MINIREVIEW

Cyclic Di-GMP Signaling in Bacteria: Recent Advances and New Puzzles[▽]

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Cyclic di-GMP [bis-(3'-5')-cyclic di-GMP] (Fig. 1) is a novel second messenger in bacteria that was first described as an allosteric activator of cellulose synthase in *Gluconacetobacter xylinus* (49). It is now established that this nucleotide is almost ubiquitous in bacteria, where it regulates a range of functions including developmental transitions, aggregative behavior, adhesion, biofilm formation, and the virulence of animal and plant pathogens (for reviews, see references 15, 18, 30, 31, 47, and 48). The level of cyclic di-GMP in bacterial cells is influenced by both synthesis and degradation. The GGDEF protein domain synthesizes cyclic di-GMP, whereas the EAL and HD-GYP domains are involved in cyclic di-GMP hydrolysis (43, 50, 51, 53, 54, 58). Bacterial genomes encode a number of proteins with GGDEF, EAL, and HD-GYP domains; e.g., in *Pseudomonas aeruginosa*, there are 40 such proteins. The majority of these proteins contain additional signal input domains. These signaling systems are presumed to use cyclic di-GMP as a second messenger to link the sensing of specific environmental cues to appropriate alterations in bacterial physiology and/or gene expression. Some details about the operation and organization of cyclic di-GMP signaling systems are emerging. Nevertheless, many questions remain to be answered, and new puzzles have arisen as a result of experimental investigations. Here, we review the recent advances and highlight the considerable gaps in our understanding of cyclic di-GMP signaling. In particular, we discuss (i) the protein domains involved in cyclic di-GMP turnover, highlighting HD-GYP, recently described as a novel cyclic di-GMP phosphodiesterase; (ii) signals that are transduced through cyclic di-GMP signaling; (iii) the role of cyclic di-GMP signaling in bacterial virulence; (iv) recent findings addressing the organization of, and interplay between, different cyclic di-GMP signaling systems; (v) the concept of localized pools of cyclic di-GMP within the cell; and (vi) the mechanisms by which cyclic di-GMP exerts its effects on diverse cellular functions.

PROTEIN DOMAINS INVOLVED IN CYCLIC DI-GMP TURNOVER

Role of the GGDEF and EAL domains. The association of GGDEF and EAL domains with cyclic di-GMP turnover was initially made through studies of the regulation of cellulose

synthesis in *G. xylinus* (57). Biochemical studies revealed that the level of cyclic di-GMP was controlled by the opposing action of the enzymes diguanylate cyclase (DGC), which catalyzes its formation from GTP, and phosphodiesterase A (PDEA), which catalyzes its degradation. In a reverse genetic approach, amino acid sequence information derived from purified DGC and PDEA was used to clone three *cdg* genes encoding isoforms of DGC and three *pdeA* genes encoding isoforms of PDEA (57). All three *cdg* genes and all three *pdeA* genes encode hybrid proteins containing a GGDEF domain (formerly known as DUF1, for domain of unknown function) and a C-terminal EAL domain (formerly known as DUF2). The nomenclature for GGDEF and EAL relates to conserved amino acid motifs in these domains (21). The association of GGDEF and EAL domains with cyclic di-GMP turnover was further strengthened by the later demonstration of cyclic di-GMP phosphodiesterase activity of the recombinant PdeA1 protein (12). The GGDEF domain was first identified in PleD, a regulatory protein controlling swarmer-to-stalked-cell transition in *Caulobacter crescentus* (24). The EAL domain was first described for BvgR, a repressor of virulence gene expression in *Bordetella pertussis* (40). In light of the work with *G. xylinus* that implicated fused GGDEF-EAL domain proteins in cyclic di-GMP turnover, these findings gave the first indication that cyclic di-GMP signaling might be more widespread in bacteria and may control functions other than cellulose synthesis.

Although the work with *G. xylinus* associated GGDEF and EAL domains with cyclic di-GMP turnover, the precise biochemical role of these domains was not resolved. Indirect evidence for the role of the GGDEF domain in cyclic di-GMP synthesis came from *in silico* studies indicating some structural conservation with the proposed nucleotide-binding loop of eukaryotic adenylyl cyclases (44). This suggestion was supported by genetic experiments in which the expression of *dgcI* from *G. xylinus*, which encodes a GGDEF-EAL hybrid protein, as well as the expression of genes encoding proteins with GGDEF but no EAL domain from other bacteria were shown to complement a *celR2* mutant of *Rhizobium* for defects in cellulose production (5). Subsequently, it was shown that the expression of genes encoding GGDEF proteins could increase the cellular levels of cyclic di-GMP in several bacteria (43, 54, 60). Direct evidence for the role of the GGDEF domain was obtained by biochemical studies of purified GGDEF domain proteins including PleD (43, 51) and of isolated GGDEF domains from proteins from different bacterial phyla (51). Each of these GGDEF domains/proteins converted two molecules of GTP to cyclic di-GMP with Mg^{2+} as a cofactor but had no activity with other nucleotides. The conservation of function of GGDEF

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FIG. 1. Chemical structure of the intracellular second messenger bis-(3'-5')-cyclic di-GMP (cyclic di-GMP).

domains from divergent bacteria was further demonstrated by reciprocal complementation of *hmsT*, which is involved in biofilm formation in *Yersinia pestis*, and *adrA*, which is involved in cellulose synthesis in *Salmonella enterica* serovar Typhimurium (55). Furthermore, in many of the experiments cited above, site-directed mutagenesis was used to establish that the conserved GGDEF motif residues were critical for cyclic di-GMP synthesis.

Indirect support for the role of the EAL domain in cyclic di-GMP degradation was provided by the demonstration that heterologous expression of genes encoding proteins with EAL but not GGDEF domains could reduce cellular levels of cyclic di-GMP (54, 60, 61) and, conversely, that a mutation of an EAL domain protein increased cellular cyclic di-GMP levels (26). Moreover, the EAL domain protein HmsP of *Yersinia pestis* was shown to possess activity against the model phosphodiesterase substrate bis-(*p*-nitrophenol) phosphate (8). Direct biochemical evidence for the role of the EAL domain as a cyclic di-GMP phosphodiesterase came from studies of intact proteins as well as isolated EAL domains (13, 50, 53, 58). The major product of the enzymatic action of the EAL domain was the linear nucleotide pGpG, which was more slowly converted to GMP. This activity of the EAL domain was absolutely dependent on the presence of Mg^{2+} or Mn^{2+} (53). Again, in a number of these cases, mutational analysis indicated the essential role of the conserved EAL motif in enzymatic activity.

Amino acid variations within the GGDEF and EAL motifs. Variations in the amino acids within the GGDEF and EAL motifs occur naturally. Phylogenetic analysis of the GGDEF/EAL domain proteins in *Pseudomonas aeruginosa* indicates that GGDEF domains from almost all proteins that do not have an associated EAL domain are related in a single class (family I) in which the variant GGEEF motif is found (35). This alteration does not affect the cyclic di-GMP synthase activity (35). GGDEF domains from almost all *P. aeruginosa* proteins that also contain an EAL domain fall into two further classes, families II and III. This may suggest that the proteins with a GGDEF alone have evolved separately from those in which this domain is linked to an EAL domain. Family II sequences have the canonical GGDEF motif and are likely to be active in cyclic di-GMP synthesis. Conversely, family III sequences are poorly correlated with the consensus GGDEF sequence (for example, SPTRF in PA2567 and GDSIF in PA4959 [FimX]), suggesting that they may be enzymatically inactive (35). In contrast, several variations within the EAL motif (specifically, ETL and EVL) still allow cyclic di-GMP

hydrolysis (34, 35, 53). However, there are other motifs that are conserved in EAL domains that have enzymatic activity but show variations in EAL domains that are predicted to be inactive (53).

Regulatory roles for enzymatically inactive GGDEF and EAL domains. Although domains with sequences that diverge from the consensus may have lost enzymatic activity, they may still play a regulatory role. A GGDEF-EAL domain protein (CC3396) from *Caulobacter* that contains an altered active-site motif (GEDEF) lacks the ability to synthesize cyclic di-GMP. However, this altered GGDEF domain is able to bind GTP, leading to the activation of the attached EAL domain in cyclic di-GMP hydrolysis (13). This activation, which is specific for GTP, involves a lowering of the K_m of the EAL domain for cyclic di-GMP. A similar activation mechanism may occur in FimX from *P. aeruginosa*, which has the variant sequence GDSIF in the GGDEF domain (34). It is plausible that, in a converse fashion, inactive EAL domains may be capable of binding cyclic di-GMP to allosterically affect the enzymatic activity of a linked GGDEF domain. The binding of cyclic di-GMP to PleD at a noncatalytic site of the GGDEF domain regulates the diguanylate cyclase activity (11). In this way, cyclic di-GMP regulates its own synthesis. Whether the binding of cyclic di-GMP to an EAL domain has any allosteric effect on the enzymatic activity remains to be tested. As well as intramolecular effects, enzymatically inactive EAL or GGDEF domains could conceivably regulate cellular processes through intermolecular interactions with other proteins in a fashion influenced by the binding of cyclic di-GMP or GTP.

The biochemical conundrum of GGDEF-EAL domain functions. The definition of the biochemical functions of GGDEF and EAL domains presents a conundrum: what determines the overall activity of proteins that contain both domains? One possible resolution is that one of the two domains is not enzymatically functional, as discussed above. A second resolution could be that the proteins can have both activities but switch between states that are able to synthesize and hydrolyze cyclic di-GMP. One possible mechanism could be related to the oligomerization state. Structural analysis of the PleD regulator suggests that the GGDEF domain acts in cyclic di-GMP synthesis as a dimer (11), whereas EAL activity is apparently independent of protein oligomerization (53). Regulation of the oligomerization state of the GGDEF-EAL proteins, perhaps influenced by the sensory input domains, may then serve to determine which activity is expressed. A third alternative is that the two activities may be independently but dynamically set to ensure a very precise concentration emanating from a point source.

HD-GYP is a novel cyclic di-GMP phosphodiesterase. Bioinformatic studies have suggested that a third domain, HD-GYP, is also involved in cyclic di-GMP hydrolysis (20, 21). HD-GYP is a subgroup of the HD superfamily of metal-dependent phosphohydrolases. The association of the HD-GYP domain with a CheY-like two-component receiver domain in many bacterial proteomes indicates a role in signaling (20, 21). A role for HD-GYP in cyclic di-GMP hydrolysis was proposed based on an examination of the distribution and numbers of GGDEF, EAL, and HD-GYP domains encoded by different bacterial genomes, where several genomes encode proteins

with the GGDEF and HD-GYP domains but no EAL domain (20, 21, 22).

In the plant pathogen *Xanthomonas campestris*, the HD-GYP domain regulator RpfG positively regulates the synthesis of extracellular enzyme virulence factors and negatively regulates biofilm formation (17, 56). Expression of genes encoding heterologous EAL domain proteins in the *X. campestris* *rpfG* mutant restored extracellular enzymes and blocked biofilm formation. In contrast, expression of genes encoding a heterologous GGDEF domain protein in wild-type *X. campestris* gave a phenocopy of the *rpfG* mutant (50). These indirect observations were consistent with a role for the HD-GYP domain in cyclic di-GMP hydrolysis. This conclusion was supported by biochemical studies that demonstrated that the isolated domain could hydrolyze cyclic di-GMP to GMP via the linear intermediate pGpG (50). This reaction depended upon Mn^{2+} for which Mg^{2+} could not substitute. Mutation of the HD residues comprising the presumed catalytic diad of the HD-GYP domain abolished both the regulatory activity and enzymatic activity against cyclic di-GMP (50).

This recent finding of the occurrence of a second cyclic di-GMP phosphodiesterase unrelated to the EAL domain raises a number of questions for which there are currently no answers. Why did such an alternative activity arise? Is there any significance to the different relative activities of the EAL and HD-GYP domains against pGpG or for the different requirements for divalent metal ions? It has been suggested, for example, that pGpG may have a regulatory role in cyclic di-GMP signaling or may itself act as a signal molecule (47). The HD-GYP domain could serve to regulate pGpG levels, although this task may be performed by other, nonspecific, nucleases. Intriguingly, recent findings from yeast two-hybrid analyses have revealed a physical interaction between the HD-GYP domain of RpfG and GGDEF domain proteins of *Xanthomonas* (4). Although the biological relevance of such interactions has yet to be tested, these observations suggest a scenario whereby the HD-GYP domain may have evolved to modulate the activity of GGDEF domain proteins that are not associated with an EAL domain.

Bacterial genomes encode multiple proteins with GGDEF, EAL, and HD-GYP domains. Large-scale sequencing of bacterial genomes has revealed that GGDEF and EAL domains are highly abundant and widely distributed, although they are not found in all archaeal genomes sequenced thus far (21, 22). In July 2006, there were over 4,200 GGDEF domains and over 2,500 EAL domains in the Pfam protein family database. The HD-GYP domain is also widely distributed although less abundant, with over 200 HD-GYP domains in over 70 genomes. Most bacterial genomes have zero to three genes encoding HD-GYP domain proteins, although they can be more highly represented; the genomes of various delta-proteobacteria, for example, have 6 to 14 genes, *Vibrio* spp. have 5 to 13 genes, and *Thermotoga maritima* has 10 genes. (*T. maritima* is one of a few bacterial genomes that encode HD-GYP but not EAL domain proteins.) The significance of this pattern of distribution of genes encoding HD-GYP domain proteins in different genomes is unclear. The number of proteins with a potential role in cyclic di-GMP signaling encoded by bacterial genomes increases with genome size but in a nonlinear fashion (22). For example, the genome of the plant pathogen *Xylella fastidiosa*

FIG. 2. Domain analysis of the cyclic di-GMP signaling proteins of *Xylella fastidiosa* illustrates the complexities of the system. The proteins contain a variety of input domains including response receiver (REC), PAS, transmembrane domains possibly involved in carbohydrate binding (7TMR-DISMED2 and 7TMR-DISM-7TM), other transmembrane helices (TM), and an uncharacterized periplasmic domain in Xf0470. There is considerable sequence variation both in the EAL motif of the various EAL domains and in the GGDEF motif of the various GGDEF domains.

(~2.7 Mb) encodes only five proteins with a potential role in cyclic di-GMP signaling, whereas the genome of *Pseudomonas aeruginosa* at ~6.3 Mb encodes 40 proteins. These large numbers in the larger genomes indicate that there must be considerable complexity in the organization of cyclic di-GMP signaling within a single organism, which is poorly understood. This will be discussed below. Examination of the domain structures of the proteins implicated in cyclic di-GMP signaling in *Xylella fastidiosa* (Fig. 2) indicates that these complexities extend to systems with fewer components where, in addition to a range of different sensory input domains, there is considerable sequence variation both in the EAL motif of the various EAL domains and in the GGDEF motif of the various GGDEF domains.

CYCLIC DI-GMP SIGNALING AND BACTERIAL BEHAVIOR

Many reports have linked cyclic di-GMP signaling to aspects of communal behavior, e.g., biofilm development and architecture, colony morphology, and aggregation. Those reports showed the effects of mutations or overexpression of genes encoding GGDEF, EAL, or HD-GYP domain proteins on such behaviors. However, it is clear that cyclic di-GMP can also regulate a range of other phenotypes including motility, virulence factor synthesis, and cell differentiation. An emerging theme from many studies is that high cellular levels of cyclic di-GMP promote sessility, biofilm formation, and aggregative behavior, whereas low cyclic di-GMP levels promote motility and the synthesis of virulence factors in animal, human, and plant pathogens. However, recent analyses of *Pseudomonas aeruginosa* show phenotypic effects of mutations of some genes encoding cyclic di-GMP signaling systems that do not correspond to this generalization (see below).

Signal transduction leads to alteration in cyclic-di-GMP levels

FIG. 3. Cyclic di-GMP signaling systems for which environmental cues are known or suspected. The RpfC/RpfG system of *Xanthomonas campestris* is implicated in the sensing of the cell-cell signal DSF (18, 50, 56). NspS and MbaA of *Vibrio cholerae* are implicated in the sensing of norspermidine; the role of a third protein (VC0702) encoded by the *nspS-mbaA-VC0702* operon is currently unclear (9, 33). PdeA of *Gluconacetobacter xylinus* binds molecular oxygen to influence its activity (12). The influence of tobramycin on biofilm formation in *Pseudomonas aeruginosa* requires Arr (27), although it is not known if the antibiotic binds to Arr.

Diverse environmental signals or cues are transduced through cyclic di-GMP signaling. Many GGDEF, EAL, and HD-GYP domain proteins have additional domains that may directly sense environmental cues. These domains include PAS, which binds flavin or heme and may sense molecular oxygen or redox potential; GAF, which binds cyclic mononucleotides and other small-molecular-weight effectors; various membrane-associated or periplasmic domains that may be involved in sensing small molecules; and BLUF, a flavin adenine dinucleotide-dependent blue-light sensor (reviewed in reference 47). The binding of effectors to the sensory input domain is believed to affect the enzyme activity of the protein. In addition, a number of GGDEF and EAL domain proteins contain a CheY-like receiver (REC) domain. These proteins can be part of two-component signal transduction systems, and their activity may be altered by phosphorylation (1, 25, 43, 56). In a number of cases, multiple sensory input domains are found, suggesting complex regulation of individual enzymes in response to a range of environmental cues.

Although bioinformatic analysis can give some clues to environmental signals or cues that are recognized by the sensory domains, in only a few cases are the cognate signals known (Fig. 3). The best-studied example is PdeA1, a cyclic di-GMP phosphodiesterase from *G. xylinus*, which has a PAS-GAF-GGDEF-EAL domain structure (12, 57). The PAS domain of this protein contains a heme moiety that binds molecular oxygen. The consequences of oxygen sensing are a reduction in the degradative activity against cyclic di-GMP, which could conceivably lead to an elevation in the cellular levels, promoting cellulose production and pellicle formation (12). PdeA1 is highly homologous over its entire length to the Dos protein of *Escherichia coli*, which is also has a heme-binding PAS domain involved in oxygen sensing (16).

In the plant pathogen *Xanthomonas campestris*, recognition of the cell-cell signaling (quorum sensing) molecule DSF (diffusible signal factor) is linked to cyclic di-GMP degradation through a two-component system comprising the sensor histidine kinase RpfC and the CheY-HD-GYP domain regulator

RpfG (6, 14, 50, 56). DSF was characterized as the unsaturated fatty acid *cis*-11-methyl-2-dodecanoic acid (62). The *rpf*/DSF regulatory system positively controls the synthesis of extracellular plant cell wall-degrading enzymes and virulence and negatively controls biofilm dispersal. It has recently been suggested that cyclic di-GMP signaling and quorum sensing converge in the regulation of diverse bacterial behaviors including multicellular behavior, biofilm formation, and virulence (10). These findings on DSF signal transduction suggest that a direct link between the two regulatory systems can occur. However, the activation of a GGDEF/EAL domain protein by the binding of a quorum-sensing molecule has yet to be reported, and it remains likely that in many cases, the regulatory interplay between cyclic di-GMP signaling and cell-cell signaling is more indirect.

The polyamine norspermidine, which is present in a wide range of prokaryotes and eukaryotes, has been shown to activate the formation of biofilms in *Vibrio cholerae* (33). This activity depends upon NspS, a periplasmic binding protein, and MbaA, a GGDEF-EAL domain protein, previously characterized as a repressor of *V. cholerae* biofilm formation (9, 33). It was proposed that NspS acts as a sensor for norspermidine and that the interaction of a norspermidine-NspS complex with the periplasmic portion of MbaA reduces its ability to inhibit biofilm formation. This may occur through the destabilization of an MbaA dimer or through conformational changes affecting MbaA activity (33).

Subinhibitory concentrations of the antibiotic tobramycin have been shown to trigger biofilm formation in *P. aeruginosa* (27). This effect depends upon Arr (for aminoglycoside response regulator; PA2818), a membrane-associated EAL domain protein with a periplasmic domain. An *arr* mutant showed reduced membrane cyclic di-GMP phosphodiesterase activity but no apparent enhancement in biofilm formation in the absence of tobramycin. A variant Arr protein with a mutation in the conserved EAL domain was not able to restore tobramycin-induced biofilm formation to an *arr* mutant of *P. aeruginosa*. It is proposed that tobramycin, either directly or indirectly, enhances the phosphodiesterase activity of Arr, leading to cyclic di-GMP degradation and increased biofilm formation, through a localized effect on a discrete pool of cyclic di-GMP (27) (see below).

This effect of tobramycin is not due to the enhanced appearance antibiotic-resistant small variants that arise when *P. aeruginosa* is plated on a number of antibiotics (19). Such small-colony variants are also found in clinical samples from cystic fibrosis patients. Rough small-colony variants can revert to the wild-type phenotype in the absence of antibiotic. Intriguingly, a CheY-EAL domain two-component regulator, PvrR (for phenotype variant regulator), positively influences the rate of conversion (18), although the mechanistic basis of these phenotypic variations and the role of cyclic di-GMP are unknown.

Regulation by transcription and signal protein degradation. In addition to the modulation of the activity of the signaling proteins by ligand binding, cyclic di-GMP signaling is also subject to regulation at the level of transcription of the cognate genes and by signal protein degradation. For example, the transcriptional regulator CsgD of *Salmonella* regulates the transcription of *adrA*, a gene that encodes a GGDEF domain

FIG. 4. Cellulose synthesis in *Salmonella* is regulated by a hierarchical arrangement of cyclic di-GMP signaling systems. Two GGDEF-EAL domain proteins (STM3388 and STM2123) additively contribute to the expression of the transcriptional regulator CsgD. This protein regulates the transcription of *adrA*, which encodes a GGDEF domain protein that is directly implicated in the regulation of cellulose synthesis but not in the expression of *csgD* (32). These different systems can operate independently, perhaps by influencing discrete pools of cyclic di-GMP. The activities of the AdrA, STM3388, and STM2123 may also be influenced by environmental cues.

protein that is required for cellulose synthesis in the multicellular rdar phenotype (46). Interestingly, the expression of *csgD* is itself regulated by two further GGDEF-EAL cyclic di-GMP synthases that operate in a hierarchical fashion with respect to AdrA (32) (Fig. 4). Work with *P. aeruginosa* has revealed an environmental impact on the transcription of a gene encoding a GGDEF-EAL domain protein that is mediated by quorum sensing. Whole-genome microarray technology has identified a number of genes in *P. aeruginosa* whose expression responds to signals found in mucopurulent airway liquids collected from chronically infected cystic fibrosis patients (64). PA2567, encoding a cyclic di-GMP phosphodiesterase (50), is down-regulated by more than fivefold. This repression requires the *P. aeruginosa* quorum-sensing system, since these effects are not observed in an *rhIR lasR* double mutant (64).

Further regulation of cyclic di-GMP signaling occurs at the level of protein degradation. In *Yersinia pestis*, the Congo red binding Hms⁺ biofilm phenotype occurs at temperatures up to 34°C but not at higher temperatures. Biofilm formation depends upon the GGDEF domain protein HmsT as well as other Hms proteins (45). The cellular level of the HmsT protein is very low at 37°C, although transcription from the *hmsT* promoter or the level of *hmsT* mRNA is not significantly affected by growth temperature. However, HmsT at 37°C is sensitive to degradation by cellular proteases, thus contributing to the temperature regulation of the Hms⁺ phenotype (45).

Role of cyclic di-GMP signaling in virulence in animal and plant pathogens. It is now established that cyclic di-GMP signaling contributes to the pathogenesis of both animal and plant pathogens. In *Vibrio cholerae* and *Pseudomonas aeruginosa*, specific GGDEF and/or EAL domain proteins are implicated in virulence in different mouse models (35, 58, 60). In *Vibrio cholerae*, the CheY-EAL-HTH domain protein VieA positively activates the expression of the virulence genes *toxT*, which encodes a transcriptional regulator, and *ctxAB*, which encode cholera toxin (58, 59), and negatively influences exopolysac-

charide production and biofilm formation (57). A *vieA* mutant is attenuated for colonization in the infant mouse model (59). All of these effects require the cyclic di-GMP phosphodiesterase activity of VieA and are abolished by a mutation of the EAL amino acid motif.

In *Salmonella*, the “stand-alone” EAL domain protein CdgR is required for the bacterium to resist host phagocyte oxidase in vivo and contributes to virulence in mice (26). The HD-GYP domain regulator RpfG of the plant pathogen *Xanthomonas campestris* is a cyclic di-GMP phosphodiesterase that positively regulates the synthesis of extracellular enzyme virulence factors and motility, negatively regulates biofilm formation, and is required for full virulence in plants (14, 17, 50, 56).

These findings extend previous work that implicated specific EAL or GGDEF domain proteins in bacterial disease but that did not address their biochemical function. In *Bordetella pertussis*, the activation of virulence factors by the BvgAS two-component system is accompanied by the repression of the transcription of a further set of genes, which involves the “stand-alone” EAL domain protein BvgR (39, 40, 41). BvgR-mediated regulation of gene expression contributes to respiratory infection of mice (41). In *Vibrio anguillarum*, the GGDEF domain protein VirC contributes to virulence in fish (42).

The above-described examples illustrate that the effect of cyclic di-GMP on bacterial virulence is not restricted to those circumstances in which high levels promote biofilm formation; the synthesis of some virulence determinants is activated only under low cellular levels of the nucleotide. It is possible that bacteria within the population in infected tissue may adopt a planktonic, motile lifestyle in which virulence factors are expressed or a sessile lifestyle of residence in aggregates or biofilms, depending on the environmental conditions. The ability to undergo transitions between these physiological states may favor both the spread and persistence of bacterial disease within host tissues.

THE CELLULAR ORGANIZATION OF CYCLIC DI-GMP SIGNALING SYSTEMS

As outlined above, the large numbers of cyclic di-GMP signaling systems encoded by many bacterial genomes indicate that there must be considerable complexity in the organization of cyclic di-GMP signaling within a single organism. One pertinent issue is whether signaling systems are part of regulatory networks or are dedicated to specific cellular tasks. A related question is whether cyclic di-GMP exists as a general pool, as a set of discrete localized pools, or as both. We address these issues in the following sections.

Localization studies of the PleD regulator (CheY-CheY-GGDEF), which influences swarmer-to-stalk-cell transitions and pole development in *Caulobacter crescentus* (1), have shown that upon phosphorylation, the protein locates to the pole of the cell, where the new stalk will be formed (43). Phosphorylation also activates the protein for cyclic di-GMP synthesis (43). The EAL domain protein TipF of *C. crescentus* localizes to the division septum and the newborn pole after division (29). TipF is a flagellum assembly factor that relies on a second protein, TipN, for proper positioning. In the absence of TipN, flagella are assembled at ectopic locations, and TipF is mislocalized to such sites. The GGDEF-EAL domain pro-

tein FimX of *Pseudomonas aeruginosa* has also been shown to locate to a single pole of the cell (28), an effect that depends upon both the EAL and GGDEF domains (34). Previously, it was shown that the DgcA and PdeA proteins of *G. xylinus* copurified with the cellulose synthase (49). These findings have led to the suggestion that the alteration of localized pools of cyclic di-GMP by specific components in cyclic di-GMP signaling may activate processes that are determined by colocalizing proteins. In other words, certain cyclic di-GMP signaling systems are dedicated to specific cellular tasks.

An alternative, but not mutually exclusive, view is that a number of signaling systems form a surveillance network to integrate information about various aspects of the cellular environment and to process this information by determining a cellular level of cyclic di-GMP, which may influence bacterial functions. Observations that different GGDEF, EAL, or HD-GYP domain proteins have significant roles in the regulation of specific bacterial processes under different environmental conditions are consistent with the notion of an environmentally responsive network. This has been reported previously for the role of GGDEF domain proteins in cellulose synthesis in *Salmonella* (23, 46, 54).

More recent studies of *Salmonella* have revealed a hierarchical arrangement of cyclic di-GMP signaling in the regulation of cellulose synthesis (32). Two GGDEF-EAL domain proteins (STM3388 and STM2123) additively contribute to the expression of the transcriptional regulator CsgD. This protein regulates the transcription of *adrA*, which encodes a GGDEF domain protein that is directly implicated in the regulation of cellulose synthesis but not in the expression of *csgD* (32) (Fig. 4). These findings point to the coexistence of both networks of signaling systems and dedicated signaling systems in the same cell and indicate that different systems can operate independently, perhaps by influencing discrete pools of cyclic di-GMP. Interplay between different signaling systems in the regulation of the same phenotypes is evident from studies of *Vibrio cholerae*, where the actions of three cyclic di-GMP signaling proteins, CdgC, RocS, and MbaA, converge to regulate rugosity and polysaccharide production (36).

The existence of localized pools of cyclic di-GMP has been proposed previously to explain the varied effects of mutations of genes encoding GGDEF and EAL domain proteins on biofilm formation in *P. aeruginosa* (27, 35). In *P. aeruginosa* PAO1, the EAL domain protein Arr is required for biofilm formation in response to subinhibitory concentrations of the antibiotic tobramycin. This suggests that cyclic di-GMP degradation is required for biofilm formation, which contradicts the consensus view from work with a number of bacterial systems. Similarly, an examination of the effects of mutations of genes encoding other GGDEF and/or EAL domain proteins in *P. aeruginosa* PAO1 and PA14 reveals a complex relationship to biofilm formation (27, 35). In general, the results are consistent with the concept that enhanced cellular levels of cyclic di-GMP promote biofilm formation. There is, however, no strict correlation; for example, in *P. aeruginosa* PA14, a mutation of the gene encoding the GGDEF protein PA3343, which is active in cyclic di-GMP synthesis, leads to hyperbiofilm formation, whereas the overexpression of PA2870 and PA3343, which both lead to increases in the cellular level of cyclic di-GMP, has no effect on biofilm formation in the wild type (35). An added

complexity is that the nucleotide adopts different but related structures with different counterions and under different concentrations (65). A bimolecular intercalated structure found at low concentrations with Na^+ and Mg^{2+} is also the form in which cyclic di-GMP binds to PleD and may be the active form for signaling. At higher concentrations, in the presence of K^+ , octameric complexes form, which may serve to sequester cyclic di-GMP in an inactive state (65).

The findings for *P. aeruginosa* and *Salmonella* described above support the notion of localized effects of elements involved in cyclic di-GMP signaling, where synthesis or hydrolysis of the nucleotide is intimately related to its site of action (27, 32, 35). By inference, some functions contributing to biofilm formation in *P. aeruginosa* could be activated by low levels of cyclic di-GMP. However, if elements involved in cyclic di-GMP signaling form complexes with proteins that influence biofilm formation, the possibility that the loss or overexpression of the signaling component may adversely affect complex assembly and function cannot be overlooked. In this case, the function of the GGDEF/EAL domain protein may not be restricted to its action on cyclic di-GMP. It is clear that much research is needed to resolve the role of individual signaling systems, taking into account issues of cellular localization, interactions with other proteins, and the effects of gene disruption on specific cellular activities.

MECHANISM OF CYCLIC DI-GMP ACTION

Almost nothing is known about the mechanisms whereby cyclic di-GMP exerts its action on the diverse cellular functions under its control. The best-studied effect of cyclic di-GMP is its allosteric activation of cellulose synthesis in *G. xylinus*, where cyclic di-GMP was shown to bind to BcsB, the β subunit of cellulose synthase, and to an uncharacterized 200-kDa protein (37, 63). Recent bioinformatic studies have suggested that in contrast, cyclic di-GMP binds to BcsA, the α subunit of cellulose synthase, and that PilZ, a domain at the C terminus of BcsA, is part of the binding site (3). PilZ was originally described as being a protein involved in the assembly of functional pili in *Pseudomonas aeruginosa* (2). Several lines of evidence support the proposal that PilZ is also involved in the wider cellular activities of cyclic di-GMP. PilZ can be present as a "stand-alone" domain but can also be found associated with other domains including CheY, GGDEF, EAL, and HD-GYP, suggesting a role in regulation and signaling. The phylogenetic distribution of the PilZ domain is generally similar to those of GGDEF and EAL, and some phenotypes of mutations of genes encoding PilZ domain proteins are consistent with a role in cyclic di-GMP regulation (3, 28).

These bioinformatic predictions for the role of PilZ domain proteins are open to experimental verification. Indeed, very recent findings have shown that the PilZ domain protein YcgR of *Escherichia coli* can bind cyclic di-GMP (52). Furthermore, the isolated PilZ domain of YcgR and the PilZ domain from *G. xylinus* BcsA can also bind cyclic di-GMP, albeit with lower affinity (52). Studies of *E. coli* and *Salmonella* showed that YcgR regulates flagellum-based motility in a cyclic di-GMP-dependent manner. Proteins that interact with YcgR have, however, yet to be identified (52).

The full extent of cellular functions under the control of

cyclic di-GMP has been investigated by transcriptome or proteome analysis of bacteria in which the levels of cyclic di-GMP have been manipulated by mutation or ectopic expression of genes encoding GGDEF/EAL proteins and/or by the exogenous addition of cyclic di-GMP (7, 25, 38). An elevation in the level of cyclic di-GMP alters the expression of a substantial number of genes in *Pseudomonas aeruginosa* (25) and *Escherichia coli* (38). The *V. cholerae* response to an elevated level of c-di-GMP includes increases in the transcription of the *vps* genes encoding polysaccharide biosynthesis, *eps* genes involved in the extracellular protein secretion system, and *msh* genes required for mannose-sensitive hemagglutinin type IV pilus biogenesis as well as a decrease in the expression of *fla* genes required for flagellum biogenesis (7).

Although caution should be exercised when interpreting experiments where the ectopic expression of diguanylate cyclases or the addition of exogenous dinucleotide could lead to non-physiological levels of cyclic di-GMP in the cell, these observations indicate that cyclic di-GMP may exert a regulatory action at the level of transcription. This contention is supported by other findings. For example, deletion of the *rpfG* gene of *Xanthomonas campestris*, which encodes an HD-GYP domain regulator, leads to a reduction in the levels of transcript for *engXCA*, which encodes the major extracellular endoglucanase (56). A major question is whether these effects of cyclic di-GMP on gene transcription are direct or indirect. By analogy with cyclic AMP, cyclic di-GMP may affect transcription by binding to transcriptional regulators, although as far as we are aware, there have been no descriptions of such binding. An alternative view is that cyclic di-GMP works primarily at the posttranslational level and that effects on transcription are indirect. Only further experimental work will resolve these issues.

CONCLUDING REMARKS

The appreciation of the role of cyclic di-GMP as an important and almost ubiquitous signaling molecule in bacteria has built upon the seminal findings of the group of Moshe Ben-Ziman both in the discovery of cyclic di-GMP in 1987 and in the cloning of genes encoding proteins involved in cyclic di-GMP turnover in 1998. Subsequently, the biochemical roles of domains involved in the turnover of cyclic di-GMP have been defined, and proteins with GGDEF/EAL/HD-GYP domains have been implicated in a number of important aspects of bacterial behavior. Despite this significant progress, there are many questions that remain unanswered. Principal among these is the mechanism(s) by which cyclic di-GMP exerts its action on different cellular functions, about which almost nothing is known. We might also expect that investigations of the cell biology and spatial aspects of the various signaling systems will come to the fore, as researchers address the issue of specificity of individual signaling systems. The ultimate goal of this research is a deeper understanding of a signaling system whose importance to the lifestyle of diverse bacteria is now emerging.

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