REVIEW

The HD-GYP Domain, Cyclic Di-GMP Signaling, and Bacterial Virulence to Plants

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Cyclic di-GMP is an almost ubiquitous second messenger in bacteria that was first described as an allosteric activator of cellulose synthase but is now known to regulate a range of functions, including virulence in human and animal pathogens. Two protein domains, GGDEF and EAL, are implicated in the synthesis and degradation, respectively, of cyclic di-GMP. These domains are widely distributed in bacteria, including plant pathogens. The majority of proteins with GGDEF and EAL domains contain additional signal input domains, suggesting that their activities are responsive to environmental cues. Recent studies have demonstrated that a third domain, HD-GYP, is also active in cyclic di-GMP degradation. In the plant pathogen *Xanthomonas campestris* pv. *campestris*, a two-component signal transduction system comprising the HD-GYP domain regulatory protein RpfG and cognate sensor RpfC positively controls virulence. The signals recognized by RpfC may include the cell-cell signal DSF, which also acts to regulate virulence in *X. campestris* pv. *campestris*. Here, we review these recent advances in our understanding of cyclic di-GMP signaling with particular reference to one or more roles in the bacterial pathogenesis of plants.

Additional keyword: biofilm formation.

The extracellular environment undoubtedly influences many aspects of bacterial behavior, including the production of virulence determinants by pathogens. An array of signal transduction systems links the sensing of specific environmental cues to appropriate alterations in either bacterial physiology, gene expression, or both. In one or more of these signal transduction mechanisms, perception of a primary signal alters the level of a second intracellular signal also known as a second messenger. Cyclic di-GMP (bis-(3'-5')-cyclic di-guanosine monophosphate) is a second messenger in bacteria that is implicated in regulation of diverse processes including developmental transitions, aggregative behavior, adhesion, biofilm formation, and virulence in human pathogens. Here, we review recent advances in our understanding of cyclic di-GMP signaling with particular reference to one or more potential roles in the bacterial pathogenesis of plants. The reader is also directed to several other recent reviews of this area (D'Argenio and Miller 2004; Jenal 2004; Römling et al. 2005).

Cyclic di-GMP was originally described in 1987 as an allosteric regulator of cellulose synthesis in *Acetobacter xylinum*

(now *Gluconacetobacter xylinus*) (Ross et al. 1987). It was subsequently shown that enzymes from *G. xylinus* involved in cyclic di-GMP synthesis and degradation contained two protein domains, GGDEF (formerly known as DUF1, for domain of unknown function) and EAL (formerly known as DUF2) (Tal et al. 1998). The GGDEF and EAL nomenclature relates to conserved amino-acid motifs in these domains (Galperin et al. 2001). The biochemical roles of these domains in cyclic di-GMP turnover have been recently described. Direct evidence for the enzymatic role of the GGDEF domain has been obtained by studies of the purified PleD regulatory protein from *Caulobacter crescentus* (Paul et al. 2004) and of isolated GGDEF domains and intact proteins from different bacterial phyla (Ryjenkov et al. 2005). Each of these GGDEF domain proteins converts two molecules of GTP to cyclic di-GMP but has no activity with other nucleotides. Biochemical evidence that the EAL domain is a cyclic di-GMP phosphodiesterase has come from studies of intact proteins as well as isolated EAL domains (Christen et al. 2005; Schmidt et al. 2005; Tamayo et al. 2005). In these studies, the EAL domain hydrolyzed cyclic di-GMP to generate the linear nucleotide pGpG.

The definition of the biochemical functions of GGDEF and EAL domains presents a conundrum; what determines the activity of proteins that contain both domains? One possible resolution to this paradox is that one of the two domains is nonfunctional (Schmidt et al. 2005). There is evidence that some variant GGDEF domains with alterations within the signature motif are enzymatically inactive and may act in a regulatory capacity (Christen et al. 2005). The same considerations may also apply to inactive EAL domains (Schmidt et al. 2005). A second resolution of the paradox could be that the proteins can have both activities but switch between states able to synthesize and hydrolyze cyclic di-GMP in response to environmental cues.

Whole-genome sequencing has revealed an abundance of GGDEF and EAL domains containing proteins across the majority of bacterial species (both gram-positive and gram-negative). Most bacterial genomes encode a number of proteins with these domains. Most GGDEF and EAL domain proteins contain additional signal input domains, suggesting that their activities are responsive to environmental cues. An increased interest in the molecular analysis of communal behavior and biofilm formation in bacteria has uncovered roles for proteins containing GGDEF or EAL domains, or both, in a range of bacteria (Amikam and Galperin 2006; D'Argenio and Miller 2004; Jenal 2004; Römling et al. 2005). In addition, cyclic di-GMP signaling is implicated in developmental transitions in *Caulobacter crescentus* (Aldridge et al. 2003; Paul et al. 2004), motility (Simm et al. 2004), and in the virulence of the human pathogens, *Bordetella pertussis*, *Vibrio cholerae*, *Salmonella enterica*

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var. Typhimurium, and *Pseudomonas aeruginosa* (Hisert et al. 2005; Kulesekara et al. 2006; Merkel et al. 1998; Tischler and Camilli 2005). An emerging theme from a number of studies is that high levels of cyclic di-GMP promote biofilm formation and sessility but low levels promote motility and the synthesis of virulence factors in a range of human and animal pathogens (Römling et al. 2005; Simm et al. 2004).

The HD-GYP domain is a second cyclic di-GMP phosphodiesterase.

Bioinformatic studies suggested that a third domain, HD-GYP, is also involved in cyclic di-GMP hydrolysis (Galperin et al. 1999, 2001). HD-GYP is a subgroup of the HD superfamily of metal dependent phosphohydrolases that contain an additional GYP motif. The HD residues are the presumed catalytic diad and are known to be required for the multiple enzymatic activities of the HD domain protein tRNA nucleotidyl transferase (Yakunin et al. 2004). The association of the HD-GYP domain with a CheY-like two-component receiver domain in many bacterial proteomes indicates a role in signaling (Galperin et al. 1999, 2001). 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 in which several genomes encode proteins with the GGDEF and HD-GYP domains but no EAL domain (Galperin 2005; Galperin et al. 1999, 2001).

In the plant pathogen *Xanthomonas campestris* pv. *campestris*, the HD-GYP domain two-component regulator RpfG positively regulates synthesis of extracellular enzymes and extracellular polysaccharide (EPS) virulence factors and negatively regulates biofilm formation (Dow et al. 2003; Slater et al. 2000). Expression of genes encoding EAL domain (cyclic di-GMP phosphodiesterase) proteins in the *X. campestris* pv. *campestris* *rpfG* mutant restored extracellular enzyme and EPS synthesis to

near wild-type levels and blocked biofilm formation. In contrast, expression of a gene encoding a GGDEF domain (cyclic di-GMP synthase) protein in wild-type *X. campestris* pv. *campestris* caused a reduction in extracellular enzyme and EPS synthesis and promoted biofilm formation, thus giving a phenocopy of the *rpfG* mutant (Ryan et al. 2006). These observations offered indirect support for a role for the HD-GYP domain in cyclic di-GMP hydrolysis. This conclusion was further supported by biochemical studies that demonstrated that the isolated domain could hydrolyze cyclic di-GMP to GMP via the linear intermediate pGpG. Mutation of the HD residues in the presumed catalytic diad abolishes both the enzymatic activity against cyclic di-GMP and the regulatory activity in controlling extracellular enzyme synthesis (Ryan et al. 2006). Overall, these findings established the HD-GYP domain as a novel cyclic di-GMP phosphodiesterase. Although the cellular levels of cyclic di-GMP were not measured in these experiments, the results are also consistent with the notion that high levels of cyclic di-GMP promote biofilm formation but, conversely, inhibit the synthesis of virulence determinants in *X. campestris* pv. *campestris*.

As outlined above, GGDEF and EAL domains are highly abundant and widely distributed in bacteria, although they are not found in archaea (Galperin 2005). The HD-GYP domain is also widely distributed although less abundant, with over 200 HD-GYP domains in over 70 genomes (NCBI Computational Biology Branch website). Most bacterial genomes encode 0 to 3 HD-GYP domain proteins, although they can be more highly represented; different *Vibrio* spp., for example, contain five to 13 cognate genes. HD-GYP domain proteins are found in genomes of bacterial plant pathogens from the genera *Agrobacterium*, *Ralstonia*, *Pseudomonas*, *Erwinia*, *Xanthomonas*, and *Xylella*. In these bacteria, the domain is found in association with the CheY-like response receiver regulatory domain as well as in association with other uncharacterized protein domains (Table 1).

Table 1. HD-GYP domain proteins encoded by the genomes of selected plant pathogens, symbionts and plant-associated bacteria

Organism	Protein designation ^a	Domain organization ^b
<i>Bradyrhizobium japonicum</i>	blr0842 bli2340 bli4192	HD-GYP ^c CheY-HD-GYP HD-GYP ^c
<i>Mesorhizobium loti</i>	mlt1989	HD-GYH-GER ^c
<i>Pseudomonas putida</i> KT2440	PP2599 PP2942	HAMP-GAF-HD//GYP ^c CheY-YN-GYP
<i>Pseudomonas fluorescens</i> Pf5	PFL_2536 PFL_3324 PFL_4240 PFL_4895	HD-GYP ^d CheY-YN-GYP HAMP-GAF-HD//GYP ^c CheY-HN-GFP
<i>Pseudomonas fluorescens</i> Pf-01	Pfl_3976 Pfl_4550	HAMP-GAF-HD//GYP ^c CheY-YN-GFP
<i>Agrobacterium tumefaciens</i>	Atu2291	HD-GYP ^c
<i>Ralstonia solanacearum</i>	Rsc0592 Rsc2515	HD-GYP ^d HD-GYP ^c
<i>Erwinia carotovora</i> subsp. <i>atroseptica</i>	ECA3548	PilZ-HT-GYP
<i>Pseudomonas syringae</i> pvs. <i>tomato</i> , <i>syringae</i> , <i>phaseolicola</i>	PSPT01152 Psyn_0994 PSPPH_1043	All HD-GYP ^d
<i>Xanthomonas campestris</i> pv. <i>campestris</i>	XCC0350; XC0362; XCV0363; XAC0350; XOO4220	All HD-GYP ^d
<i>Xanthomonas campestris</i> pv. <i>vesicatoria</i>		
<i>Xanthomonas axonopodis</i> pv. <i>citri</i>		
<i>Xanthomonas oryzae</i> pv. <i>oryzae</i>	XCC1854; XC2335 (RpfG); XCV1917; XAC1877; XOO2871 XCC2361; XC1755; XCV2672; XAC2493; XOO2798	All CheY-HD-GYP All CheY-HD-GYP
<i>Xylella fastidiosa</i> 9a5c	Xf1113 (RpfG)	CheY-HD-GYP

^a Protein designations are from genome sequencing projects. XCC and XC annotations refer *Xanthomonas campestris* 33913 and 8004 respectively.

^b Key to domains: CheY = response regulator receiver domain; HAMP = Signal transduction through conformational changes; GAF = Role in binding of cyclic nucleotides and other small molecules; GER = helix-turn-helix DNA binding domain; PilZ = putative cyclic di-GMP binding domain. Variations within the amino acids within the HD-GYP motif are also indicated. HD//GYP indicates a domain in which the separation of the two signature motifs of HD and GYP is approximately 230 amino-acid residues, rather than the 51 residues in the canonical HD-GYP domain. More detailed description of domains can be found at the Pfam database.

^c Proteins contain additional N-terminal domains that are uncharacterized.

^d Proteins contain additional N-terminal and C-terminal domains that are uncharacterized.

The genomes of *X. campestris* pv. *campestris* 33913 and 8004 both encode three HD-GYP domain proteins; RpfG and XCC2361/XC1755 have the CheY-HD-GYP domain organization, whereas in XCC0350/XC0362, the HD-GYP domain comprises the central region of an uncharacterized protein. The genomes of *X. axonopodis* pv. *citri*, *X. campestris* pv. *vesicatoria*, and *X. oryzae* each encode three proteins that are almost identical to those in *X. campestris* pv. *campestris*. The single HD-GYP protein in *Xylella fastidiosa* is highly related to RpfG of *X. campestris* pv. *campestris*. Intriguingly, the single HD-GYP domain proteins found in different pathovars of *Pseudomonas syringae* and in *Agrobacterium tumefaciens* and both HD-GYP domain proteins found in *Ralstonia solanacearum* show considerable amino-acid sequence similarity to each other and to XCC0350/XC0362. This sequence similarity is not restricted to the HD-GYP domain, although the proteins do differ considerably in a region of low complexity and variable length. In *Erwinia carotovora* subsp. *atroseptica*, the single HD-GYP domain protein (ECA 3548) has the variant HT-GYP motif and is attached to a PilZ domain, which is proposed to be involved in cyclic di-GMP binding (Amikam and Galperin 2006) (discussed below). Whether this variant of the HD-GYP domain or the other motif variants, such as YN-GYP, have enzymatic activity is not known. Conceivably, these domains could act in a regulatory capacity as seen or proposed for variant GGDEF and EAL domains (Christen et al. 2005; Schmidt et al. 2005). Interestingly, in a class of HD-GYP proteins in *Pseudomonas* spp., the two signature motifs are separated by approximately 230 amino acids, whereas in the canonical domain, this separation is 51 amino acids. Since the role of the GYP motif is as yet unknown, the significance of this difference is unclear.

The HD-GYP domain regulator RpfG of *X. campestris* links cell-cell signaling to cyclic di-GMP turnover.

The HD-GYP domain regulator RpfG and the complex sensor histidine kinase RpfC comprise a two-component system that acts to positively regulate virulence, the synthesis of extracellular enzymes, and biofilm dispersal in *X. campestris* pv. *campestris* (Dow et al. 2003; Ryan et al. 2006; Slater et al. 2000). The *rpfG* and *rpfC* genes are transcribed as the *rpfGHC* operon (Fig. 1), although no function has yet been ascribed to RpfH, which is related in amino-acid sequence to the input

domain of RpfC. Extracellular enzyme synthesis and biofilm dispersal are also regulated by cell-cell signaling, mediated by the diffusible signal molecule DSF (Barber et al. 1997; Slater et al. 2000). DSF has been characterized as the unsaturated fatty acid *cis*-11-methyl-2-dodecanoic acid (Wang et al. 2004). The synthesis of the DSF signal is partially dependent on *rpfB*, which encodes a long chain fatty acyl CoA ligase and is fully dependent on *rpfF*, which encodes a protein with some similarity to enoyl CoA hydratase (Barber et al. 1997). The *rpfB* and *rpfF* genes are contiguous and adjacent to the *rpfGHC* operon, which is convergently transcribed (Barber et al. 1997; Slater et al. 2000; Fig. 1).

Addition of DSF can restore extracellular enzyme synthesis to *rpfF* mutants but not to strains with mutations in *rpfG* or *rpfC*. Mutation of *rpfC*, *rpfG*, or *rpfF* all lead to the formation of biofilms by *X. campestris* pv. *campestris* in certain media, although the wild type grows in a dispersed planktonic fashion (Dow et al. 2003). Addition of DSF causes dispersal of biofilms produced by the *rpfF* mutant but not those by other *rpf* mutants. These findings are consistent with a role for RpfC/RpfG in perception and transduction of the DSF signal (Crossman and Dow 2004). Further support for this model has come from experiments in which the RpfC/RpfG two-component system has been reconstructed in *Pseudomonas aeruginosa* and has been shown to confer responsiveness to exogenously added DSF as seen through effects on swarming motility (Ryan et al. 2006). It has been proposed that recognition of DSF by RpfC leads to phosphorylation of RpfG and its activation in cyclic di-GMP hydrolysis (Ryan et al. 2006; Fig. 2), although this has not been directly demonstrated.

Indirect evidence suggests that cyclic di-GMP regulates a wide range of functions in *X. campestris* pv. *campestris*, as has been shown in other bacteria (Hickman et al. 2005). Transcriptome profiling has revealed that perception of DSF has a widespread influence on *X. campestris* pv. *campestris* gene expression to include effects on genes implicated in multidrug resistance, motility, chemotaxis, TCA cycle, and iron uptake, as well as the previously established effects on genes encoding extracellular enzymes and the synthesis of EPS (He et al. 2006). A similar transcriptome analysis of the role of RpfG on gene expression has yet to be reported, although comparison of the effects of *rpfF* and *rpfG* mutation would provide a further test for the model that DSF is recognized via the RpfG/RpfC two-component system. The possibilities that RpfC may sense additional environmental cues to activate RpfG phosphorylation and that one or more other perception systems for DSF occur in *X. campestris* pv. *campestris* cannot currently be excluded, however.

It has been recently suggested that cyclic di-GMP signaling and extracellular cell-cell signaling (quorum-sensing) converge in the regulation of diverse bacterial behaviors including multicellular behavior, biofilm formation, and virulence (Camilli and Bassler 2006). The recent findings from the *rpf*/DSF system in *X. campestris* pv. *campestris* suggest that a direct link between the two regulatory systems can occur, in which cyclic di-GMP acts as a second messenger in cell-cell signaling. However, activation of a GGDEF and EAL domain protein by 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 *rpf*/DSF regulatory system occurs in other *Xanthomonas* spp. and the related pathogen *Xylella fastidiosa*.

Genome sequencing indicates the presence of an *rpf* gene cluster with the *rpfB-rpfF-rpfC-rpfH-rpfG* gene organization of *X. campestris* pv. *campestris* 8004 in *X. campestris* pv.

Fig. 1. Organization of genes involved in DSF synthesis and perception in the genomes of different *Xanthomonas* spp. and *Xylella fastidiosa*. The top line shows the gene organization in *X. campestris* pv. *campestris* together with the experimentally determined transcriptional organization of this region (Slater et al. 2000). No role for the *rpfH* gene has been found to date. The gene organization in *X. axonopodis* pv. *vesicatoria* is the same as in *X. campestris* pv. *campestris*. In *X. axonopodis* pv. *citri* and *X. oryzae* pv. *oryzae*, the *rpfH* gene is missing (second line). In *Xylella fastidiosa*, *rpfH* is missing and *rpfB* appears elsewhere in the chromosome, as indicated by the dotted line.

campestris strain 33913 and *X. campestris* pv. *vesicatoria*. In *X. oryzae* pv. *oryzae* and *X. axonopodis* pv. *citri*, the *rpfH* gene is missing. In *Xylella fastidiosa*, *rpfH* is missing and *rpfB* is located elsewhere in the chromosome. (Representative gene organizations are given in Figure 1). DSF activity has been detected in a number of *Xanthomonas* spp. (Barber et al. 1997; Jacques et al. 2005) and in *Xylella fastidiosa* (Newman et al. 2004; Scarpari et al. 2003). A role for DSF signaling in the virulence of a number of these bacteria has now been described.

Mutation of *rpfC* or *rpfF* in different strains of *X. oryzae* pv. *oryzae* leads to a loss of virulence on rice (Chatterjee and Sonti 2002; Tang et al. 1996). The *rpfC* mutant of *X. oryzae* pv. *oryzae* 13751 showed a defect in EPS production, but no effects on extracellular enzyme synthesis. In contrast, the *rpfF* mutant of *X. oryzae* pv. *oryzae* BXO4 showed no alteration in EPS synthesis but a defect in iron uptake-related functions. These latter findings are consonant with the recent results of transcriptome profiling from *X. campestris* pv. *campestris* that show that DSF regulates a number of iron-uptake related functions (He et al. 2006). Even in the absence of strain-dependent effects, similarity in the effects of mutation of *rpfC* and *rpfF* might not be expected, since RpfC may recognize additional environmental factors than DSF and it cannot be excluded that additional mechanisms of DSF signal transduction occur.

Xylella fastidiosa is a xylem-restricted bacterium that requires an insect vector to be transmitted between plants. In *Xylella fastidiosa*, mutation of *rpfF* leads to a loss of the DSF signal, which influences the interaction of *Xylella fastidiosa* with both its insect vector and host plant (Newman et al. 2004). The absence of the DSF signal leads to the loss of biofilm formation in the insect vector but an increased virulence when the bacteria are mechanically inoculated into plants. These effects in plants are, thus, apparently opposite to those seen after mutation of *rpfF* in *X. campestris* pv. *campestris*, which leads to a reduction in virulence. Newman and colleagues (2004) have proposed that progression of disease caused by *Xylella fastidiosa* requires transitions between an aggregated state and a state able to synthesize extracellular enzymes that degrade the primary cell wall in bordered pit membranes, allowing the bacteria to colonize new xylem vessels. Newman and colleagues (2004) envisage that these transitions occur in response to the DSF signal. The hypovirulent phenotype of the *rpfF* mutant may result either from the inability of mutant bacteria to release from an aggregated state, to degrade the pit membranes, or both, with a consequent blockage of the vascular system and increased symptom production. The biofilms produced by *Xylella fastidiosa* in insects differ architecturally from those formed in planta and, in contrast to the effects in planta, mutation of *rpfF* leads to a loss of biofilm formation in insects (Newman et al. 2004).

The above examples illustrate the importance of the *rpf*/DSF regulatory system to the virulence of a number of plant-pathogenic bacteria. By extension of the work in *X. campestris* pv. *campestris*, DSF perception may be linked to the turnover of cyclic di-GMP in these related bacteria. Importantly, this suggests that there may be considerable interplay between this signaling system and others involving GGDEF, EAL, and other HD-GYP domain proteins in the regulation of bacterial pathogenesis to plants.

These findings complement a body of work that has implicated cyclic di-GMP signaling in the virulence of animal and human pathogens. In *Vibrio cholerae* and *Pseudomonas aeruginosa*, specific GGDEF or EAL domain proteins, or both, are implicated in virulence in different mouse models (Kulesekara et al. 2006; Tischler and Camilli 2005; Tischler et al. 2002). In *Vibrio cholerae*, the CheY-EAL-HTH domain protein VieA positively activates expression of virulence genes *toxT*, which

encodes a transcriptional regulator, and *ctxA* and *ctxB*, which encode cholera toxin (Tischler and Camilli 2005; Tischler et al. 2002) and negatively influences exopolysaccharide production and biofilm formation (Tischler and Camilli 2005). A *vieA* mutant is attenuated for colonization in the infant mouse model (Tischler et al. 2002). All of these effects require the cyclic di-GMP phosphodiesterase activity of VieA and are abolished by mutation of the EAL amino-acid motif. In *Bordetella pertussis*, activation of virulence factors by the BvgAS two-component system is accompanied by repression of transcription of a further set of genes, which involves the EAL domain protein BvgR (Merkel and Stibitz 1995; Merkel et al. 1998). BvgR-mediated regulation of gene expression contributes to respiratory infection of mice (Merkel et al. 1998). The “stand-alone” EAL domain protein CdgR of the genus *Salmonella* is required for the bacterium to resist host phagocyte oxidase in vivo and contributes to virulence in mice (Hisert et al. 2005). Thus far, the role in virulence of the HD-GYP domain proteins found in animal pathogens has not been examined.

Diverse environmental signals may be transduced through alterations in cyclic di-GMP levels.

As indicated above, 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

Fig. 2. Model for DSF perception and signal transduction in *Xanthomonas campestris*. The synthesis of the DSF signal requires RpfF and is partially dependent on RpfB. DSF perception and signal transduction involves the complex sensor RpfC and HD-GYP domain regulator RpfG, which is a cyclic di-GMP phosphodiesterase. By analogy with a number of related sensor proteins, signal transduction may involve autophosphorylation of RpfC in response to ligand (DSF) binding, followed by phosphorelay and phosphotransfer to the cognate regulator, in this case RpfG (indicated by arrows). Phosphorylation of RpfG leads to its activation as a cyclic di-GMP phosphodiesterase, an activity associated with the HD-GYP domain. The consequent alterations in the level of cyclic di-GMP affect the synthesis of virulence factors such as extracellular enzymes, biofilm dispersal and motility by as yet unknown mechanisms. RpfC may recognize additional environmental cues. Boxes indicate putative transmembrane helices; REC = CheY-like two-component receiver domain; HPT = histidine phosphotransfer; and HK = histidine kinase.

small molecular weight effectors, and various membrane-associated or periplasmic domains that may be involved in sensing small molecules. Binding of effectors to the sensory input domain is believed to affect the enzyme activity of the protein in cyclic di-GMP turnover. As with RpfG, a number of GGDEF, EAL, and HD-GYP domain proteins contain a CheY-like response receiver domain. These proteins can be part of either two-component signal transduction systems or of modified chemotaxis systems. In both of these cases, the environmental signal is presumably sensed by another element of the system, either a sensory histidine kinase or methyl-accepting chemotaxis protein.

Very little is known about the environmental signals or cues that are recognized by the sensory domains or which activate two-component or chemotaxis-like signal transduction pathways involving these proteins. Perhaps the best-studied example is PdeA1, the cyclic di-GMP phosphodiesterase from *Gluconacetobacter xylinus*, which has a PAS-GAF-GGDEF-EAL domain structure (Chang et al. 2001). The PAS domain of this protein contains a heme moiety that binds molecular oxygen. The effect of oxygen binding is to reduce the ability of PdeA1 to degrade cyclic di-GMP. This is thought to contribute to an elevation in the cellular level of the nucleotide, with consequent activation of cellulose synthesis. PdeA1 is homologous over its entire length to proteins from *Xanthomonas* spp., although it is not known whether these proteins also bind molecular oxygen.

Work on the human bacterial pathogens *Vibrio cholerae* and *Pseudomonas aeruginosa* has indicated a role for specific cyclic di-GMP signaling systems in activation of the formation of biofilms in response to the polyamine norspermidine and subinhibitory concentrations of the antibiotic tobramycin (Karatan et al. 2005; Hoffman et al. 2005). These or related environmental cues may exist within the soil, since norspermidine is found in lower plants and tobramycin is one of a number of antibiotics produced by *Streptomyces* spp. However, it is not known whether these compounds influence biofilm formation or any other behavior of plant pathogens.

It is well-established that plant-derived signals such as phenolic secondary metabolites, sugars, and cell wall components influence many aspects of microbial interaction with plant hosts, including the induction of factors contributing to the virulence of pathogens. Two-component systems that act in the perception of a number of these signals have been described. However, it is plausible that these or related signals are also environmental cues that influence bacterial behavior through cyclic di-GMP signaling.

Is the RpfG/RpfC system part of a regulatory network?

Although some small genomes like that of *Xylella fastidiosa* (approximately 2.7 Mb) encode only five proteins with a potential role in cyclic di-GMP signaling, *X. campestris* pv. *campestris*, at approximately 5 Mb, encodes 37 and *Pseudomonas syringae* pv. *tomato*, at approximately 6 Mb, encodes 40. These large numbers indicate that there must be considerable complexity in the organization of cyclic di-GMP signaling within a single organism, some details of which are beginning to emerge (Jenal 2004; Römmling et al. 2005). One pertinent issue is whether signaling systems are part of regulatory networks or are dedicated to specific cellular tasks.

Localization studies of the PleD regulator (CheY-CheY-GGDEF), which influences swarmer to stalk cell transitions and pole development in *Caulobacter crescentus* (Aldridge et al. 2003), have shown that upon phosphorylation the protein locates to the pole of the cell where the new stalk will be formed (Paul et al. 2004). Phosphorylation also activates the protein for cyclic di-GMP synthesis (Paul et al. 2004). Previously, it was shown that the proteins of *G. xylinus* involved in

cyclic di-GMP turnover copurified with the cellulose synthase (Ross et al. 1987). These findings have led to the suggestion that the generation of localized pools of cyclic di-GMP by specific components in cyclic di-GMP signaling may activate processes that are determined by colocalizing proteins. Work on GGDEF-EAL domain proteins in *Salmonella enterica* and *Pseudomonas aeruginosa* also supports the idea that certain cyclic di-GMP synthesis or degradation systems are dedicated to specific cellular tasks (Hoffman et al. 2005; Kader et al. 2006; Kulesekara et al. 2006).

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 regulation of specific bacterial processes under different environmental conditions would support the notion of an environmentally responsive network. This has been reported for the role of several GGDEF domain proteins in cellulose synthesis in *Salmonella* spp. (Garcia et al. 2004; Römmling 2005).

We have recently carried out a comprehensive mutational analysis of the role of all 37 proteins with HD-GYP, GGDEF, and EAL domain proteins in regulation of extracellular enzyme synthesis and motility in *X. campestris* pv. *campestris* (R. P. Ryan, Y. Fouhy, J. Lucey, Y.-Q. He, J.-X. Feng, J.-L. Tang, and J. M. Dow, *unpublished*). A number of proteins in addition to RpfG act to regulate extracellular enzyme synthesis, although different proteins have significant roles under different growth conditions. RpfG is the only protein to have an influence under all growth conditions tested, and loss of RpfG has the biggest effect on extracellular enzyme synthesis. These findings are consistent with the concept of a signaling network that includes the RpfC/RpfG system and that responds to and integrates information from a number of cues, including the DSF cell-cell signal. Conversely other signaling elements in *X. campestris* pv. *campestris* regulate motility but have no effect on extracellular enzyme production. This is consistent with the concept of localized action of certain elements in cyclic di-GMP signaling.

Overall, these observations, taken together with other recent findings in *Salmonella* spp. (Garcia et al. 2004; Kader et al. 2006; Simm et al. 2004), indicate the coexistence in bacteria of networks of interacting cyclic di-GMP-linked signaling systems and signaling systems dedicated to specific cellular functions. These latter systems would be associated with discrete, perhaps microlocalized, pools of cyclic di-GMP. In *X. campestris* pv. *campestris*, RpfG is the only cyclic di-GMP signaling protein that affects both motility and enzyme production. One rationalization for these broader effects of RpfG is that, as a soluble protein, phosphorylated (active) RpfG is able to influence a number of pools of cyclic di-GMP within the cell. However cellular localization studies, such as those reported for the PleD regulator (Paul et al. 2004), have not been reported for RpfG and this remains speculative. Clearly many questions about the detailed interactions and regulatory circuits within cyclic di-GMP signaling in *Xanthomonas* spp. (and other bacteria) remain to be resolved.

How does cyclic di-GMP exert its action?

Almost nothing is known about the mechanisms whereby cyclic di-GMP exerts its action on diverse cellular functions including virulence factor synthesis and biofilm formation. The best-studied effect of cyclic di-GMP is its allosteric activation of cellulose synthesis in *G. xylinus*, in which cyclic di-GMP has been shown to bind to BcsB, the β -subunit of cellulose synthase, and to an uncharacterized 200-kDa protein. Re-

cent 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 (Amikam and Galperin 2006). PilZ was originally identified as a regulator of fimbrial biosynthesis in *Pseudomonas aeruginosa* (Alm et al. 1996). 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 (Amikam and Galperin 2005). The PilZ proteins may act as a cyclic di-GMP “adaptors,” binding to other proteins to affect cellular functions. Other mechanisms of action are also plausible. By analogy with cyclic AMP, cyclic di-GMP may affect transcription of genes involved in virulence and biofilm formation by binding to transcriptional regulators. Interestingly, the PilZ domain has also been found linked to a HTH domain (Amikam and Galperin 2006), indicating a potential link between cyclic di-GMP binding and transcription. Where cognate regulators of virulence genes have been identified, it may be possible to examine the effects of cyclic di-GMP by in vitro promoter-binding assays.

Concluding remarks.

The appreciation of the role of cyclic di-GMP as an important and almost ubiquitous signaling molecule in bacteria has occurred relatively recently and has been strongly promoted by the increased interest in microbial biofilm formation and by microbial genome sequencing. It is now known that cyclic di-GMP acts to regulate diverse bacterial properties that include developmental transitions, aggregative behavior, adhesion, biofilm formation, and virulence. Identification of the role of the HD-GYP domain in cyclic di-GMP degradation increases our understanding of a signaling system whose importance to the lifestyle of diverse bacteria, including plant-associated pathogens, symbionts, and beneficial bacteria is now emerging. However, our knowledge of this domain is still very limited and there are a host of questions. What is the role of GYP and other conserved motifs and residues? Does the HD-GYP domain have other cellular functions, for example, in protein-protein interactions? Do HD-GYP domain proteins show specific cellular localizations, for example, after activation? What are the cues that activate or inactivate the signaling proteins? What is the interplay between the different signaling systems that use cyclic di-GMP as a second messenger? A systems biology approach, integrating information from biochemistry and genetics with cell biology and physiology will clearly be required to achieve a deeper understanding of the role of cyclic di-GMP signaling in diverse bacterial behaviors that include virulence.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

NCBI Computational Biology Branch signal transduction protein table:
www.ncbi.nlm.nih.gov/Complete_Genomes/SignalCensus.html
 Pfam database: pfam.wustl.edu