poorly with c-di-GMP. The ability of BrpT to activate P_{brpA} expression under low intracellular c-di-GMP concentrations together with its low affinity for c-di-GMP suggested that signal binding was not absolutely required for BrpT to function.

We next tested the ability of BrpT^{P124T} to bind c-di-GMP. BrpT^{P124T} was purified to homogeneity, and as with BrpT^{WT}, only a low concentration (nanomolar) of c-di-GMP was detected in the preparation (Fig. S2). ITC data suggested that BrpT^{P124T} bound c-di-GMP with a K_d of ~ 1.4 μ M and a stoichiometry of 1:1 (Fig. S4), metrics very similar to those of c-di-GMP binding by VpsT. Electromobility shift assays (EMSAs) with BrpT^{P124T} were conducted to determine if promoter binding was now c-di-GMP dependent (Fig. 7). BrpT^{P124T} was unable to shift the P_{brpA} fragment (Fig. 7, compare lanes 1 and 2) unless exogenous c-di-GMP was added to the reaction (lanes 1 to 3). Binding was specific, since BrpT^{P124T} was not able to bind P_{flpA} (lanes 4 and 5). These results suggested that the P124T substitution altered the DNA binding behavior of BrpT such that stable complex formation was now c-di-GMP dependent. Together, these data support our contention that BrpT^{WT} likely does not directly sense c-di-GMP and so can remain active at low intracellular c-di-GMP concentrations.

FIG 8 The VpsT^{T133P} is active at lower intracellular c-di-GMP concentrations than VpsT^{WT}. VpsT^{WT} and VpsT^{T133P} were expressed alone or together with CdgJ in strain FY-Vc618ΔvpsT. Colony morphology was monitored for a switch from the smooth (S) to the rugose (R) phenotype as an indicator of VpsT activity.

To verify the importance of the third position of the motif, we next introduced the reciprocal change into VpsT (T133P), in anticipation that this would render it less dependent on intracellular c-di-GMP levels for activation than VpsT^{WT}. VpsT^{WT} and VpsT^{T133P} were expressed alone or together with CdgJ in a *V. cholerae* vpsT-null mutant derived from a rugose parental strain (FY-Vc618ΔvpsT). This mutant appears smooth on solid medium (Fig. 8, top), and the development of rugosity was used as a visual indicator of activator function. VpsT^{WT}, when expressed alone, induced rugose-colony formation but failed to do so when coexpressed with CdgJ (Fig. 8, middle). FY-Vc618ΔvpsT cells expressing VpsT^{T133P} were also rugose. Although it was subtle, the cells exhibited a partially rugose phenotype when coexpressed with CdgJ (Fig. 8, bottom). This suggested that VpsT^{T133P}, unlike VpsT^{WT}, was able to suppress to some extent the CdgJ-induced smooth phenotype. This demonstrated that the VpsT^{T133P} allele remained functional at intracellular c-di-GMP concentrations too low to support VpsT^{WT} activity and supported the notion that the WLPR variant of the c-di-GMP binding motif plays an important role in moderating c-di-GMP-dependent regulator function.

DISCUSSION

In *V. vulnificus*, increased intracellular c-di-GMP concentrations trigger biofilm development, a process that includes expressing the *brp*-encoded EPS (15, 16). We have shown that the *brp* locus is organized as two linked operons of five and four genes, respectively, and that its c-di-GMP dependent expression is mediated by BrpT, which binds to promoters upstream of *brpA* and *brpH*. We also showed that *brp* expression was temperature dependent, similar to the expression of the *vps* locus in *V. cholerae*, in which it has been reported that intracellular c-di-GMP concentrations increase with decreasing growth temperature (24). Although we did not demonstrate that the intracellular c-di-GMP concentration of *V. vulnificus* varied with temperature, the change in rugosity may be of ecological and pathological relevance. *V. vulnificus* is most prevalent in oysters at estuary temperatures just below 20°C (25–27), a range that supports *brp* expression, biofilm formation, and host colonization. The CPS is an essential virulence factor in *V. vulnificus* (28–30). Decreased *brp* expression at 37°C, such as during disease progression in the human body, might help to ensure that important cellular resources for polysaccharide synthesis are invested in CPS production rather than being diverted to a competing Brp-EPS biosynthesis pathway.

BrpT belongs to the subclass of VpsT-type regulators that bear a proline in the 3rd

FIG 9 Inferred evolutionary relationship of VpsT-type transcriptional regulators in 9 species of *Vibrio*. Phylogenetic tree of VpsT-type regulators bearing a W[F/L/M][T/S/P]R motif. The protein name or locus tag is shown followed by the host species. The *V. harveyi* (Vha) genome coded for five VpsT homologs, while *V. vulnificus* (Vvu), *V. parahaemolyticus* (Vpa), and *V. alginolyticus* (Val) each encoded four. Two representatives were identified in *V. cholerae* (Vch), *V. anguillarum* (Van), *V. coralliilyticus* (Vco), and *V. splendidus* (Vsp), and a single homolog was found in *V. fischeri* (Vfi). Eco, *Escherichia coli*. Shading is as follows: blue, regulators with a 3rd-position proline; yellow, host strains that also exhibit homology/synteny in their *eps* loci (see Fig. S5); green, BrpT shares aspects of the adjacent blue and yellow subgroups; red, putative c-di-GMP-regulated repressors encoded by the genomes of strains with activators that cluster with BrpT and CpsQ (connected by a red line and bar).

position of the canonical W[F/L/M][T/S]R c-di-GMP binding motif (19). We demonstrated that the WLPR variant of the BrpT c-di-GMP binding motif diminished its dependence on signal binding for activator function. Thus, BrpT defines a new class of VpsT-like transcriptional regulators, and we anticipate that other members of the WLPR motif subclass may behave similarly. In support of this notion, a VpsT mutant bearing a WLPR motif (VpsT^{T133P}) also exhibited activity at intracellular c-di-GMP concentrations too low to support the activity of wild-type VpsT. These data suggest that the 3rd position of the WLTR motif plays a key role in moderating the degree to which activator function is regulated by direct signal binding. Obtaining a detailed structure of BrpT^{WT} and BrpT^{P124T} (free and c-di-GMP bound) should shed light on the manner in which BrpT activates gene expression.

VpsT-type regulators bearing a 3rd-position proline motif were identified in 8 of 9 *Vibrio* species examined. The exception was the squid symbiont *Vibrio fischeri*, whose genome coded for only one VpsT-type regulator and no identifiable *brp*-type locus. Estimation of the maximum likelihood phylogeny for VpsT-type regulators from these species revealed that regulators with a 3rd-position proline grouped separately from other W[F/L/M][T/S]R motif members (Fig. 9). Notably, BrpT clustered with CpsQ on a separate branch from VpsT. This is particularly interesting since BrpT is not absolutely

FIG 10 Model for BrpT- and c-di-GMP-dependent regulation of *brp* expression. Environmental cues activate DGCs and/or PDEs that alter the internal c-di-GMP concentration. Increased c-di-GMP levels invoke an effector(s) that activates *brpR* expression, or c-di-GMP may bind directly to BrpR already present in the cell. BrpR drives expression of P_{brpT} and functional BrpT oligomers bind to promoters upstream of *brpA* and *brpH* to activate *brp* expression. Concomitant derepression of *brp* expression by specific repressors occurs, possibly via physical interaction of the repressor with c-di-GMP or its displacement from the DNA by BrpT. UE, unknown effector; Rep, repressor; green arrows, known (solid) and unknown (dashed) steps in the activation of *brp* expression.

dependent on signal binding for activity and the apo form of CpsQ is reported to bind c-di-GMP so tightly that, once bound, it may in effect be constitutively active (21). As a result, both *V. parahaemolyticus* and *V. vulnificus* would likely require additional systems to decrease expression of their respective *eps* loci, since the activity of CpsQ and BrpT would be challenging to quickly moderate via further direct interactions with c-di-GMP. *V. parahaemolyticus* codes for additional VpsT-type regulators not found in *V. cholerae*. One of these, CpsS, is more closely related to VpsT than the CpsQ activator (Fig. 9, red shading). CpsS functions to repress *cps* expression, although it is not known if its activity is c-di-GMP dependent. *V. vulnificus* also codes for additional VpsT-type regulators not found in *V. cholerae*. It is thus conceivable that c-di-GMP also regulates the activity of systems that counter BrpT activity to keep *brp* expression low under low intracellular c-di-GMP conditions.

The synteny and homology of the *eps* loci from species encoding a similar number of VpsT-type regulators were conserved (see Fig. S5 in the supplemental material). We suspect that species coding for only two VpsT-type regulators (*V. cholerae*, *V. anguillarum*, *V. coralliilyticus*, and *V. splendidus*) moderate activator function by direct molecule binding, whereas the regulatory network of those with four or more VpsT-type regulators may use additional VpsT-type repressors to counter activator function. This notion is supported by our observation that although the expression of BrpT alone in *V. vulnificus* induced P_{brpA} expression, it was not sufficient to promote biofilm formation. Maximal and timely *brp* expression would thus be dependent on the simultaneous activation and relief of *brp* repression (Fig. 10).

VpsT and BrpT share only 33% identity (57% similarity [Fig. S3]), yet a single nucleotide transversion was sufficient to produce the proline/threonine mutation that shifted their dependence on c-di-GMP binding for activity. It is not known which activity (signal-dependent or liberated) is ancestral or if they evolved independently;

however, both pathways remain dependent on an increased intracellular c-di-GMP concentration to initiate biofilm formation; in *V. vulnificus*, the expression of *brpT* itself is c-di-GMP regulated. The third-position proline variant of the c-di-GMP binding motif simply suppresses the barrier of regulator-signal association before downstream target promoter activation occurs. An obvious advantage is that, once expressed, BrpT is competent for activation. However, a tenet of this arrangement is that the activity of BrpT would require counterbalancing by repressors, whereas the activity of VpsT can be directly mitigated. Direct signal binding by VpsT-type regulators may also permit them to behave as c-di-GMP sentinels, promoting their roles from specific to global regulators that are capable of influencing multiple networks. Conversely, the liberated activity of BrpT likely restricts its impact to a few biofilm-related pathways.

MATERIALS AND METHODS

Media and strains. LB and LB agar were purchased from BD Difco. Antibiotics and additives were purchased from Sigma and used at the following concentrations: ampicillin (Ap), 100 μ g/ml; chloramphenicol (Cm), 25 μ g/ml; gentamicin (Gm), 10 (*E. coli*) or 35 (*V. vulnificus*) μ g/ml; IPTG, 100 μ M; X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside), 20 μ g/ml; L-arabinose, 0.1%. Strains and plasmids are listed in Table S1 in the supplemental material.

Colony morphology, CV assays, and RT-PCR. Colony morphology and crystal violet (CV) assays were conducted as previously described (16). Colony morphology images were captured on a Leica M55 dissecting scope equipped with a Leica DC300F camera. The following reverse transcription (RT)-PCR primer pairs (data not shown) were used to monitor expression of *wzc*, *brpA*, and *rplT*: *wzc*seq2 and *wzcR*, *wcrART1* and *wcrART2*, and *vvuL20RT1* and *vvuL20RT2*, respectively. PCR products were visualized by agarose gel electrophoresis and quantified by densitometry using the ImageLab gel analysis software package (Bio-Rad). The transcript levels for *wzc* and *brpA* were normalized relative to the level of *rplT* (ribosomal L20) transcript in the same sample. Assays were performed in triplicate on three biological samples. Plots show the means, and error bars represent the standard deviations. *P* values were calculated using the Student *t* test.

Construction of the Δ *brpT* mutant. PCR fragments (3 kb) upstream and downstream of *brpT* were fused together by Gibson assembly (New England BioLabs), such that only the start and stop codons of the *brpT* coding region remained (data not shown). The fragment was cloned into pRE112 (31) and conjugated to *V. vulnificus*. Cointegrants were selected on LB Cm plates, gridded onto LB 10% sucrose plates for *sacB* counterselection, and confirmed to have a deletion in *brpT* by PCR.

Construction of reporter plasmids. A 325-bp *PbrpA* PCR product was fused to *gfp* or the *E. coli lacZ* (*lacZ^{Ec}*) and inserted into pTX1*KebgA* by Gibson assembly. Plasmids were maintained in *E. coli* S17.1 λ π and conjugated to *V. vulnificus* for integration into *ebgA* (a cryptic *lacZ*). The same approach was used to fuse *brpT^{WT}* or *brpT^{P124T}* to *gfp* for cloning into the IPTG-inducible pC2X6HT expression plasmid for subcellular localization studies. In order to monitor β -galactosidase activity in *V. vulnificus* without interference due to expression of its endogenous *lacZ* (*lacZ^{Vv}*), pTX1*KebgA-PbrpAlacZ^{Ec}* was conjugated to a *V. vulnificus* strain bearing a markerless deletion of the promoter for *lacZ^{Vv}* that was created using the pRE112 sucrose counterselection plasmid (32).

Fluorescence and reporter fusion analysis. Strains were grown overnight, diluted in fresh medium containing inducer, and inoculated in triplicate into 96-well plates. Optical density at 600 nm (OD_{600}) and fluorescence measurements were taken at 30-min intervals on a Biotek Synergy H1 microplate reader (excitation wavelength, 485 nm; emission wavelength, 528 nm). Data for three technical replicates from three biological replicates were collected for all experiments. Plots show the means, and error bars represent the standard deviations. *P* values were calculated using the Student *t* test.

Protein expression and purification. His-tagged PCR products of the wild-type and mutated *brpT* genes were cloned into pACYC-Duet (Novagen) by Gibson assembly and transformed into *E. coli* BL21(DE3) (Novagen). Cells were grown overnight at 37°C with shaking (200 rpm) in LB containing Cm. The cultures were diluted 1:100 into 4 liters fresh LB Cm and grown to an OD_{600} of 0.5 at 37°C with shaking. IPTG was added to 250 μ M, and incubation was continued at 25°C with shaking for 6 h. The cells were harvested by centrifugation, and pellets were frozen at -80°C for later purification.

Cell pellets were thawed on ice, resuspended in lysis buffer (20 mM imidazole, 550 mM NaCl, 1 mg/ml lysozyme, and 50 mM NaH_2PO_4 [pH 7.5]), and disrupted by sonication. Lysates were clarified by centrifugation at $12,000 \times g$ for 15 min at 4°C and then passed through a 0.22- μ m filter. The cleared lysates were loaded onto a 1-ml HisTRAP HP column (GE Healthcare) connected to an AKTA FPLC (Amersham Pharmacia) with a flow rate of 1 ml/min. The column was washed with 20 column volumes of lysis buffer minus lysozyme, and bound proteins were eluted using a linear imidazole gradient from 20 mM to 500 mM over 20 column volumes. The eluted fractions were separated by SDS-PAGE (12%), and those containing greater than 95% His-tagged BrpT (judged by Coomassie blue staining) were pooled. The pooled sample was applied to a 5-ml HiTrap desalting column and eluted in 50 mM NaH_2PO_4 (pH 7.5) and 550 mM NaCl to exchange the buffer. Purified protein was recovered in the flowthrough. Protein purity was analyzed by SDS-PAGE. If necessary, the protein was concentrated using an Amicon Ultracel 10K column (Milipore) and stored at 4°C for up to 14 days.

To demonstrate that purified BrpT (Fig. S2A) was, in effect, free of c-di-GMP for subsequent ITC experiments, it was extracted and quantified by liquid chromatography-tandem mass spectrometry

(LC-MS/MS). Bovine serum albumin (BSA) spiked with a known quantity of c-di-GMP was treated in parallel and served as a control for recovery of the signaling molecule. Nearly 86% of the c-di-GMP added to the BSA sample was recovered (Fig. S2B), proving the efficacy of the extraction method. The amount of c-di-GMP detected in our purified BrpT was in the low nanomolar range and approached the limit of detection. Western blots were probed with Sigma monoclonal anti-polyhistidine-peroxidase primary conjugate antibody and detection was performed by using the Invitrogen Novex ECL HRP chemiluminescent substrate reagent kit.

Quantification of the intracellular c-di-GMP concentration. For extraction of intracellular c-di-GMP from whole cells, strains carrying pSU38GT-*dcpA*, pSU38GT-*cdgJ*, or the empty vector were grown overnight in LB with Gm. The next day, cells were diluted 1:100 in fresh medium and grown to an OD₆₀₀ of 0.5. Cells were again diluted to an OD₆₀₀ of 0.05 into fresh LB containing Gm with 0.2% L-arabinose and grown to an OD₆₀₀ of 1.0. Six milliliters of each sample was centrifuged at 4°C for 10 min at 12,000 × *g*. The pellet was resuspended in 200 μl of 40% (vol/vol) acetonitrile–40% (vol/vol) methanol–0.1 N formic acid. This suspension was incubated at –20°C for 30 min and then centrifuged at 4°C for 5 min. The supernatant was recovered, and 10 μl of each sample was analyzed by LC-MS/MS on a Quattro Premier XE mass spectrometer (Waters) coupled with an Acquity Ultra Performance LC system (Waters). c-di-GMP was detected with electrospray ionization using multiple-reaction monitoring in the negative-ion mode at *m/z* 689.16→344.31. The MS parameters were as follows: capillary voltage, 3.5 kV; cone voltage, 50 V; collision energy, 34 V; source temperature, 110°C; desolvation temperature, 350°C; cone gas flow (nitrogen), 50 liters/h; desolvation gas flow (nitrogen), 800 liters/h; collision gas flow (nitrogen), 0.15 ml/min; and multiplier voltage, 650 V. Chromatography separation was reverse phase using a Waters BEH C₁₈ 2.1- by 50-mm column with a flow rate of 0.3 ml/min with a gradient of 10 mM tributylamine plus 15 mM acetic acid in 97:3 water-methanol (solution A) to methanol (solution B) with the following parameters: *t* = 0 min; A, 99%, and B, 1%, *t* = 2.5 min; A, 80%, and B, 20%, *t* = 7.0 min; A, 35%, and B, 65%, *t* = 7.5 min; A, 5%, and B, 95%, *t* = 9.01 min; A, 99%, and B, 1%, *t* = 10 min. A c-di-GMP standard curve for calculating the c-di-GMP concentration in each extract was generated by dissolving synthesized c-di-GMP (Axxora) in extraction buffer to 250 nM followed by 2-fold serial dilutions to 0.975 nM.

Isothermal titration calorimetry. Apparent dissociation constants (*K_d*) and interaction stoichiometry of BrpT^{WT} and BrpT^{P124T} with c-di-GMP were measured by isothermal titration calorimetry (ITC) using a Nano ITC instrument from TA Instruments. Calorimetric titrations were done using 2 μM c-di-GMP and 3-μl injections spaced 180 s apart for BrpT and using 300 μM c-di-GMP with 2-μl injections spaced 180 s apart for BrpT^{P124T}. The cuvette contained 30 μM protein, and reactions were conducted at 25°C in 50 mM NaH₂PO₄ (pH 7.5), 550 mM NaCl, and 0.3 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP). The data were analyzed by integrating heat effects normalized to the amount of injected c-di-GMP. Curves were fit based on a single-site binding model using the TA Instruments NanoAnalyze data analysis software (v 3.4.0), and the *K_d* was derived from the data using standard methods.

EMSAs. EMSAs were performed using purified BrpT^{WT} and BrpT^{P124T} and infrared 700 (IR700)-labeled probes (IDT). IR700-labeled primers (data not shown) were used to generate *P_{brpA}*, *P_{brpH}*, and *P_{flpA}* probes from *V. vulnificus* genomic DNA. Reaction mixtures contained 5 μM BrpT^{WT} or BrpT^{P124T}, 0.2 mg/ml BSA, 0.1 μg/ml poly(dI-dC) in binding buffer (10 mM Tris-HCl [pH 7.5], 50 mM KCl, 75 mM NaCl, 10 mM MgCl₂, 5% glycerol, 1 mM dithiothreitol [DTT], and 0.1 mM EDTA). When indicated, c-di-GMP was added to a final concentration of 50 μM. After a 30-min incubation at room temperature in the dark, reaction mixtures were loaded onto a 5% Tris-borate-EDTA (TBE)-polyacrylamide gel with 10% glycerol and run in 0.5× TBE. Gels were prerun for 1 h at 50 V before loading, and samples were resolved at 50 V for 2 h. Gels were visualized on an Odyssey FC imaging system (Licor).

Structural modeling, phylogenetic analysis, and genomic comparisons. The crystal structure of VpsT (PDB 3KLO) was used as a scaffold to build structural models for wild-type and mutant BrpT using PHYRE² (33) and MODELLER (34). Chimera (35) was used for molecular visualization. Five homology models were generated for each protein, and the model with the best overall agreement score was analyzed further. Protein sequences were aligned using T-Coffee (36), and maximum likelihood trees were generated with PhyML (37). Mauve alignments (38) were viewed using Geneious (Biomatters).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00344-17>.

SUPPLEMENTAL FILE 1, PDF file, 0.8 MB.

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