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Roles of Cyclic Diguanylate in the Regulation of Bacterial Pathogenesis

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Abstract

Cyclic diguanylate (c-di-GMP) is a bacterial second messenger of growing recognition involved in the regulation of a number of complex physiological processes. This review describes the biosynthesis and hydrolysis of c-di-GMP and several mechanisms of regulation of c-di-GMP metabolism. The contribution of c-di-GMP to regulating biofilm formation and motility, processes that affect pathogenesis of many bacteria, is described, as is c-di-GMP regulation of virulence gene expression. Finally, ways in which c-di-GMP may mediate these regulatory effects are proposed.

Keywords

second messenger; biofilm; motility; virulence; life cycle

INTRODUCTION

Classical cytoplasmic second messengers relay signals received at the cell surface (i.e., the first messengers) to target molecules within the cell. Often, this process allows the amplification of the original signal into an intracellular signal capable of eliciting massive biochemical changes in the cell. Nucleotides, such as cyclic AMP (cAMP), cyclic GMP (cGMP), and guanosine 5'-diphosphate 3'-diphosphate (ppGpp), are employed in this capacity and are some of the best-characterized intracellular signaling molecules.

c-di-GMP: cyclic diguanylate

Biofilm: surface-attached community of microorganisms embedded in an extracellular matrix

Nucleotide second messengers have been extensively studied, primarily for their regulatory functions in eukaryotic cells, yet some play critical roles in regulating basic processes in bacteria. For example, cAMP allosterically activates the catabolite regulation protein, a transcriptional regulator of genes involved in the use of alternative carbon sources and other cellular functions (26). Other second messengers, such as cGMP, are commonly used in eukaryotes but have little or no use in bacteria. Conversely, other second messengers are utilized almost exclusively by bacteria. The polyphosphate nucleotide ppGpp, although

sometimes present in lower eukaryotes, predominantly functions in the regulation of the bacterial stringent response to nutrient limitation (36).

The cytoplasmic second messenger cyclic diguanylate (c-di-GMP) (Figure 1) is used by most bacteria, but not eukaryotes or Archaea, to regulate a myriad of biological processes. In recent years, a burst of interest in this molecule has led to the discovery of complex systems for regulating the intracellular concentration of c-di-GMP, and an equally multi-farious set of regulatory targets of c-di-GMP. Cellular functions regulated by c-di-GMP include cell-cell signaling, biofilm formation, motility, differentiation, and virulence. This review focuses on c-di-GMP-mediated regulation of these processes and how such regulation pertains to elaboration of disease. The bacteria discussed have diverse niches in the environment and the host and so likely experience a variety of signals in these milieus. It is becoming apparent that many of these signals can feed into the regulation of c-di-GMP concentration, in turn affecting the above phenotypes. Therefore, mechanisms of regulation of c-di-GMP level are described as well. In some cases the extracellular stimuli that result in changes in c-di-GMP are known or predicted, but in most cases these first messengers have not been identified.

BIOSYNTHESIS AND HYDROLYSIS OF c-di-GMP

c-di-GMP was first identified as an allosteric activator of cellulose synthase in the grape-associated bacterium *Gluconacetobacter xylinus*, and it was in this context that the c-di-GMP biosynthetic and hydrolytic enzymes were first identified (65). Later, the diguanylate cyclase (DGC) and phosphodiesterase (PDE) enzymes capable of synthesizing and degrading c-di-GMP were purified, and reverse genetics was used to identify their corresponding genes (74). Each DGC and PDE identified in *G. xylinus* contained two conserved domains, termed GGDEF and EAL, based on conserved amino acid residues. These domains were determined to be present in other bacterial proteins (74); subsequently, the phylogenetic distribution of genes encoding GGDEF and EAL domains was shown to be wide in bacteria, but absent in Archaea or Eukarya, suggesting that c-di-GMP is limited to bacteria (21).

GGDEF: conserved protein domain with DGC activity

DGC: diguanylate cyclase

EAL: conserved protein domain with PDEA activity

PDEA: phosphodiesterase A

PDE: phosphodiesterase

HD-GYP: conserved protein domain with PDE activity

EPS: extracellular polysaccharide

PilZ: conserved protein domain that binds c-di-GMP

Multiple GGDEF and EAL domain proteins have been analyzed biochemically. The biosynthesis and degradation of c-di-GMP by these proteins are depicted in Figure 1. The GGDEF domain catalyzes the formation of c-di-GMP from two GTPs; the GG(D/E)EF motif itself is the active site, and mutation of any residue in GG(D/E)E abolishes enzymatic activity (12,69). Most GGDEF domains contain an I-site immediately N-terminal to the active site, defined as an RxxD motif, allowing noncompetitive product inhibition by dimeric c-di-GMP (14). Allosteric inhibition of DGC activity by the reaction product limits the final concentration of c-di-GMP and may prevent the depletion of GTP. c-di-GMP is hydrolyzed by EAL domain phosphodiesterase A (PDEA), resulting in the linear molecule 5'-pGpG, which is presumably (though not necessarily) biologically inactive and can be rapidly hydrolyzed by other PDEs in

the cell (13,15,70,75). The EAL domain requires Mg^{2+} or Mn^{2+} for activity, but it is strongly inhibited by Ca^{2+} or Zn^{2+} . The glutamic acid of the EAL motif itself is predicted to participate in Mg^{2+} coordination (21). The active site of PDEA remains unknown, although there are several candidate residues involved on the basis of conservation of key motifs within EAL domains with confirmed PDEA activity (70).

Another protein domain, HD-GYP, has a phylogenetic distribution mirroring that of GGDEF and EAL domains and was recently shown to hydrolyze c-di-GMP (67). The HD-GYP domain is unrelated to the EAL domain and belongs to the metal-dependent phosphohydrolase superfamily; unlike the EAL domain, HD-GYP activity degrades c-di-GMP completely to two GMPs. Herein, while PDEA refers specifically to EAL domain activity, PDE includes both EAL and HD-GYP hydrolytic activities. The significance of the presence of two unrelated domains capable of hydrolyzing c-di-GMP in the same genomes is uncertain.

REGULATION OF c-di-GMP BIOSYNTHESIS AND DEGRADATION

Because c-di-GMP regulates processes critical to many aspects of bacterial survival in diverse and often dynamic environments, it stands to reason that the concentration of this second messenger is subject to tight control. Accordingly, there is evidence that DGC and PDE activities are modulated at multiple levels, including allosterically, by phosphorylation of other domains in the protein, by localization to microdomains within the cell, and at the levels of transcription and protein stability.

Regulation of c-di-GMP by Interaction of Tandem GGDEF and EAL Domains

One of the most intriguing facets of c-di-GMP metabolic enzymes is the modular nature of the protein structures. Although some proteins are composed of GGDEF or EAL domains alone, most are present in conjunction with other conserved domains. The presence of additional input domains provides multiple mechanisms by which the activities of the GGDEF, EAL, and HD-GYP domains can be controlled to elicit the appropriate c-di-GMP-regulated physiological changes. Add to this the fact that bacteria often encode multiple copies of GGDEF, EAL, and HD-GYP domains with different combinations of input and output domains, and the potential modes of regulation of c-di-GMP synthesis and degradation become numerous. For example, *Pseudomonas aeruginosa* encodes 17 GGDEF, 5 EAL, 3 HD-GYP, and 16 GGDEF-EAL domain proteins (21). *Vibrio cholerae* encodes 31 GGDEF, 22 EAL, 9 HD-GYP, and 10 GGDEF-EAL proteins (21). These and other bacteria that have an abundance of these domains have in common the ability to survive in a variety of environmental niches. Thus, c-di-GMP likely aids in the ability to adapt to changing environmental conditions.

GGDEF and EAL (or HD-GYP) domains are frequently found in tandem in the same protein. The interplay between the two domains potentially provides new modes of regulation of c-di-GMP metabolism. For example, one GGDEF-EAL protein, CC3396, in the nonpathogenic bacterium *Caulobacter crescentus* has PDEA activity in vitro; the active site of the GGDEF domain is altered (GEDEF) and thus lacks DGC activity but can still bind GTP (15). Binding of GTP to the GEDEF domain activates the PDEA activity of the EAL domain by lowering the K_m for c-di-GMP to a physiologically relevant level. This mechanism of regulation likely functions in GGDEF-EAL proteins of pathogenic bacteria as well (37,43). In other GGDEF-EAL proteins, the relationship between the two domains is less clear. Although one activity tends to prevail in the full-length protein, the dominant activity cannot always be predicted from the primary sequences of the domains.

Regulation of Activity by Input Domains

Other domains often present in conjunction with GGDEF, EAL, and HD-GYP domains include sensory and regulatory modules such as PAS, GAF, HAMP, REC, and HTH domains (21). Therefore, the potential input signals for the regulation of enzymatic activity are diverse. For example, the PAS domain has been implicated in sensing light, redox potential, or oxygen, often through an associated cofactor such as heme or flavin (23). The first PDEA to be characterized in vitro, *G. xylinus* PdeA1, has a PAS domain that contains heme (13). The reversible binding of O₂ to this cofactor regulated the PDEA activity of the EAL domain. Although *G. xylinus* itself is not pathogenic to humans, the arrangement of PAS-GGDEF and/or EAL domains is common in pathogens such as the FimX protein, which governs twitching motility of *P. aeruginosa* (33,37). The GAF domain, although it has not been investigated in the context of GGDEF and EAL domains, may modulate their activities. The GAF domain in a *Saccharomyces cerevisiae* PDE unrelated to c-di-GMP metabolism binds cGMP (32); although cGMP is not found in bacteria, it is feasible that other small molecules such as c-di-GMP or cAMP bind the bacterial GAF domain and modulate c-di-GMP synthesis or degradation.

In addition, many GGDEF, EAL, and HD-GYP domain proteins contain REC and/or HTH domains characteristic of response regulator components of bacterial signal transduction systems. In the GGDEF domain protein Rrp1 of *Borrelia burgdorferi*, the cause of Lyme disease, phosphorylation of the REC domain is required for DGC activity (69). The genetic context of *rrp1* suggests a possible source of phosphate: *rrp1* is predicted to be cotranscribed with a sensor histidine kinase, Hpk1, the likely source of phosphorylation of Rrp1. This would provide a mechanism by which activation of the sensor by extracellular signals can directly control the enzymatic activity of the cognate response regulator and thus the production of c-di-GMP. However, the presence of other domains does not necessarily imply direct regulation of enzymatic activity. In *V. cholerae*, the cause of cholera, the PDEA VieA contains REC and HTH domains flanking an EAL domain, yet phosphorylation has no effect on in vitro PDEA activity (R. Tamayo & A. Camilli, unpublished data).

PAS: conserved protein domain involved in sensing oxygen, redox, or light and named for its presence in PER, ARNT and SIM eukaryotic proteins

GAF: cGMP binding domain named for its presence in eukaryotic cGMP-regulated cyclic nucleotide phosphodiesterases, certain eukaryotic adenylyl cyclases, and the bacterial transcription factor FhlA

REC: phosphoreceiver domain

HTH: helix-turn-helix

Hms: heme storage

Regulation of c-di-GMP Metabolism by Altered Enzyme Stability

An alternative, perhaps underappreciated, mode of regulation of c-di-GMP metabolism involves altering the stability of DGC and PDE enzymes. In *Yersinia pestis*, the cause of plague, a set of six proteins controls heme storage (Hms) (63). Hms occurs below 34°C and is required for colonization of the flea, the insect vector of *Y. pestis* (9,30). Three of the Hms proteins, including HmsT, a putative DGC, are regulated by temperature (62). Whereas *hmsT* mRNA level remains unchanged between 26°C and 37°C, HmsT protein is degraded by Lon and ClpPX proteases at 37°C.

Regulation of c-di-GMP by Controlling Localization of Enzymes

There is evidence that bacteria may regulate c-di-GMP metabolism by delimiting the biosynthetic and degradative enzymes into microdomains within the cell. Eukaryotic cells can localize cAMP in such a manner (64). Some evidence of localization is indirect, such as the fact that the *G. xylinus* DGC and PDEA proteins copurify with cellulose synthase in the membrane fraction (66). Other studies provide direct evidence, most notably the localization of *C. crescentus* PleD, a REC-GGDEF domain protein, from a diffuse cytoplasmic location to the flagellated pole of cells undergoing differentiation from a swarmer to a stalked cell (1, 61). Both the localization and DGC activity of PleD require phosphorylation of the REC domain (61). DGC activity is needed for ejection of the flagellum, stalk formation, and synthesis of the holdfast (1,49). Thus, this mechanism spatially and temporally restricts the activity of the DGC domain to specifically modulate the nature of the cell pole. Spatial localization of c-di-GMP may turn out to be the norm and not the exception.

Tfp: type IV pili

Regulation of Transcription of *dgc* and *pde* Genes

Control of DGC and PDE expression at the transcriptional level is likely to be a major mode of c-di-GMP regulation in bacteria. In *V. cholerae*, VieA, a dual function protein with independent transcriptional activator and PDEA activities, is coexpressed with the putative cognate sensor histidine kinase VieS. Expression of *vieSA* is autoregulatory (46) and is likely dependent on phosphorylation of VieA in order to activate its transcriptional regulatory activity. Other *dgc* and *pde* genes in *V. cholerae* are regulated at the transcriptional level as well (51,86).

The diversity of mechanisms of regulation of DGC and PDE activity indicates that tight spatial and temporal management of c-di-GMP in the cell is critical for proper control of downstream effects. Future genetic and biochemical studies of the plethora of DGC and PDE proteins may elucidate additional modes of regulating the expression and activity of these enzymes.

PHENOTYPES REGULATED BY c-di-GMP AND THEIR ROLES IN PATHOGENESIS

Although the genes and proteins affected by c-di-GMP vary between bacterial species, one common theme that has emerged is that c-di-GMP activates biofilm formation while inhibiting motility, thus regulating the transition between sessile and motile lifestyles. The effects of c-di-GMP on biofilm formation and motility have major repercussions on the ability of a number of pathogens to cause disease. In addition, c-di-GMP also affects the expression of virulence factors. The clearest pictures of c-di-GMP regulation of pathogenicity have developed in *P. aeruginosa*, *Y. pestis*, *V. cholerae*, and *Salmonella enterica* serovar Typhimurium.

Regulation of Motility by c-di-GMP

c-di-GMP inhibits bacterial locomotion of various types, including swimming, swarming, and twitching motility. These mechanisms of movement are mediated by the functions of different surface organelles: Swimming abilities are provided by flagella; swarming motility across surfaces is aided by flagella in some bacteria; and twitching motility across surfaces is provided by a cycle of extension, attachment, and retraction of Type IV pili (Tfp) (55).

The first direct evidence of c-di-GMP regulation of twitching motility came from mutation of the *P. aeruginosa fimX* gene, which encodes a protein containing REC, PAS, GGDEF, and EAL domains (33). Production of pilin subunits remained normal in the *fimX* mutant, but fewer

pili were assembled on the cell surface. Tfp are localized to one pole of *P. aeruginosa* (53), and a fluorescent FimX fusion protein similarly localizes to a single pole by virtue of a localization sequence adjacent to the REC domain (33). Biochemical studies showed that FimX could degrade c-di-GMP but not synthesize it (37). By removing the PDEA activity of FimX, and thus increasing c-di-GMP in the cell, twitching motility was inhibited. However, mutation of the degenerate GGDEF motif, GDSIF, to AASIF negatively affected PDEA activity, implying an important role for the GGDEF domain in regulating motility. The authors hypothesize that the GDSIF domain regulates the PDEA domain, possibly by binding GTP as described for CC3396 (see above).

Twitching motility of *P. aeruginosa* can be affected by other enzymes controlling the c-di-GMP level. The Wsp chemosensory system includes WspR and WspF, which are response regulator-like proteins with DGC and CheB-like methylesterase activities, respectively (28). Whereas loss of WspR has no phenotype, a *wspF* mutation results in increased biofilm formation, decreased twitching motility, and decreased swimming (17,28). A *wspR* mutation in the *wspF* mutant background suppressed all three phenotypes. The authors proposed that mutation of *wspF* causes constitutive activation of WspR and, as a result, elevated the c-di-GMP level (17). The extracellular signals that activate the Wsp system are unknown, but activation of WspR DGC activity promotes sessility by inhibiting multiple types of motility and activating biofilm formation (17,28).

Downregulation of flagellar motility by c-di-GMP also has been demonstrated in *Salmonella* Typhimurium and *V. cholerae*. In *Salmonella* Typhimurium, ectopic expression of a DGC, AdrA, significantly inhibited both swarming motility and swimming; conversely, expression of the PDEA YhjH enhanced these phenotypes (72). The same pattern of regulation was seen when AdrA or YhjH was expressed in *P. aeruginosa* or *Escherichia coli*. The equivalent experiments were done in *V. cholerae*: overexpression of the DGC VCA0956 abolished swimming, whereas expression of the PDEA VieA greatly enhanced it (6). In the classical biotype of *V. cholerae*, a VieA PDEA mutant has an increased level of c-di-GMP and lacks motility (77; A. Tischler & A. Camilli, unpublished data). Accordingly, transcriptional profiling showed a clear repression of genes involved in flagellum biosynthesis, motility, and chemotaxis in *V. cholerae* with ectopically increased c-di-GMP (6).

It is evident that bacteria can regulate different types of motility through regulation of DGC and PDE activity. Because motility commonly contributes to pathogenesis, often necessary in early steps of colonization of the host, the c-di-GMP-mediated regulation of this process is important for pathogenesis. A case in point is twitching motility of *P. aeruginosa*, an opportunistic pathogen that causes chronic lung infections in cystic fibrosis patients. First, the Tfp themselves are major adhesions that aid in colonization of host epithelial cells and play a role in virulence in animal models of infection (18,84,89). Second, mutations that abolish twitching motility, but still allow surface expression of Tfp, caused reduced cytotoxicity to epithelial cells and decreased adherence (7,16,83). In addition, although these nonmotile mutants still colonized the lung and caused mortality, they had decreased colonization of the liver in a mouse model of acute pneumonia (16). Consistent with its role in regulating twitching motility, the FimX mutant, which had decreased surface-expressed Tfp and twitching motility owing to loss of PDEA activity, exhibited dramatically decreased cytotoxicity toward tissue culture cells but had no defect in virulence in a mouse model of pneumonia (33,37).

VPS: *Vibrio* exopolysaccharide

Regulation of Biofilm Formation by c-di-GMP

Another important process regulated by c-di-GMP is the production of extracellular polysaccharides (EPS), specifically those that serve as an extracellular matrix for formation and support of biofilm architecture. Biofilms are complex communities of one or more species of microorganisms attached to a surface and to one another embedded in a hydrated, often polysaccharide matrix. c-di-GMP activates biofilm formation in a variety of bacteria, including many pathogens such as *P. aeruginosa*, *Salmonella* Typhimurium, *Vibrio* spp., and *Y. pestis* (22,28,39,72,77).

c-di-GMP activates the production of different EPS components depending on the species. *Vibrio* exopolysaccharide (VPS) is used by *V. cholerae* to form one type of biofilm. Enzymes for biosynthesis of VPS are encoded by two operons that are under the control of two transcriptional activators, VpsR and VpsT (11,85). Mutation of the PDEA gene *vieA* in the classical biotype is sufficient to dramatically increase transcription of *vps* genes and augment biofilm formation (77). Later work in the El Tor biotype, which is responsible for all current cholera cases, showed that overexpression of the DGC VCA0956 similarly increased biofilm formation, and conversely overexpression of the VieA PDEA impeded biofilm development (6). Regulation of VPS biosynthesis by c-di-GMP occurs through transcriptional activation of *vpsR* and *vpsT* (6,77).

Rugose: wrinkly colonies due to hyperproduction of extracellular polysaccharide

For production of VPS-dependent biofilm, *V. cholerae* uses a complex set of intersecting signaling pathways. These pathways include phase variation between smooth and rugose colony morphologies, signaling through the sodium-driven flagellar motor, and quorum sensing (25,45,82,87). There is evidence that c-di-GMP can regulate all three of these mechanisms, implying that c-di-GMP is a major factor controlling biofilm formation.

Flagella are essential for initiation of *V. cholerae* biofilm formation but interfere with development of a mature biofilm (56,81). *V. cholerae* becomes nonmotile during the early (monolayer) stage of biofilm formation (81). The results of one study suggest that signaling through the flagellar motor regulates VPS production, potentially by sensing changes in motility or the presence or absence of a flagellum (45). Because biofilm is activated by c-di-GMP while motility and flagellar gene expression are repressed, it is tempting to speculate that, analogous to regulation of the motility to virulence transition, c-di-GMP can mediate the transition between a motile, planktonic lifestyle and a sessile, community-based existence.

Taken together, there is strong evidence that *V. cholerae* uses c-di-GMP to increase *vps* transcription and augment biofilm formation. Factors other than motility and VPS that contribute to biofilm formation may be regulated by c-di-GMP. For example, the mannose-sensitive hemagglutinin Type IV pilus, MSHA, is involved in attachment of *V. cholerae* to abiotic surfaces in the initial steps of biofilm development (80,81); the MSHA biosynthetic operon includes a GGDEF-EAL domain gene with putative PDEA activity (20).

P. aeruginosa also uses c-di-GMP to regulate biofilm formation, which in this organism involves multiple factors, including EPS production, chemotaxis, quorum sensing, and twitching motility. *P. aeruginosa* EPS can be composed of alginate, a known virulence factor often associated with bacteria isolated from the cystic fibrosis lung, as well as other polysaccharides (19,24,35). As mentioned above, activation of the DGC WspR through loss of WspF increased biofilm formation (28). Presumably this is due to increased expression of the *psl* and *pel* operons, which contribute to EPS and biofilm production (19,35). In addition, a comprehensive study that analyzed phenotypes associated with mutations in all putative

DGC- and PDEA-encoding genes of *P. aeruginosa* found a strong correlation between high intracellular c-di-GMP and hyperbiofilm formation (43).

Regulation of biofilm development in *P. aeruginosa* incorporates c-di-GMP as a signaling molecule at different stages of maturation. The SadARS three-component signal transduction system includes a response regulator, SadR, with an EAL domain and putative PDEA activity. A transposon insertion negatively affecting transcription of *sadRS* resulted in increased biofilm at an early stage of development (42), consistent with increased c-di-GMP enhancing biofilm formation. Deletion of *sadARS* genes, however, led to a reduction of early-stage biofilm formation. Thus, regulation of *P. aeruginosa* biofilm development by c-di-GMP is complex. Dispersion of bacteria from a mature biofilm is another target for c-di-GMP regulation. Indirect evidence of this in *P. aeruginosa* comes from analysis of a mutation in BdlA, a PAS domain-containing chemotaxis regulator (57). The BdlA mutant had elevated c-di-GMP and showed defective detachment in response to environmental signals such as sudden changes in nutrient availability (57). Whereas BdlA itself lacks DGC or PDE function, BdlA may activate a PDE or repress a DGC, accounting for the increased c-di-GMP in the BdlA mutant.

Twitching motility is also essential for biofilm formation in *P. aeruginosa* (40,41,60). This seemingly contradicts the results of the WspR studies that showed an inverse effect of c-di-GMP on biofilm production and twitching motility. It is possible that c-di-GMP is maintained at a level high enough to promote the initial stages of biofilm maturation, such as aggregation into microcolonies, which requires twitching motility (60,73), but is then reduced to allow the biofilm to mature.

In *Y. pestis*, an EAL domain protein, HmsP, and a GGDEF domain protein, HmsT, inversely control Hms-dependent biofilm formation (39). HmsT positively regulates biofilm formation and expression of the hemin storage operon *hmsHFRS*, which is predicted to encode genes required for EPS production. Conversely, HmsP represses biofilm and *hmsHFRS* expression. The effects on biofilm and Hms were attributed to putative DGC and PDEA activities of HmsT and HmsP, respectively. Consistent with this, a point mutation in the HmsT GGDEF domain reduced biofilm formation, and an equivalent mutation in the EAL motif of HmsP dramatically increased biofilm formation (39).

The contribution of biofilm formation to pathogenesis varies with the biology and lifestyle of the pathogen; therefore there is not always a direct relationship between the ability of the bacteria to grow as a biofilm and to cause disease in humans. The role of Hms in *Y. pestis* pathogenesis is intriguing. Hms was found to have no effect on virulence in a mouse model of plague (50), suggesting no role for Hms, nor presumably for c-di-GMP, in virulence in humans. However, Hms and biofilm formation are essential for colonization and blockage of the flea proventriculus, a food storage and predigestion compartment (30). Colonization of the flea is essential for transmission of plague to humans (4,5). In addition, as discussed above, the HmsT DGC is more susceptible to proteolysis at 37°C, suggesting a mechanism by which c-di-GMP and consequently biofilm formation are reduced in a mammalian host.

Two-component signal transduction system: sensor histidine kinase and response regulator phosphorelay

In *V. cholerae*, biofilm formation and virulence are inversely related. *V. cholerae* that overproduce VPS are defective in the production of virulence factors such as cholera toxin (CT), the primary cause of the profuse diarrhea in humans, and in infectivity (27,45,82). Although biofilm somehow enhances the initial colonization by *V. cholerae* (88), biofilm formation itself is thought to be most critical for the maintenance of *V. cholerae* in the aquatic environment. There is evidence that *V. cholerae* forms biofilms on the chitinous exoskeletons

of aquatic organisms such as copepods (34,76). It has been hypothesized that biofilm may enhance colonization of the human host by protecting the ingested *V. cholerae* from antimicrobial conditions in the upper gastrointestinal tract (88).

Regulation of Virulence Gene Expression

Not only has c-di-GMP been shown to regulate phenotypes that affect pathogenesis indirectly, such as biofilm formation and motility, c-di-GMP can also directly modulate virulence properties and virulence factor expression. The effect of c-di-GMP on virulence gene expression is best understood in *V. cholerae*, where it has been demonstrated to inhibit virulence gene expression. The *vieSAB* operon, which encodes the VieSA putative two-component signal transduction system containing the VieA response regulator/PDEA, was identified in two different genetic screens: *vieB* was identified in a screen for genes induced during infection of infant mice, and *vieS* was identified in a screen for positive regulators of CT (10,47). VieA is required for virulence in the mouse and for virulence gene expression in vitro (78,79). Specifically, VieA was required for full expression of *toxT*, which encodes a transcriptional activator of toxin-coregulated pili, the major colonization factor of *V. cholerae*, and CT (27,29,54). The detrimental effect of the VieA mutation on virulence gene expression was attributed to loss of VieA PDEA activity and thus to increased intracellular c-di-GMP in the mutant. Consistent with this, inhibition of CT production in vitro could be stimulated by ectopic expression of a DGC (78).

These data provide an interesting model by which *V. cholerae* can transition from an environmental state, such as biofilm, to survival and colonization in the host (Figure 2). Upon entering the small intestine, host signals activate the VieSA system, leading to increased expression of VieA PDEA and a reduction in intracellular c-di-GMP, allowing for optimal virulence gene expression (see above). Other PDE may also be activated in vivo (59), or alternatively, DGC activity may be repressed. Because cholera inevitably involves dissemination of bacteria back into the environment, and because *V. cholerae* exiting patients via watery stool have turned off virulence gene expression (44,58), c-di-GMP may be increased at a late stage of infection, perhaps giving the bacteria some advantage upon exiting the host.

Also showing an inhibitory effect of c-di-GMP on virulence is a study in *Salmonella* Typhimurium in which genes essential for virulence were screened for in a mouse model of systemic infection. This screen led to the identification of an EAL domain-encoding gene, *ydiV* (*cdgR*) (31), that appears to play a role in bacterial survival of killing by host phagocyte oxidase. The *cdgR* mutant had increased c-di-GMP relative to the wild type, suggesting c-di-GMP was inhibitory to mechanisms of resistance to reactive oxygen species. The levels of the major enzymes involved in this resistance, catalase and alkyl-hydroperoxide reductase (71), were unaffected, so the target of c-di-GMP regulation in this case remains unknown.

A systematic study of DGC and PDEA in *P. aeruginosa* uncovered a similar trend of c-di-GMP inhibition of virulence. Mutations in two PDEA genes, *pvrR* and PA3947, attenuated pathogenicity of *P. aeruginosa* in a murine model of pneumonia, and two mutations in GGDEF-EAL domain proteins with degenerate GGDEF domains and predicted PDEA activity also reduced pathogenicity (43). However, one DGC mutant, PA4332, also had reduced virulence; therefore regulation of virulence by c-di-GMP in this organism may not be straightforward. In addition, the panel of DGC and PDEA mutants was tested for Type III secretion-mediated cytotoxicity to epithelial cells, which is often indicative of a role in pathogenesis. No clear connection between changes in c-di-GMP and cytotoxicity could be made, as both a DGC and a PDEA were defective in this assay.

A number of studies using genetic screens or transcriptional profiling have implicated c-di-GMP in the regulation of virulence of a number of other bacteria, including *Legionella*

pneumophila, *Brucella melitensis*, *Bordetella pertussis*, and *Vibrio vulnificus* (3,8,38,48). Overall, there is abundant evidence that c-di-GMP represses virulence in a number of bacteria by affecting a variety of pathways, in some cases inhibiting expression of virulence genes. Yet there is evidence that DGC enzymes also contribute to infection. Thus regulation of c-di-GMP and its control of pathogenicity involve a complex interaction of DGC, PDE, and the regulatory targets of c-di-GMP.

MODES OF ACTION OF c-di-GMP

With the exception of allosteric regulation of cellulose synthase in *G. xylinus* and DGC enzymes containing the I-site, the means by which c-di-GMP regulates physiological processes is unknown. It has been hypothesized that protein sensors of c-di-GMP must exist that, upon binding c-di-GMP, mediate output activities such as targeted proteolysis, protein complex formation, or changes in gene expression. Several recent reports lend weight to this hypothesis.

Type III secretion: proteinaceous pore extending across bacterial membranes and host cell membrane through which virulence proteins are transported into host cells

Based on the c-di-GMP-binding α -subunit of *G. xylinus* cellulose synthase, Amikam & Galperin (2) reported the in silico identification of a putative c-di-GMP binding domain. The domain was named PilZ for the *P. aeruginosa* protein by the same name, which is required for assembly of functional Tfp. The PilZ domain has a phylogenetic distribution generally similar to those of GGDEF and EAL domain proteins. Like GGDEF, EAL, and HD-GYP, the PilZ domain is found in a variety of protein configurations: singularly, in tandem, and with an assortment of additional domains including REC, HTH, GGDEF, and EAL domains.

Several recent studies have demonstrated c-di-GMP binding by PilZ domain proteins from different bacteria. YcgR is one of two PilZ proteins encoded by *E. coli*, and the other is cellulose synthase. YcgR specifically binds c-di-GMP, and its PilZ domain alone was sufficient for c-di-GMP binding (68). Mutation of the YcgR PilZ domain revealed that not all highly conserved residues are required for c-di-GMP binding. Mutation of conserved residue R118 blocked c-di-GMP binding completely, whereas mutation of S147 improved binding. Consistent with a role in c-di-GMP-regulated processes, YcgR is involved in swimming in *E. coli* and *Salmonella* Typhimurium (68). Specifically, deletion of the PDEA *yhjH* greatly reduces swimming in either bacterium, but deletion of *ycgR* in the Δ *yhjH* background restores motility to 80% of wild type. Complementation of Δ *yhjH* Δ *ycgR* with YcgRS147A restores the reduced motility phenotype, but complementation with YcgRR118D does not, suggesting that the ability to bind c-di-GMP is critical to YcgR function as a regulator of motility in *E. coli* and *Salmonella* Typhimurium.

Riboswitch: part of messenger RNA that can bind a small target molecule and modulate translation and/or stability of the transcript

In *C. crescentus*, two PilZ domain proteins, DgrA and DgrB, bind c-di-GMP, and binding was dependent upon conserved PilZ domain residues (14a). Deletion of *dgrA* or *dgrB* restored motility in a strain lacking motility owing to an ectopically elevated c-di-GMP. Additionally, overexpression of either PilZ protein in the wild-type background reduced motility, but overexpression of PilZ domain point mutants incapable of binding c-di-GMP did not. Taken together, these data indicate that DgrA and DgrB negatively regulate motility in a PilZ domain-dependent manner. Overexpression of either PilZ protein did not affect transcription of genes involved in flagellar biosynthesis. Instead, overexpression of DgrA negatively affected the

level of FliL, a protein required for flagellar rotation, providing a posttranscriptional mechanism by which c-di-GMP can regulate motility.

Two of the five PilZ proteins encoded by *V. cholerae*, PlzC (VC2344) and PlzD (VCA0042), specifically bind c-di-GMP in vitro (63a). PlzC contains tandem PilZ domains, and PlzD is similar in structure to YcgR with an unknown N-terminal domain and a C-terminal PilZ domain. Mutational analysis of PlzD confirmed that several conserved PilZ domain residues are required for c-di-GMP binding. Additionally, PlzC and PlzD are involved in the c-di-GMP-regulated processes of biofilm formation and virulence. Mutation of the PDEA *VieA* results in increased biofilm formation, but deletion of *plzC* in the *VieA* mutant background abrogated this effect and restored biofilm formation to the level of wild type. Thus PlzC is a positive regulator of biofilm formation when the concentration of c-di-GMP is high. The role of PilZ domain proteins in virulence of *V. cholerae* is more complex. Deletion of either *plzC* or *plzD* had no effect on colonization of the infant mouse small intestine, but deletion of both led to a significant decrease in colonization, indicative of redundancy. A similar decrease in colonization was observed when a conserved PilZ domain residue required for c-di-GMP binding by PlzD was mutated in the $\Delta plzC$ background ($\Delta plzC plzDR140A$), suggesting that this attenuation was dependent on the PilZ domain.

The discovery of the PilZ domain provides a starting point for understanding the mechanisms of c-di-GMP-mediated regulation, but much remains to be elucidated. Thus far, there have been no reports suggesting how c-di-GMP binding alters PilZ protein activity, nor is there any evidence to suggest a model of how specific PilZ domain proteins act within the c-di-GMP regulatory circuit. Therefore, additional work is required to understand how these proteins function.

While the PilZ domain may be one pathway by which c-di-GMP exerts control over bacterial processes, there likely are additional c-di-GMP binding domains or molecules, as evidenced by the fact that not all c-di-GMP-producing bacteria encode PilZ domains. One additional mechanism of c-di-GMP activity that can be envisaged is binding to a riboswitch that then controls translation of specific mRNA transcripts. Although no such riboswitch has been identified as yet, one specific for guanine has been identified and shown to regulate purine metabolism and transport (52). If a riboswitch regulatory mechanism exists, it could function as an autoregulator whereby c-di-GMP binds to a DGC mRNA and blocks translation, leading to reduced c-di-GMP production. Alternatively, such a riboswitch could function to regulate expression of genes involved in other processes.

CONCLUDING REMARKS

Although the specific components involved vary between organisms, it is clear that regulatory pathways have evolved to use c-di-GMP as a second messenger and as an allosteric regulator to inhibit motility and activate sessility and biofilm formation. Because these processes have a profound effect on the virulence of many bacterial pathogens, and because c-di-GMP can also regulate virulence factor expression, this second messenger is a bona fide regulator of pathogenicity. Two areas require much additional research in order to further our understanding of c-di-GMP regulation of pathogenicity. First, what is the extent of regulation of pathogenicity by c-di-GMP? The vast majority of research on c-di-GMP regulation to date has been done on gram-negative bacteria. Because the GGDEF, EAL, and HD-GYP domains are present in the genomes of many gram-positive bacteria as well, it will be interesting to see whether the same patterns of regulation emerge. Second, much remains to be learned concerning the molecular mechanisms by which c-di-GMP exerts its regulatory effects.

SUMMARY POINTS

1. The allosteric regulator and second messenger cyclic dinucleotide, c-di-GMP, is present in most bacteria, where it commonly regulates motility and biofilm formation.
2. In many facultative pathogens, c-di-GMP regulates pathogenesis through its control of motility and biofilm formation. In addition, there are increasing data to indicate that, in these same pathogens, c-di-GMP also regulates the expression of virulence factors.
3. We are at an early stage in our understanding of how c-di-GMP mediates its regulatory effects. At least two c-di-GMP binding domains are present in bacteria, the I-site and PilZ domains. The latter appears to serve as an intermediary between c-di-GMP and regulation of downstream physiological processes including virulence, although how it does this remains to be understood.

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Figure 1. c-di-GMP regulatory pathway. c-di-GMP is synthesized from two GTPs by GGDEF domain DGCs. c-di-GMP is hydrolyzed by EAL domain PDEAs into the linear pGpG before being hydrolyzed by other PDEs into two GMPs. HD-GYP domain PDEs hydrolyze c-di-GMP completely into two GMPs. Numerous studies have shown that c-di-GMP activates EPS production and biofilm formation but inhibits motility and virulence. These processes are regulated by c-di-GMP through a variety of pathways, as discussed in the text. PilZ domain proteins are one class of c-di-GMP sensor, although others likely exist. The mechanism(s) by which PilZ domain proteins transduce changes in c-di-GMP into downstream physiological effects is unknown.

Figure 2.

(a) Model of the role of c-di-GMP in the transition of *V. cholerae* from persistence in aquatic reservoirs to survival in the human host. c-di-GMP is predicted to be high in *V. cholerae* existing in biofilms attached to biotic and abiotic surfaces in the pond environment. Upon entry into the human host, induction of PDE and/or repression of DGC activities can lower c-di-GMP and allow dispersion from the biofilm and maximal expression of virulence genes. Conversely, c-di-GMP must be elevated once again through activation of DGC and/or repression of PDE to resume the biofilm lifestyle. There is evidence that c-di-GMP may begin to be elevated during the later stage of *V. cholerae* infection, potentially giving the bacteria an advantage once they have exited the host. (b) The VieSA signal transduction system provides one mechanism by which *V. cholerae* can lower c-di-GMP concentration in the small intestine. An unknown extracellular signal present in the small intestine activates the VieS sensor, leading to autophosphorylation of VieS and phosphotransfer to the dual-function protein VieA. The phosphorylated, activated VieA autoactivates *vieSA* transcription, resulting in higher VieA PDEA in the cell, reduced c-di-GMP, and full expression of virulence factors.