
Cyclic di-GMP as an Intracellular Signal Regulating Bacterial Biofilm Formation

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Abstract

Cyclic di-GMP is a novel second messenger in bacteria that was first described as an allosteric activator of cellulose synthase in *Gluconacetobacter xylinus*. It is now established that this nucleotide regulates a range of functions including developmental transitions, aggregative behavior, adhesion, biofilm formation and virulence in diverse bacteria. 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 EAL and HD-GYP domains are involved in cyclic di-GMP hydrolysis. Bacterial genomes encode a number of proteins with GGDEF, EAL and HD-GYP domains. The majority of these proteins contain additional signal input domains, suggesting that their activities are responsive to environmental cues. An emerging theme is that high cellular levels of cyclic di-GMP promote biofilm formation and aggregative behavior whereas low cellular levels promote motility. The mechanism(s) by which cyclic di-GMP exerts its effects on these cellular functions is however poorly understood.

Introduction

The extracellular environment undoubtedly influences many aspects of bacterial behavior including the formation, maturation and dissolution of biofilms. An array of signal transduction systems links the sensing of specific environmental cues to appropriate alterations in bacterial physiology and/or gene expression. In some of these signal transduction mechanism(s), perception of a primary signal alters the level of a second intracellular signal also known as a second messenger. In this chapter we discuss the role of cyclic di-GMP (bis-(3'-5')-cyclic di-guanosine monophosphate) (Figure 5.1) as a second messenger that is implicated in regulation of processes associated with biofilm formation and communal behavior in diverse bacteria.

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 turnover contained two protein domains, GGDEF and EAL, of previously unknown function (Tal *et al.*, 1998). Whole genome sequencing has revealed an abundance of GGDEF and EAL domain containing proteins across the majority of bacterial species (both Gram-positive and Gram-negative). Most GGDEF/EAL domain proteins contain additional

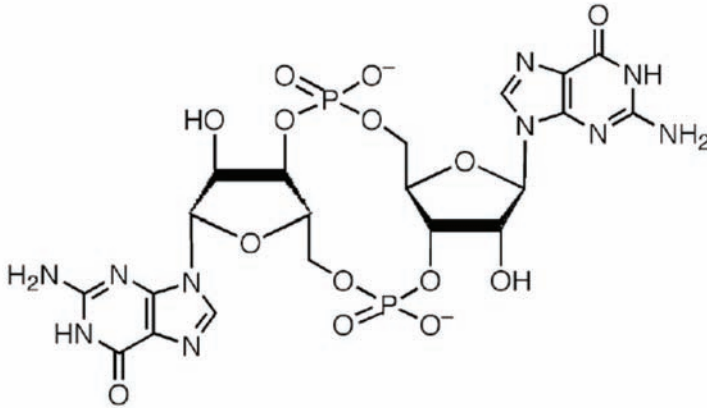


Figure 5.1 Structure of the second messenger bis-(3'-5')-cyclic di-guanosine monophosphate (cyclic di-GMP).

signal input domains, suggesting that their activities are responsive to environmental cues. Importantly, molecular analysis of communal behavior and biofilm formation has uncovered roles for a number of proteins containing GGDEF and/or EAL domains in developmental transitions, aggregative behavior, adhesion, biofilm formation and virulence in a number of bacteria. Taken together these observations indicate that signaling systems involving cyclic di-GMP as a second messenger are of potential importance in the regulation of biofilm formation in many bacteria.

In the following sections we review the discovery of cyclic di-GMP and the definition of the biochemical activities of protein domains involved in its synthesis and degradation. We go on to catalogue some proteins with an established role in biofilm or aggregative behavior and to consider the primary environmental cues to which these proteins may respond. Finally we address possible mechanisms for the poorly understood processes by which cyclic di-GMP may exert its influence on bacterial behavior and biofilm formation. The reader is also directed to several recent reviews of this area (D'Argenio and Miller, 2004; Jenal, 2004; Römling *et al.*, 2005).

The discovery of cyclic di-GMP through studies of bacterial cellulose biosynthesis

The occurrence of cyclic di-GMP in bacteria was first revealed through studies of regulation of cellulose synthesis in *Gluconacetobacter xylinus* (formerly *Acetobacter xylinum*) (Ross *et al.*, 1987). In this organism, cyclic di-GMP acts as a reversible and highly specific allosteric activator of cellulose synthase. *In vitro* the rate of cellulose synthesis is increased by up to 200 fold in the presence of cyclic di-GMP with half maximal activation at 0.35 μM . Original reports suggested that cyclic di-GMP bound to BcsB, the β -subunit of cellulose synthase (Mayer *et al.*, 1991). Subsequent work showed cyclic di-GMP binding at high affinity (K_D 20 nM) to a 200 kDa membrane-associated protein or protein complex that had no apparent cellulose synthase activity but that associated with the synthase complex (Weinhouse *et al.*, 1997). It was proposed that this protein introduced an additional level of

regulation into cellulose biogenesis *in vivo*, by preventing enzymatic degradation of the effector and by targeting its release to the synthase complex. However the 200 kDa protein(s) was not further characterized.

Recent bioinformatic studies (Amikam and Galperin, 2006) have suggested that the true binding site for cyclic di-GMP is a C-terminal domain in the α -subunit of cellulose synthase (BcsA), and propose that the 200 kDa protein comprises either a BcsA dimer or a second form of cellulose synthase in which a single polypeptide contains both subunits (Saxena *et al.*, 1995). This issue of targets for cyclic di-GMP binding will be discussed in detail later. Following the work in *G. xylinus*, cyclic di-GMP was shown to regulate bacterial cellulose synthesis in the plant pathogen *Agrobacterium tumefaciens* (Amikam and Benziman, 1989) and plant symbiotic *Rhizobium* spp. (Ausmees *et al.*, 1999; 2001).

What is the role of cellulose synthesis in *G. xylinus*? Association of cellulose microfibrils in this bacterium leads to the assembly of a multicellular pellicle in static liquid cultures. This allows the organism to colonize a niche that may be inaccessible to other microbes. Furthermore cells within the pellicle stay near the surface, a better-aerated environment with access to nutrients that may be important for the growth of these obligate aerobes (Cook and Colvin, 1980). It has also been suggested that cellulose production may contribute to the attachment of the bacteria to the decaying plant materials on which they grow, providing protection from competitors for the same nutrient source (Williams and Cannon, 1989). Bacterial cellulose production contributes to the initial attachment to plants of symbiotic *Rhizobium* spp. and the pathogen *Agrobacterium tumefaciens* (reviewed in Römling, 2002).

Identification of enzymes involved in cyclic di-GMP synthesis and degradation in *Gluconacetobacter xylinus*

Biochemical studies revealed that the level of cyclic di-GMP in *G. xylinus* were controlled by the opposing action of the enzymes diguanylate cyclase (DGC), which catalyzes its formation from two molecules of 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 (Tal *et al.*, 1998). These genes are organized in three unlinked operons, each containing a *pdeA* gene immediately upstream of a *dcg* gene (Tal *et al.*, 1998). Each of the proteins contains a GGDEF domain (formerly known as DUF1, for domain of unknown function) and a C-terminal EAL domain (formerly known as DUF2). The GGDEF and EAL nomenclature relates to conserved amino acid motifs in these domains. In addition to GGDEF and EAL domains each of these proteins has an N-terminal PAS sensory input domain, which typically bind heme and flavin (Taylor and Zhulin, 1999). The GGDEF domain was first identified in PleD, a regulatory protein controlling swarmer-to-stalked-cell transition in *Caulobacter crescentus* (Hecht and Newton, 1995). The EAL domain was first described in BvgR, a repressor of virulence gene expression in *Bordetella pertussis* (Merkel *et al.*, 1998a).

The findings in *G. xylinus* were of seminal importance; they linked GGDEF and EAL domains to cyclic di-GMP turnover and gave the first indication that cyclic di-GMP signaling might be widespread in bacteria where it might control functions other than cellulose

synthesis (Tal *et al.*, 1998). In addition the findings posed a number of questions as to the regulation of cyclic di-GMP levels, in particular what are the specific roles of the GGDEF and EAL domains, and how are the activities of enzymes that have opposite effects on cyclic di-GMP levels regulated so as to give appropriate changes in the cellular level of the nucleotide. 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 (Chang *et al.*, 2001).

The GGDEF and EAL domains catalyze cyclic di-GMP synthesis and degradation respectively

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 (Pei and Grishin, 2001). This suggestion was supported by genetic experiments in which expression of *dgc1* from *A. xylinum* as well as genes encoding proteins with GGDEF but no EAL domain from other bacteria were shown to complement a mutant of *Rhizobium* for defects in cellulose production (Ausmees *et al.*, 2001). Subsequently it was shown that expression of genes encoding GGDEF proteins could increase the cellular levels of cyclic di-GMP in several bacteria (Paul *et al.*, 2004; Simm *et al.*, 2004; Tischler and Camilli, 2004). Direct evidence for the role of the GGDEF domain has been obtained by biochemical studies of the purified PleD regulatory protein (Paul *et al.*, 2004) and of isolated GGDEF domains (Ryjenkov *et al.*, 2005). In the latter study, the GGDEF domains selected came from proteins from different bacterial phyla (Ryjenkov *et al.*, 2005; Römling *et al.*, 2005). Each of these GGDEF domain proteins converted two molecules of GTP to cyclic di-GMP but had no activity with other nucleotides (Figure 5.2). The conservation of function of GGDEF domains from divergent bacteria has been further demonstrated by reciprocal complementation of *bmsT* which is involved in biofilm formation in *Yersinia pestis* and *adrA*, which is involved in cellulose synthesis in *Salmonella enterica* serovar *typhimurium* (Simm *et al.*, 2005). In many of the experiments

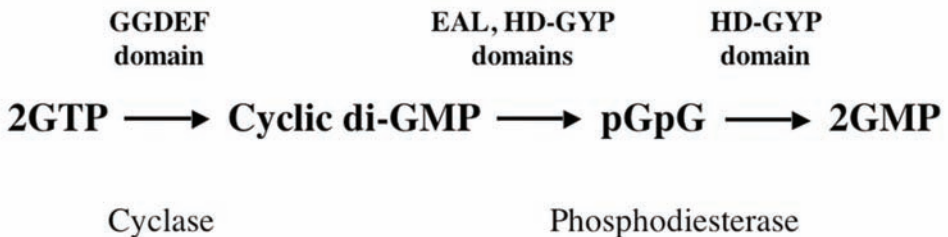


Figure 5.2 The role of GGDEF, EAL and HD-GYP domains in the synthesis and degradation of cyclic di-GMP. Synthesis of cyclic di-GMP from two molecules of GTP is catalyzed by the GGDEF domain and is predicted to occur in two steps, with pppGpG as intermediate. Each step releases a molecule of inorganic pyrophosphate. The degradation of cyclic di-GMP to GMP also occurs via a two-step reaction, with the linear dinucleotide pGpG as intermediate. EAL domains characterized thus far characterized catalyze only the first step, whereas the HD-GYP domain catalyzes both steps. Other, perhaps non-specific, phosphodiesterase enzymes may also convert pGpG to 5'GMP.

described, it was shown by site directed mutagenesis 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 (Simm *et al.*, 2004; Tischler and Camilli 2004; 2005) and conversely that mutation of an EAL domain protein increased cellular cyclic di-GMP levels (Hisert *et al.*, 2005). The EAL domain protein HmsP of *Yersinia pestis* was shown to possess activity against the model phosphodiesterase substrate *bis*-(*p*-nitrophenol) phosphate, an activity that was required for the negative regulation of biofilm formation (Bobrov *et al.*, 2005). Direct biochemical evidence for the role of the EAL domain as a cyclic di-GMP phosphodiesterase has come from studies of the intact proteins as well as isolated EAL domains (Christen *et al.*, 2005, Schmidt *et al.*, 2005). Again in many of these cases mutational analysis indicated the essential role of the conserved EAL residues in enzymatic activity and regulation. The activities of GGDEF and EAL domains in cyclic di-GMP turnover are summarized in Figure 5.2.

The biochemical conundrum of GGDEF–EAL domain fusions

The definition of the biochemical functions of GGDEF and EAL domains presents a conundrum; what determines the activity of proteins such as the PDEA and DGC from *G. xylinus*, which contain both domains. One possible resolution to this paradox is that one of the two domains is non-functional (Schmidt *et al.*, 2005). There is evidence that some GGDEF domains are enzymatically inactive and may act in a regulatory capacity. In a GGDEF-EAL domain protein from *Caulobacter*, binding of GTP to an enzymatically inactive GGDEF domain acts to regulate the activity of the protein in cyclic di-GMP hydrolysis (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. 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 (Chan *et al.*, 2004) whereas EAL activity is apparently independent of protein oligomerization (Schmidt *et al.*, 2005). 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.

Phylogenetic analysis of the GGDEF/EAL domain proteins in *Pseudomonas aeruginosa* indicates that GGDEF domains from almost all proteins that do not have an EAL domain are related in a single class (family I) in which the variant GGEEF motif is found (Kulesekara *et al.*, 2006). GGDEF domains from almost all 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 III sequences are poorly correlated with the consensus GGDEF sequence, suggesting that they may be enzymatically inactive (Kulesekara *et al.*, 2006).

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

Bioinformatic studies have 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. 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, where several genomes encode proteins with the GGDEF and HD-GYP domains but no EAL domain (Galperin *et al.*, 1999; 2001; Galperin, 2005).

In the plant pathogen *Xanthomonas campestris*, the HD-GYP domain regulator RpfG positively regulates synthesis of extracellular enzyme virulence factor and negatively regulates biofilm formation (Slater *et al.*, 2000; Dow *et al.*, 2003). Expression of genes encoding EAL domain proteins in the *X. campestris* *rpfG* mutant restored extracellular enzymes and blocked biofilm formation. In contrast expression of genes encoding a GGDEF domain protein in wild-type *X. campestris* gave a phenocopy of the *rpfG* mutant (Ryan *et al.*, 2006). 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 a linear intermediate (Figure 5.2). Mutation of the HD residues comprising the presumed catalytic diad of the HD-GYP domain abolishes both the regulatory activity and enzymatic activity against cyclic di-GMP (Ryan *et al.*, 2006).

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 archaea (Galperin, 2005). At the time of writing there were over 2400 GGDEF domains and over 1400 EAL domains in the Pfam protein family database. The HD-GYP domain is also widely distributed although slightly less abundant, with over 200 HD-GYP domains in over 70 genomes. Most bacterial genomes encode a number of proteins with these domains. For example, the *Pseudomonas aeruginosa* PAO1 proteome has 17 predicted proteins with a GGDEF but no EAL domain, 5 proteins with an EAL but no GGDEF domain, 16 proteins with both GGDEF and EAL domains, and 3 proteins with an HD-GYP domain (Galperin, 2005).

Many GGDEF, EAL, and HD-GYP domain proteins have associated regulatory/sensory input domains

As indicated above, many GGDEF, EAL and HD-GYP domain proteins have additional domains that may directly sense environmental cues (Zhulin *et al.*, 2003). 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 and various membrane-associated or periplasmic domains that may be involved in sensing small

molecules (Table 5.1). As an example, PdeA1 of *G. xylinus* has a PAS-GAF-GGDEF-EAL domain structure (Tal *et al.*, 1998; Chang *et al.*, 2001). Binding of effectors to the sensory input domain is believed to affect the enzyme activity of the protein. A number of GGDEF, EAL and HD-GYP domain proteins contain a CheY-like REC domain (Table 5.1). 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. Signal transduction involves autophosphorylation of the sensory histidine kinase or of a CheA-like histidine kinase and subsequent phosphotransfer to the REC domain, which alters the activity of the enzymatic domain in cyclic di-GMP synthesis or degradation. Examples of proteins belonging to two component systems are PleD of *C. crescentus* and RpfG of *X. campestris*, whereas WspR of *P. aeruginosa* and *P. fluorescens* are part of chemotaxis-like signal transduction systems (Aldridge *et al.*, 2003; Paul *et al.*, 2004;

Table 5.1 Selected signaling and regulatory domains that are found in association with GGDEF and/or EAL domains in bacterial proteins

Domain	Proposed signaling or regulatory function
Cytoplasmic domains	
REC	CheY-homologous response regulator receiver domain, phosphorylated by sensory or CheA histidine kinases
PAS (PAC)	Oxygen, redox potential and light sensing
GAF	Binding of cyclic nucleotides, other small molecules
HAMP	Signal transduction through conformational changes?
Extracytoplasmic domains	
PBPb	Bacterial periplasmic substrate binding protein
Reg_Prop	Proteins with multiple Reg_Prop domains may form a beta-propeller structure, unknown function
CHASE	Extracellular/periplasmic sensory domain, also associated with cyclases and histidine kinases
Transmembrane domains	
7TMR-DISMED2	Carbohydrate binding?
7TMR-DISM-7TM	Carbohydrate binding?
MHYT	Sensor of oxygen, CO or NO?
MASE1	Membrane-associated sensor
MASE2	Membrane-associated sensor
BLUF	Blue light sensor, FAD-dependent

More detailed description of domains can be found at the Pfam data base at <http://pfam.wustl.edu>

Crossman and Dow, 2004; Goymer, 2002; Hickman *et al.*, 2005; Table 5.1). 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. Published analyses of the domain organization of all GGDEF/EAL domain proteins from *Salmonella typhimurium* (Römling, 2002) and *P. aeruginosa* (Kulesekara *et al.*, 2006) exemplify the diversity of domains within proteins with a proposed role in cyclic di-GMP signaling within a single organism.

GGDEF, EAL, and HD-GYP domain proteins with a role in biofilm formation and dispersal

Molecular genetic analysis has now implicated a number of proteins with GGDEF, EAL and HD-GYP domains, and by extension cyclic di-GMP, in the regulation of communal bacterial behavior including biofilm formation, aggregation and other multicellular phenotypes (Table 5.2). However it is clear that cyclic di-GMP can also regulate a range of other phenotypes including motility, virulence factor synthesis and cell differentiation (Merkel *et al.*, 1998a,b; Hecht and Newton, 1995; Tischler *et al.*, 2002; Tischler and Camilli, 2004, 2005; Simm *et al.*, 2004; Huang *et al.*, 2003). In a number of these cases, conserved residues within the GGDEF, EAL and HD-GYP domains have been shown to be required for regulation indicating a functional link to the enzymatic activity. Here we address cyclic di-GMP regulation of biofilm formation and of the synthesis of biofilm matrix components. For more detailed discussion of the components of the extracellular matrix of biofilms, the reader is directed to Pamp *et al.*, this volume.

Table 5.2 Proteins with GGDEF, EAL, and HD-GYP domains that are implicated in biofilm formation and aggregative behavior in bacteria

Protein	Organism	Domain organization	Regulated phenotype/function	Reference
DGC1,2,3	<i>Gluconacetobacter xylinus</i>	PAS-GGDEF-EAL	Cellulose synthesis	Tal <i>et al.</i> , 1998
PDEA1,2,3	<i>Gluconacetobacter xylinus</i>	PAS-GGDEF-EAL	Cellulose synthesis	Tal <i>et al.</i> , 1998
HmsT	<i>Yersinia pestis</i>	GGDEF	Positive regulation of biofilm formation, aggregation	Jones <i>et al.</i> , 1999; Kirillina <i>et al.</i> , 2004
HmsP	<i>Yersinia pestis</i>	TM-GGDEF-EAL	Negative regulation of biofilm formation, aggregation	Kirillina <i>et al.</i> , 2004; Bobrov <i>et al.</i> , 2005
PleD	<i>Caulobacter crescentus</i>	CheY-CheY*-GGDEF	Attachment/dispersal; developmental transitions	Hecht and Newton, 1985; Aldridge <i>et al.</i> , 2003

Table 5.2 continued

Protein	Organism	Domain organization	Regulated phenotype/function	Reference
WspR	<i>Pseudomonas fluorescens</i>	CheY-GGDEF	Wrinkled colonies, synthesis of modified cellulose	Goymer, 2000; Spiers <i>et al.</i> , 2003
WspR	<i>Pseudomonas aeruginosa</i>	CheY-GGDEF	Wrinkled colonies, biofilm formation, regulation of polysaccharide synthesis operons	D'Argenio <i>et al.</i> , 2002; Hickman <i>et al.</i> , 2005
CelR2	<i>Rhizobium leguminosarum</i> bv. <i>trifolii</i>	CheY-GGDEF	Cellulose production	Ausmees <i>et al.</i> , 1999
AdrA	<i>Salmonella enterica</i> bv. <i>typhimurium</i>	TM-GGDEF	Rdar colonies	Römling <i>et al.</i> , 2000
ScrC	<i>Vibrio parahaemolyticus</i>	GGDEF-EAL	Rugose colonies, capsular polysaccharide synthesis	Boles and McCarter, 2002; Güvener and McCarter, 2003
PvrR	<i>Pseudomonas aeruginosa</i>	CheY-EAL	Repression of autoaggregation, phase variation, virulence	Drenkard and Ausubel, 2002; Kulesekara <i>et al.</i> , 2006
MbaA	<i>Vibrio cholerae</i>	TM-HAMP-GGDEF-EAL	Biofilm matrix architecture	Bomchil <i>et al.</i> , 2003; Karatan <i>et al.</i> , 2005
VieA	<i>Vibrio cholerae</i>	CheY-EAL-HTH	Repression of biofilm formation, activation of cholera toxin production	Tischler <i>et al.</i> , 2002; Tischler and Camilli, 2005
RocS	<i>Vibrio cholerae</i>	PAS-GGDEF-EAL	Rugose colonies	Rashid <i>et al.</i> , 2003
RpfG	<i>Xanthomonas campestris</i>	CheY-HD-GYP	Repression of biofilm formation, activation of virulence factor synthesis	Slater <i>et al.</i> , 2000; Dow <i>et al.</i> , 2003
RocR	<i>Pseudomonas aeruginosa</i>	CheY-EVL	Antagonism of expression of <i>cup</i> genes encoding fimbrial adhesins	Kulasekara <i>et al.</i> , 2005
PPO165	<i>Pseudomonas putida</i>	TM-GGDEF-EAL	Biofilm dispersal	Gjermansen <i>et al.</i> , 2005

HTH: helix-turn-helix DNA binding domain; TM: transmembrane helix or helices; CheY*: inactive version of the CheY receiver domain.

As in *G. xylinus*, the synthesis of cellulose or of an acetylated cellulose derivative has been shown to contribute to the multicellular behavior of a number of bacteria including *Escherichia coli*, *Salmonella enterica* bv. typhimurium, *Rhizobium leguminosarum* and *Pseudomonas fluorescens* (Ausmees *et al.*, 1999; Zogaj *et al.*, 2001; Solano *et al.*, 2002; Spiers *et al.*, 2003; Römling, 2005). These multicellular behaviors are apparent through alterations in colony morphology and in some cases through staining by Congo Red (which binds to β -linked glucans like cellulose) to give phenotypes that have been termed rdar (for red, dry and rough), wrinkly spreader and rugose. In each of these cases a GGDEF domain protein has been implicated in positive regulation of the multicellular phenotype, consistent with the earlier work on *G. xylinus* that showed activation of cellulose synthesis by cyclic di-GMP. The rdar phenotype of Enterobacteriaceae depends not only on cellulose synthesis but also on the expression of thin aggregative fibers termed curli (Römling *et al.*, 2000; Römling, 2005). The two-component regulator CsgD, also called AgfD, regulates transcription of an operon encoding structural components curli and of *adrA*, a gene that encodes a GGDEF domain protein that is membrane-associated. CsgD has no effect on the expression of genes encoding bacterial cellulose synthesis. The synthesis of the cellulose component of the extracellular matrix is thus presumably responsive to environmental cues at a least two levels; those recognized by the cognate histidine kinase for CsgD, which has not yet been identified, as well as those recognized by AdrA.

The wrinkly spreader phenotype of *Pseudomonas fluorescens* depends upon activation of the CheY-GGDEF domain protein WspR, which is part of a chemotaxis-like sensory transduction system, and the consequent synthesis of cellulose that is modified by acetylation (Goymer, 2002; Spiers *et al.*, 2003). The available evidence suggests that WspR is activated for cyclic di-GMP synthesis by phosphorylation of the CheY domain. Two classes of mutation give rise to the wrinkly spreader phenotype; those in genes encoding other elements of the sensory transduction pathway that result in enhanced or constitutive activation of WspR and those introducing amino acid alterations in WspR giving a “locked-on” conformation (Goymer, 2002). A homologous system is found in *Pseudomonas aeruginosa*, where mutations in *wspF* that likely result in constitutive phosphorylation of WspR give rise to aggregation and altered colony morphology (Hickman *et al.*, 2005). There are however some important differences between the two bacteria in the mechanisms underlying alterations in colony morphology and aggregation. *P. aeruginosa* does not have *bcs* genes for cellulose biosynthesis. Instead two operons called *psl* and *pel* are involved in extracellular polysaccharide production and biofilm formation (Friedman and Kolter, 2004). In the *wspF* mutant expression of both of these operons is elevated, which may suggest that cyclic di-GMP levels can influence the transcription of genes involved in biofilm polysaccharide formation in *P. aeruginosa*, in contrast to its (presumed) post-translational effects on cellulose synthesis in *P. fluorescens*. These effects on *psl* and *pel* gene expression are however only part of a much wider influence of elevated cyclic di-GMP levels on gene transcription; expression levels of at least 560 genes are affected by a *wspF* deletion (Hickman *et al.*, 2005). Similar broad effects on transcription in response to elevated levels of cyclic di-GMP are seen in *Escherichia coli* (Méndez-Ortiz *et al.*, 2006).

Biofilm formation in *P. aeruginosa* is also mediated by a family of adherence factors termed CupA, CupB and CupC that are orthologues of the chaperone/usher family of

E. coli adhesins (Vallet et al., 2001). Expression of two of these clusters, *cupB* and *cupC*, is regulated by a variant of a classical two-component signal transduction pathway comprising three components RocS1, RocR and RocA1 (Kulasekara et al., 2005). RocS1 is a sensor kinase, RocA1 is a DNA binding response regulator that activates *cup* genes, and RocR acts as an antagonist of RocA1 activity. RocS1 can phosphorylate both RocR and RocA1. RocR comprises a response regulator receiver domain attached to a variant of the EAL domain in which the EAL motif is replaced by EVL. It is not known whether this variant is still active in hydrolysis of cyclic di-GMP, although the *Pseudomonas aeruginosa* protein PA3947, which also has the EVL motif, does have activity (Kulasekara et al., 2006). One hypothesis is that binding of cyclic di-GMP to the (inactive) EVL domain modulates the ability of RocR to compete with RocA1 for binding to the RocS1 sensor, thus affecting *cup* gene expression (Kulasekara et al., 2005). It is also plausible that antagonism of RocS1 is not the only function of RocR and that phosphorylated RocR can be active in regulation of expression of other genes (Kulasekara et al., 2005).

A role for cyclic di-GMP in transcription of genes involved in polysaccharide synthesis associated with rugose colony morphology or biofilm formation has been demonstrated in *Vibrio* spp. In *Vibrio cholerae*, the CheY-EAL-HTH domain protein VieA acts to negatively regulate transcription of *vps* genes, required for exopolysaccharide synthesis and biofilm formation (Tischler and Camilli, 2004). These effects require the cyclic di-GMP phosphodiesterase activity of VieA, as shown by mutations in the EAL motif. In *Vibrio parahaemolyticus*, mutation of *scrC*, which encodes a GGDEF-EAL domain protein, leads to elevated levels of capsular polysaccharide production and increased transcription of *cpsA*, the first gene in an operon directing capsular polysaccharide biosynthesis (Boles and McCarter, 2002). The *scrC* gene is part of the *scrABC* operon. Transposon insertions in the *cps* operon or in an unlinked gene, *cpsR*, encoding a transcriptional regulator caused reversion of a strain with a polar mutation in *scrA* to a smooth colony phenotype (Güvener and McCarter, 2003). *CpsR* is not required for capsular polysaccharide production in *scrABC*⁺ strains however.

Elevated levels of cyclic di-GMP promote biofilm formation but repress motility and virulence factor synthesis

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, animal and plant pathogens (Figure 5.3). Elevation of the cellular level of cyclic di-GMP by expression of different GGDEF domain proteins promotes biofilm formation and reduces motility in a number of bacterial species including enteric bacteria such as *Salmonella* spp. and *Pseudomonas aeruginosa* (Simm et al., 2004). Conversely expression of EAL domain proteins reduces biofilm formation and enhances motility. In *Vibrio cholerae*, the CheY-EAL-HTH domain VieA positively activates expression of virulence genes *toxT*, which encodes a transcriptional regulator and *ctxAB*, which encode cholera toxin (Tischler et al., 2002; Tischler and Camilli, 2005) 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

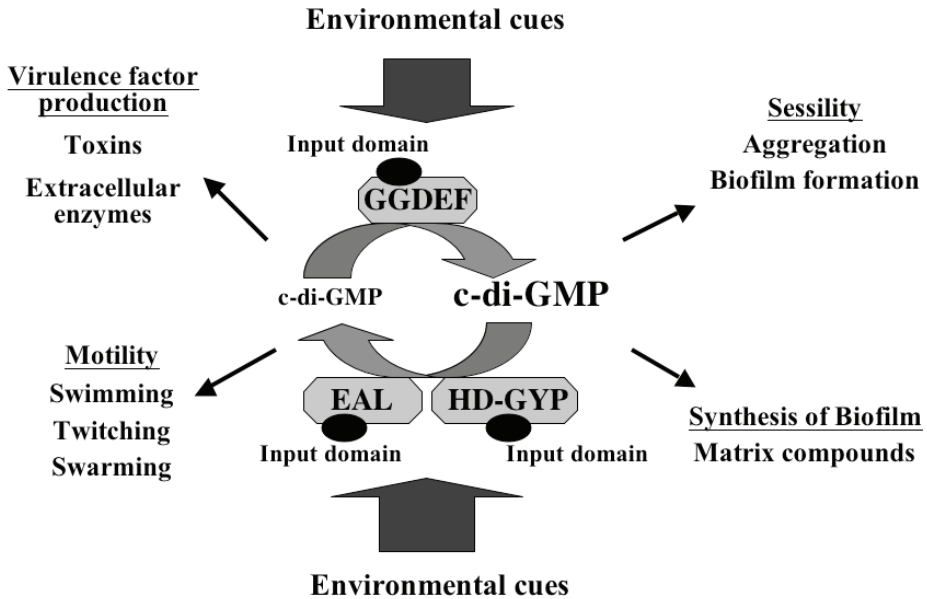


Figure 5.3 Cyclic di-GMP as a second messenger links the perception of environmental cues to changes in bacterial physiology and behavior. Proteins with GGDEF, EAL or HD-GYP domains have additional sensory input domains that are involved in the perception of diverse environmental cues. Alteration in the activity of the protein in cyclic di-GMP synthesis or hydrolysis occurs upon signal perception and leads to changes the cellular levels of cyclic di-GMP. In general elevated levels of cyclic di-GMP promote biofilm formation and sessility whereas low levels promote motility and virulence factor synthesis. However localized effects on specific functions may not follow these general rules. Further levels of regulation occur at the level of transcription of the genes encoding the signaling proteins and in their degradation by proteolysis (see text). Figure adapted from Simm *et al.* (2004).

and are abolished by mutation of the EAL amino acid motif. Similarly the CheY-HD-GYP domain regulator RpfG of the plant pathogen *Xanthomonas campestris*, which is required for full virulence, positively regulates the synthesis of extracellular enzyme virulence factors and motility, and negatively regulates biofilm formation (Slater *et al.*, 2000; Dow *et al.*, 2003; Crossman and Dow, 2004).

Cyclic di-GMP may contribute to bacterial pathogenesis in a number of other pathosystems. The GGDEF domain protein VirC contributes to the virulence of *Vibrio anguillarum* to fish (Milton *et al.*, 1995). 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 “stand-alone” EAL domain protein BvgR (Merkel and Stibitz, 1995; Merkel *et al.*, 1998a,b). BvgR-mediated regulation of gene expression contributes to respiratory infection of mice (Merkel *et al.*, 1998b). The “stand-alone” EAL domain protein CdgR of *Salmonella* is required for the bacterium to resist host phagocyte oxidase *in vivo* and contributes to virulence in mice (Hisert *et al.*, 2005).

These 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.

Although work on a number of bacteria has linked increased cyclic di-GMP levels with augmented biofilm formation, this generality is challenged by more recent work on biofilm formation in *P. aeruginosa* (Hoffman *et al.*, 2005; Kulesekara *et al.*, 2006). Mutation or over-expression of particular genes encoding GGDEF and EAL domain proteins has effects on biofilm formation that are not strictly correlated with the observed or predicted effects on cyclic di-GMP levels. These findings have been rationalized by suggesting that these signaling elements affect localized cytoplasmic cyclic di-GMP pools which influence specific processes contributing to biofilm formation (Hoffman *et al.*, 2005; Kulesekara *et al.*, 2006). This will be discussed in detail below.

Are cyclic di-GMP-dependent systems dedicated to specific cellular functions?

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 DgcA and PdeA proteins of *G. xylinus* co-purified 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 co-localizing proteins. In other words, certain cyclic di-GMP synthesis 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 regulation of specific bacterial processes under different environmental condition are consistent with the notion of an environmentally responsive network. This has been reported for the role of GGDEF domain proteins in cellulose synthesis in *Salmonella* (Römling *et al.*, 2000; Garcia *et al.*, 2004) and is also seen upon examination of the role of HD-GYP, GGDEF and EAL domain proteins in regulation of extracellular enzyme synthesis in *Xanthomonas campestris* (R.P. Ryan, Y. Fouhy, J. Lucey and J.M. Dow, unpublished).

The existence of localized pools of cyclic di-GMP has been proposed to explain the varied effects of mutation of GGDEF and EAL domain proteins on biofilm formation in *P. aeruginosa* (Hoffman *et al.*, 2005; Kulesekara *et al.*, 2006). In *P. aeruginosa* PAO1, the EAL domain protein Arr (for aminoglycoside response regulator) is required for biofilm formation in response to subinhibitory concentrations of the antibiotic tobramycin. A variant Arr protein with a mutation in the conserved EAL domain was not able to restore tobramycin-

cin-induced biofilm formation to an *arr* mutant of *P. aeruginosa*. This suggests that cyclic di-GMP degradation is required for biofilm formation, which contradicts the consensus view from work on a number of bacterial systems. Examination of the effects of mutation of other GGDEF and/or EAL domain proteins in *P. aeruginosa* PAO1 also reveals a complex relationship to biofilm formation (Hoffman *et al.*, 2005). A comprehensive mutational and over-expression study of the role of all 37 proteins with GGDEF and/or EAL domains in *Pseudomonas aeruginosa* PA14 has likewise shown that a number of genes with GGDEF or GGDEF/EAL domains affect biofilm formation (Kulesekara *et al.*, 2006). 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 mutation of the gene encoding the GGEEF protein PA3343, which is active in cyclic di-GMP synthesis, leads to a hyperbiofilm formation whereas overexpression of PA2879 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 (Kulesekara *et al.*, 2006).

These findings in *P. aeruginosa* have led to the suggestion 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 (Hoffman *et al.*, 2005; Kulesekara *et al.*, 2006). By inference, some functions contributing to biofilm formation 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 loss or over-expression 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 a great deal more 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.

What are the cellular levels of cyclic di-GMP?

Knowledge of the cellular levels of cyclic di-GMP is of considerable importance to an overall understanding of the role of the nucleotide in influencing bacterial behavior. Most simply it acts as a guide as to whether effects of cyclic di-GMP seen in *in vitro* experiments occur at physiologically relevant concentrations. However such measurements could also help to establish the *in vivo* role of GGDEF-EAL domain fusions, to examine any connection between cyclic di-GMP levels and the temporal expression of particular bacterial genes and to assess the physiological status of bacteria in different environments.

The measurement of cyclic di-GMP in bacterial cells presents considerable technical difficulties. It is found in only very low concentrations in most bacteria and may bind to cellular proteins thereby affecting extraction. A number of laboratories have used two-dimensional thin layer chromatography to separate labeled nucleotides extracted from growing cells in ³²P-labelled inorganic phosphate. The methodology is of low throughput however and is presumably restricted for use in specific growth conditions. MALDI-TOF and/or liquid chromatography coupled to mass spectrometric analyses are methods of choice, offering highly sensitive and accurate determinations from bacteria under a range of growth conditions. Available data show that ectopic elevation of cyclic di-GMP through the expres-

sion of GGDEF domain proteins can lead to levels of the nucleotide that are over 200 μM (Simm *et al.*, 2004). In contrast, Hisert *et al.* (2005) determined that in *Salmonella*, each cell has eight molecules of cyclic di-GMP, which if the nucleotide were freely distributed would put the cellular concentration in the pM range. There are proposals that localized pools of cyclic di-GMP may occur (see above), so the relationship between the concentration that is locally active and that measured as the cytoplasmic concentration is unclear.

In the cellulose-producing *G. xylinus*, the levels of cyclic di-GMP are estimated to be in the range of 5 to 10 μM . However free cyclic di-GMP may account for only 10% of total of intracellular pool, since the nucleotide is believed to bind to a number of cellular proteins. The level of cyclic di-GMP giving half-maximal activation of cellulose synthesis *in vitro* is 0.35 μM , which would be within the biologically relevant range however.

Environmental signals affecting biofilm formation through alterations in cyclic di-GMP levels

Very little is known about the environmental signals or cues that are recognized by the sensory domains of GGDEF, EAL and HD-GYP domain proteins or which activate two-component or chemotaxis-like signal transduction pathways involving these proteins. Although bioinformatic analysis can give some clues through the nature of the input domains (see Table 5.1), only in a few cases are the cognate signals known. The best-studied example is PdeA1, the cyclic di-GMP phosphodiesterase from *G. 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 with a K_d of approximately 10 μM . Removal of the heme results in a dramatic loss of enzymatic activity, whereas binding of oxygen to the heme reduces the activity approximately threefold. The consequences of oxygen sensing are thus a reduced ability to degrade cyclic di-GMP, which could conceivably lead to an elevation in the cellular levels, promoting cellulose production and pellicle formation. 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 (Delgado-Nixon *et al.*, 2000).

A second example of a defined environmental signal whose recognition is linked to cyclic di-GMP turnover is the cell–cell signaling molecule DSF of *Xanthomonas campestris* (Barber *et al.*, 1997; Slater *et al.*, 2000; Crossman and Dow, 2004). DSF has been characterized as a *cis*-unsaturated fatty acid (Wang *et al.*, 2004). Perception and transduction of the DSF signal is thought to involve a two-component system comprising the sensor histidine kinase RpfC and the CheY-HD-GYP domain regulator RpfG (Slater *et al.*, 2000; Crossman and Dow, 2004; Ryan *et al.*, 2006). It is proposed that recognition of DSF by RpfC leads to phosphorylation of RpfG and its activation in cyclic di-GMP hydrolysis, although this has not been directly demonstrated. Mutation of *rpfC*, *rpfG* or *rpfF* (which directs DSF synthesis), all lead to the formation of biofilms by *X. campestris* (Dow *et al.*, 2003).

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* (Karatan *et al.*, 2005). 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 (Bomchil *et al.*, 2003; Karatan *et al.*, 2005). The *nspS* and *mbaA* genes are arranged in an operon with a third gene that has little or no role under the conditions tested. It is proposed that NspS acts as a sensor for norspermidine and that interaction of a norspermidine-NspS complex with the periplasmic portion of MbaA reduces its ability to inhibit biofilm formation. This may occur through destabilization of an MbaA dimer or through conformational changes affecting MbaA activity (Karatan *et al.*, 2005).

Two laboratories have described effects of addition of antibiotics on biofilm formation in *Pseudomonas aeruginosa* that were influenced by the action of specific EAL domain proteins (Drenkard and Ausubel, 2002; Hoffman *et al.*, 2005). When plated in the presence of a number of antibiotics including kanamycin, tobramycin and tetracycline, antibiotic-resistant small colony variants of *P. aeruginosa* PA14 arise at a frequency of 10^{-7} to 10^{-6} (Drenkard and Ausubel, 2002). A percentage of these variants have a rough colony appearance and were called RSCV (for rough small-colony variant). The RSCV cells show increased attachment to surfaces and increased biofilm formation compared to the wild type. Small colony variants are also found in clinical samples from cystic fibrosis patients. RSCV cells can revert to the wild-type phenotype in the absence of antibiotic. A CheY-EAL domain two-component regulator, PvrR (for phenotype variant regulator), positively influences the rate of conversion. The mechanistic basis of these phenotypic variations and the role of cyclic di-GMP are however not known.

A more recent report (Hoffman *et al.*, 2005) shows that subinhibitory concentrations of tobramycin trigger biofilm formation in *P. aeruginosa*. This effect depends upon Arr (for aminoglycoside response regulator), a membrane-associated EAL domain protein with a periplasmic domain. The *arr* mutant phenotype is not due to altered frequency of RSCV variants. 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*. Hoffman *et al.* (2005) propose 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 (see also above).

Since biofilms and phase variation contribute to the increased resistance of *Pseudomonas aeruginosa* to antibiotics, compounds that affect PvrR or Arr function could have a role in the treatment of infections associated with cystic fibrosis (Drenkard and Ausubel, 2002; Hoffman *et al.*, 2005). Mechanisms related to those discussed above may have evolved to allow *Pseudomonas aeruginosa* to evade the action of antibiotics produced by other microorganisms co-inhabiting environments such as the soil and plant root surfaces.

The above examples illustrate the ability of bacteria to adopt different modes of growth as an adaptation to different environmental conditions. Studies of the effects of carbon starvation in *Pseudomonas putida* have shown that transitions from a biofilm to planktonic state can occur quite rapidly, within minutes of the application of the stress (Gjermansen *et al.*, 2005). A TM-GGDEF-EAL domain protein PP0165 is implicated in these transitions, although mechanistic details of the role of cyclic di-GMP in this rapid switch in lifestyle remain to be uncovered.

Regulation by transcription and protein degradation

In addition to 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. A number of studies have shown that the expression of genes encoding GGDEF and/or EAL domain proteins is differentially regulated under different environmental conditions. As we have seen above, the two-component regulator CsgD of *Salmonella* regulates transcription of *adrA*, a gene that encodes a GGDEF domain protein that is required for cellulose synthesis in the multicellular *rdar* phenotype (Römling *et al.*, 2000; Römling, 2005). CsgD is activated by as yet unidentified histidine kinase, which presumably responds to environmental cues. Whole-genome microarray technology has revealed a number of genes in *P. aeruginosa* whose expression responds to signals found in muco-purulent airway liquids collected from chronically infected cystic fibrosis patients (Wolfgang *et al.*, 2004). PA2567, encoding a cyclic di-GMP phosphodiesterase, is downregulated by more than 5-fold. This repression requires *P. aeruginosa* quorum sensing system since these effects are not observed in an *rhlRlasR* double mutant (Wolfgang *et al.*, 2004).

The formation of mixed biofilms between the hyperthermophiles *T. ermotoga maritima* and *Methanococcus jannaschii* is accompanied by increased expression in *T. maritima* of genes encoding synthesis of an extracellular polysaccharide. In addition two genes encoding GGDEF domain proteins are upregulated whereas a third gene encoding a GGDEF domain protein is down regulated (Johnson *et al.*, 2004). The synthesis of extracellular polysaccharide is also regulated by a peptide cell–cell signal (Johnson *et al.*, 2005). In *Vibrio cholerae*, the quorum-sensing regulator AphA, which influences the expression of virulence genes, also strongly represses the expression of an operon encoding proteins involved in acetoin biosynthesis (Kovacikova *et al.*, 2005). This molecule prevents intracellular acidification and is required for growth of *V. cholerae* on glucose. The acetoin biosynthesis operon also contains genes encoding a GGDEF domain protein and an EAL domain protein (Kovacikova *et al.*, 2005). It has yet to be determined whether these proteins influence biofilm formation or motility or whether cyclic di-GMP influences expression of the acetoin operon.

Several of these examples illustrate interplay between cyclic di-GMP signaling and extracellular cell–cell signaling (quorum-sensing), which also has a critical role in biofilm formation. This has led to the suggestion that the two signaling processes converge in the regulation of diverse bacterial behaviors (Camilli and Bassler, 2006). A direct connection between cell–cell signaling and cyclic di-GMP turnover has been proposed; perception and transduction of the DSF cell–cell signal in *Xanthomonas campestris* is thought to involve a two-component system involving the CheY-HD-GYP domain regulator RpfG (Slater *et al.*, 2000; Crossman and Dow, 2004), which is active in hydrolysis of cyclic di-GMP (Ryan *et al.*, 2006). Activation of a GGDEF/EAL domain protein by binding of a quorum-sensing molecule has not yet been reported however.

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 (Table 5.2, Perry *et al.*, 2004). 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 (Perry *et al.*, 2004). Regulation at the level of transcription and/or proteolytic degradation may be particularly important in determining the activity of “stand alone” EAL or GGDEF domain proteins, which lack sensory input domains. However it cannot be excluded that these EAL and GGDEF domains also bind small molecules that influence their enzymatic activity.

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 biofilm formation. The best-studied effect of cyclic di-GMP is its allosteric activation of cellulose synthesis in *G. xylinus*, where cyclic di-GMP has been shown to bind to BcsB, the β -subunit of cellulose synthase and to an uncharacterized 200 kDa protein (see above). 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 (Amikam and Galperin, 2006). PilZ was originally described as a protein involved in assembly of functional pili in *Pseudomonas aeruginosa* (Alm *et al.*, 1996). 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 phyletic distribution of the PilZ domain is generally similar to those of the GGDEF and EAL and some phenotypes of mutation of genes encoding PilZ domain proteins are consistent with a role in cyclic di-GMP regulation (Huang *et al.*, 2003; Amikam and Galperin, 2006). These bioinformatic predictions of the role of PilZ domain proteins are open to experimental verification. Key questions include what are cellular roles of PilZ proteins, do they bind cyclic di-GMP and is this binding required for their activity?

Other possible targets of cyclic di-GMP action should not be overlooked. By analogy with cyclic AMP, cyclic di-GMP may affect transcription of genes involved in virulence and biofilm formation by binding to transcriptional regulators. Since in a number of cases 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. One caveat is that transcriptome profiling reveals that elevation of the level of cyclic di-GMP alters the expression of a substantial number of genes in *Pseudomonas aeruginosa* (Hickman *et al.*, 2005) and *Escherichia coli* (Méndez-Ortiz, *et al.*, 2006). Such large-scale changes may make it difficult to distinguish direct and indirect effects of cyclic di-GMP and consequently to identify candidate transcription factors for further study.

How will an understanding of cyclic di-GMP signaling have an impact in biotechnology?

An understanding of the role of cyclic-di-GMP signaling in the regulation of bacterial biofilm formation and virulence could underpin the design of new strategies for the control of disease in both animals and plants. In the biomedical context, directed interference

to promote biofilm dispersal may improve the efficacy of antibiotic treatment to control persistent infections. This could potentially be achieved through blocking the perception of the environmental signal(s), modulation of the biochemical activity of the signaling domains or blocking the sites or targets of cyclic di-GMP action. An in-depth understanding of this sophisticated signaling system is needed to achieve the desired aims. For example knowledge of the biochemical activity of the domains involved in cyclic di-GMP turnover suggests that chemicals that inhibit GGDEF domain proteins or those that activate EAL domain proteins may have a role in triggering biofilm dispersal. However we now appreciate that there are particular signaling systems in which EAL or GG(D/E)EF proteins have opposite effects on biofilm formation from that expected from their activities against cyclic di-GMP. Furthermore potential effects on the activation of virulence factor synthesis cannot be overlooked. An understanding of how cyclic di-GMP exerts its effects on different cellular functions may allow blocking of biofilm formation without promotion of virulence factor synthesis.

The definition of those signaling elements that are key to biofilm formation and/or virulence under certain conditions may identify specific targets for intervention. Two examples from *Pseudomonas aeruginosa* illustrate this point; Arr, which positively influences tobramycin-induced biofilm formation and antibiotic resistance and PvrR, which influences phase variation, biofilm formation and virulence in thermally injured mice (Drenkard and Ausubel, 2002; Hoffman *et al.*, 2005; Kulesekara *et al.*, 2006).

Although most attention has been focused on animal and human pathogens, the ability of plant pathogenic bacteria to form and detach from biofilms may equally have considerable implications for the completion of their disease cycle. An understanding of these processes at the molecular level and their role in virulence opens a new perspective for the control of plant disease. Interference with the ability of pathogens to attach to and form biofilms during epiphytic growth phases on leaf surfaces, to attach and from aggregates on internal plant surfaces such as within xylem elements or to associate with insect vectors may all have a role in controlling disease.

More research is also required to examine potential industrial applications for cyclic di-GMP “technologies.” For example will alteration of cyclic di-GMP levels enhance the production of microbial products such as polysaccharides, secondary metabolites and enzymes of commercial importance? Will the ability to control biofilm formation or dispersal by regulated ectopic expression of GGDEF or EAL domains have a role in microbial fermentations?

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. The research in this area has built upon the seminal findings of the group of Moshe Benziman, 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 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 such as developmental transitions, adhesion and virulence, in addition to biofilm formation. Despite this significant progress, there are many questions that remain unanswered. Principal amongst these are 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 investigation 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. There are a host of other questions. Are there networks of interacting systems? What are the cues that activate or inactivate the signaling proteins? Are further domains involved in cyclic di-GMP turnover? Can the cellular level of cyclic di-GMP be regulated by export from the cell? Can bacteria take up cyclic di-GMP? 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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