c-di-GMP-mediated regulation of virulence and biofilm formation

Peggy A Cotter¹ and Scott Stibitz²

It is now apparent that the signaling molecule 3',5'-cyclic diguanylic acid (c-di-GMP) is a central regulator of the prokaryote biofilm lifestyle and recent evidence also links this molecule to virulence. Environmentally responsive signal transduction systems that control expression and/or activity of the enzymes (GGDEF and EAL domain containing proteins) that are responsible for synthesis and degradation of c-di-GMP have recently been identified. Members of the phosphorelay family feature prominently amongst these systems, which include several with hybrid polydomain sensors and one that is similar to well-characterized chemotaxis-controlling pathways. These findings support the hypothesis that c-di-GMP levels are tightly controlled in response to a broad range, in terms of both diversity and intensity, of extracellular signals. Insight into how c-di-GMP affects changes in gene expression and/or protein activity has come from the demonstration that proteins containing the PilZ domain can bind c-di-GMP and control phenotypes involved in biofilm formation and virulence. These recent developments should pave the way for researchers to answer the important question of how a vast array of extracellular signals that are sensed by multiple sensory transduction pathways which all lead to the production or destruction of c-di-GMP are coordinated such that the appropriate phenotypic response is produced.

Addresses
¹ Department of Molecular, Cellular, and Developmental Biology, University of California, Santa Barbara, Santa Barbara, CA 93106-9610, USA
² Division of Bacterial, Parasitic and Allergenic Products, Center for Biologics Evaluation and Research, Food and Drug Administration, 8800 Rockville Pike, Bethesda, MD 20892, USA

Corresponding author: Cotter, Peggy A (cotter@lifesci.ucsb.edu)

Introduction

Most, if not all, bacteria are able to live either as independent planktonic cells or as members of organized surface-anchored communities called biofilms [1]. As both lifestyles confer specific advantages and liabilities, choosing the right one in any particular environment is crucial for bacterial survival. For pathogens, it appears that this choice is often tied to virulence. Vibrio cholerae, for example, is thought to form biofilms on the chitinous exoskeleton of zooplankton and phytoplankton [2,3], but switches to a planktonic form upon arrival in the mammalian gut where it causes the profusely diarrheal disease cholera [4–6]. For the phytopathogen Xanthomonas campestris pathovar campestris, biofilm formation is important for colonization of leaf surfaces, but planktonic growth is important for vascular disease [7]. The ubiquitous environmental bacterium Pseudomonas aeruginosa is thought to exist in biofilms in ex vivo environments, and factors required for this lifestyle are expressed reciprocally with those required to cause acute infections in burns and other wounds [8]. By contrast, colonization of pacemakers and other indwelling devices by Staphylococci and chronic lung infection of cystic fibrosis patients by P. aeruginosa is facilitated by biofilms, which might also be responsible for the remarkable resistance these infections display [8,9]. Whereas much has been learned regarding control of virulence gene expression, until recently almost nothing was known about how the crucial switch between biofilm and planktonic lifestyles is regulated.

c-di-GMP is a key regulator of biofilm versus planktonic growth

Pioneering work from a handful of laboratories over the past few years has revealed the core of an evolutionarily conserved regulatory mechanism for controlling phenotypes associated with the biofilm lifestyle. The central feature of these systems is the second messenger 3',5'-cyclic diguanylic acid (c-di-GMP), which is synthesized by diguanylate cyclase (DGC) and degraded by phosphodiesterase A (PDEA; see [10,11,12] for recent excellent reviews on this topic). The basic system was actually discovered in the 1980s through the work of Benzimen and colleagues [13,14] who found that c-di-GMP was a positive allosteric activator of the cellulose synthase enzyme used by Gluconacetobacter xylinus to produce an extracellular cellulose matrix. The current explosion of interest in this signaling mechanism is fueled, in part, by our ability to recognize the genes encoding putative DGCs and PDEAs in newly sequenced bacterial genomes. DGCs share sequence similarity and are called GGDEF proteins after the conserved residues in their active site. PDEAs are members of the EAL domain family, similarly named, although recently a type II c-di-GMP-specific phosphodiesterase (HD-GYP) family member was also shown to have PDEA activity [15]. Many GGDEF and EAL proteins contain both types of domain, yet in all cases examined, these proteins possess only one enzymatic activity, with the enzymatically inactive domain potentially serving a regulatory function [16–18].
A role for c-di-GMP in controlling biofilm formation was first proposed independently in 2002 by McCarter and colleagues [19] working with *Vibrio parahaemolyticus* and D’Argenio and colleagues [20] working with *P. aeruginosa* when they identified genes encoding GGDEF and EAL domain proteins that affected biofilm formation when mutated and/or overexpressed. Although additional genes encoding similar proteins were subsequently identified and shown to affect biofilm phenotypes (e.g. see [21,22]), convincing evidence linking GGDEF and EAL domain proteins to both c-di-GMP levels and biofilm formation was provided by two studies published in 2004. Tischler and Camilli [23] showed that overexpression of an isolated GGDEF domain in *V. cholerae* resulted in increased expression of tcpA–Q genes, which encode *V. cholerae* exopolysaccharide (VPS), concomitant with increased c-di-GMP levels, and that the opposite effect was achieved by overexpression of an isolated EAL domain. Simm et al. [24] showed that AdrA of *Salmonella typhimurium*, a GGDEF domain protein, is required for activation of cellulose biosynthesis and the associated pdar (pink, dry and rough) phenotype. Overexpression of *adrA* resulted in increased c-di-GMP levels, and overexpression of *yjhH*, which encodes an EAL domain protein, resulted in decreased pdar phenotype and decreased c-di-GMP levels. This group also showed that overexpression of these *Salmonella* genes in *P. aeruginosa* had similar effects.

Thus, the core of this regulatory system appears to be that GGDEF and EAL domain proteins control intracellular c-di-GMP levels, and c-di-GMP levels control the transition between biofilm and planktonic lifestyles. How is expression and/or activity of GGDEF and EAL domain proteins regulated in response to environmental cues? And how are varying intracellular levels of c-di-GMP translated into meaningful changes in gene expression or protein activity (Figure 1)? Moreover, many bacteria have the potential to express many GGDEF and/or EAL domain proteins. Genome sequence data predicts 34 such proteins for *P. aeruginosa* and 61 for *V. cholerae*, and recent comprehensive analyses indicate that most, if not all, do in fact function as DGCs or PDEAs and play roles in controlling phenotypes that influence biofilm formation and virulence [25,26]. How is the expression and/or activity of all of these proteins coordinated? And, even more perplexing, how are the ultimate affects of altered c-di-GMP levels coordinated with the specific enzymes that caused them? Studies published in the last two years have begun to shed light on the answers to the first two of these questions.

### How is expression and/or activity of GGDEF and EAL domain proteins controlled?

#### *V. cholerae*

In *V. cholerae*, two regulatory systems have been identified in which expression or activity of GGDEF and/or EAL domain proteins is regulated in response to extracellular cues. Both systems appear to respond to signals present during intestinal colonization and both are also involved in the control of expression of virulence genes. In fact, these systems were discovered by researchers investigating bacterial virulence rather than biofilm formation.

*V. cholerae* virulence gene expression is controlled by a complex cascade of regulatory factors. Although AphA and AphB are viewed as the apex of this cascade, under conditions of high cell density *aphA* expression is repressed by the LuxR homolog HapR through a LuxO-mediated control of biofilm formation and virulence gene expression. In fact, these systems were discovered by researchers investigating bacterial virulence rather than biofilm formation.

#### Genes

The *tcpA–F* operon, which encodes the TcpPH regulatory system [28]. TcpPH, together with an analogous system, ToxRS, activates transcription of *toxT*, which encodes an AraC-like transcriptional activator (*ToxT*) that activates transcription of genes encoding the toxin co-regulated pilus (*tcpA–F*) and, together with ToxRS, cholera toxin (*ctxAB*) [29]. Skorupski and colleagues [30*] used microarray technology to search for additional *AphA*-regulated genes. Amongst those identified was an operon that encodes enzymes involved in acetoin production, and within this operon were two genes, *acgA* and *acgB*, that are predicted to encode proteins containing EAL and GGDEF domains, respectively. Overexpression of *acgA* caused decreased biofilm formation and increased motility, whereas overexpression of *acgB* had the opposite effect. Deletion of either gene, however, had no discernible effect on motility or biofilm formation and, despite the fact that their products have apparently opposing activities, both genes are regulated in the same (negative) way by AphA. Whereas it is not yet clear how this regulation is coordinated with the overall schema, these results reveal a potential link between c-di-GMP-mediated control of biofilm formation and virulence gene expression.

---

**Figure 1**

Questions regarding c-di-GMP-mediated regulation. How is expression or activity of GGDEF and EAL domain proteins controlled? How is c-di-GMP used to effect phenotypic changes?
The vieSAB locus was identified twice by Camilli and colleagues [31,32], in two different genetic screens: one designed to identify any ‘in vivo-induced’ genes, and the other to select specifically genes required for full induction of ctxAB in vivo. VieS is a polydomain sensor of the phosphorelay family that contains two periplasmic binding protein domains at its N-terminus, and histidine kinase, receiver and histidine phosphotransferase domains at its cytoplasmically located C-terminus (Figure 2). VieA is a protein that contains an N-terminal receiver domain, an EAL domain, a partial receiver domain and a C-terminal helix-turn-helix (HTH) putative DNA-binding domain. VieB is a protein that contains both receiver and tetratricopeptide repeat domains at its N-terminus and a region with no similarity to known protein domains at its C-terminus. Using Tn5lac to screen for genes regulated by VieA, Camilli and Tischler [23] found that expression of genes within the vpsA–Q locus was significantly decreased when VieA was overexpressed. Decreased vpsA–Q expression was as a result of decreased transcription of vpsR, which encodes a positive regulator of the vpsA–Q genes. Unexpectedly, the HTH domain of VieA contributed only modestly to this effect, and VieA receiver domain phosphorylation was also not required. Instead, the EAL domain was shown to be both necessary and sufficient to prevent vpsR transcription. Mutational studies demonstrated that this effect, as well as the ability of VieSAB to induce ctxAB transcription [33*], requires the PDEA activity of the EAL domain, which this group also demonstrated biochemically [34*]. Although the precise functions of the various domains are yet to be determined, a model that fits the available data is one in which phosphorylation of the VieA receiver domain activates DNA binding, resulting in increased transcription of vieA (VieA appears to function as a positive autoregulator, explaining the modest contribution of the HTH domain to repression of vpsR), and the resulting increased production of VieA.

**Figure 2**

*V. cholerae* c-di-GMP regulatory network. The two environmentally responsive regulatory systems discussed in the text are shown. Black arrows and bars represent activation or repression of the genes shown or the genes encoding the proteins shown (not necessarily direct). Blue arrows emanating from the GGDEF and EAL domain proteins indicate that these proteins perform the function to which the arrow points. The green and red arrows represent DGC and PDEA activity, respectively. Presumed phosphorylation reactions within and between domains of VieS, VieA and VieB are not represented. The thick gray line represents the cytoplasmic membrane; the peptidoglycan and outer membrane are not represented. Domain name abbreviations: HK, histidine kinase and associated histidine kinase ATPase; Hpt, histidine phosphotransferase; HTH, helix-turn-helix; PBP, periplasmic binding protein; Rec, receiver; TPR, tetratricopeptide repeat; ?, unknown.
decreases c-di-GMP levels through the PDEA activity of its EAL domain. Although also not proven, it is probable that it is VieS that is responsible for phosphorylating VieA in response to some as yet unidentified signal present within the mammalian intestine.

**P. aeruginosa**

Two environmentally responsive regulatory systems that are known or suspected to affect c-di-GMP levels have also been identified in *P. aeruginosa*. The *wsp* locus is homologous to loci encoding well-characterized chemotaxis-related signaling pathways. Predicted protein products include a methyl-accepting chemotaxis protein (MCP)-like sensor (*WspA*), CheW-like adaptor proteins (*WspB* and *WspD*), a methyl transferase (*WspC*), a methyl esterase (*WspF*), a hybrid histidine kinase that is similar to CheA but which also contains a receiver domain (*WspE*) and, in place of CheY, *WspR*, which contains a receiver domain and a GGDEF domain (Figure 3). The available data suggest that this system functions analogously to chemotaxis-controlling systems but, instead of altered flagellar rotation, the output is c-di-GMP production [20,35**]. The presence of an MCP, a methyl transferase and a methyl esterase suggest that the system responds not only to (as yet unknown) environmental cues but also to changes in the intensity of those cues over time. As suggested by Harwood and colleagues [35**], the system therefore appears to be capable of fine-tuning c-di-GMP levels, possibly as bacteria transition in and out of different niches within the complex biofilm community.

The *rocSIRA1/sadSRA* locus was identified simultaneously by Kulasekara et al. [36**] and Kuchma et al. [37**] in screens for mutants with altered biofilm-forming ability (a second locus, *rocS2A2*, was also identified, but...
was not studied further). It is predicted that rocS1/sadS encodes a hybrid sensor that is remarkably similar to V. cholerae VieS and that rocA1/sadA encodes a typical response regulator with N-terminal receiver and C-terminal DNA binding domains (Figure 3). The predicted rocR/sadR gene product contains N-terminal receiver and C-terminal EAL domains. Two-hybrid analysis indicates that both RocA1/SadA and RocR/SadR interact with RocS1/SadS, and mutational and overexpression analyses implicate RocS1/SadS and RocA1/SadA as positive regulators of cupB and cupC, which encode fimbiac involved in biofilm formation [36**]. RocR/SadR, however, appears to be involved in repression of cup gene expression. Kuchma et al. [37**] have shown that the system also controls genes encoding the P. aeruginosa T3SS and at least two of its cognate effector proteins, and that it inversely regulates genes involved in forming biofilms. Whereas it might seem paradoxical that RocA1/SadA and RocR/SadR have opposite affects on the expression of target genes — especially if, as the phosphorelay paradigm predicts, both are phosphorylated by RocS1/SadS — details regarding the signaling mechanism are still lacking and the effect that phosphorylation has on the activities of RocR/SadR and RocA1/SadA1 are unknown. Nonetheless, like the V. cholerae VieSAB system with which it bears striking similarity, this system appears to control reciprocal expression of biofilm and virulence phenotypes in response to environmental signals in a phosphorelay-dependent manner, possibly through c-di-GMP.

**How does c-di-GMP function mechanistically to control gene expression?**

Mechanisms by which variations in c-di-GMP levels are converted into changes in gene expression remain one of the least understood aspects of c-di-GMP-mediated regulation. Although c-di-GMP was shown to act as an allosteric activator of the cellulose synthase enzyme complex of G. xylinus in 1990 [38], no other c-di-GMP-binding proteins (other than DGCs and PDEA enzymes) were identified until very recently. This was partly because of the fact that cross-linking studies suggested that c-di-GMP bound the BcsB subunit of cellulose synthase, a protein with no significant similarity to other known proteins [39]. A recent bioinformatics analysis by Amikam and Galperin [40*], however, identified a ‘PilZ domain’, named after the PilZ protein from P. aeruginosa, in the cellulose synthase BcsA subunit, as well as in several proteins containing GGDEF, EAL or HD-GYP domains, implicating it as a probable candidate for a c-di-GMP regulatory target. Two groups have now shown that PilZ domains do in fact bind c-di-GMP, and with affinities appropriate for sensing the range of intracellular c-di-GMP concentrations that have been measured. Ryenkov et al. [41**] used the Escherichia coli YcgR protein and the isolated PilZ domains from YcgR and G. xylinus BcsA, whereas Pratt et al. (J Pratt, A Tischler, R Tamayo, A Camilli, unpublished data) used predicted PilZ domain proteins from V. cholerae (named PlzA-E). In both studies, mutational analyses demonstrated the importance of conserved residues within the PilZ domains for c-di-GMP binding and in vivo function, including phenotypes related to biofilm formation and, in the case of V. cholerae, virulence. The next step, of course, will be to determine what activities these PilZ domain proteins acquire upon c-di-GMP binding.

**Conclusion**

Research in the last couple of years has provided significant insight into how the transition between planktonic and communal lifestyles is regulated. Several environmentally responsive signal-transduction systems that control expression and/or activity of GGDEF and EAL domain proteins have been identified, and the PilZ domain has emerged as one mechanism by which c-di-GMP levels can be sensed and converted into changes in gene expression or protein activity. These discoveries represent major advances in our understanding of c-di-GMP-mediated signaling as it relates to both biofilm formation and virulence. However, as expected, many questions remain unanswered and even more have been generated. One issue that we find particularly intriguing is the apparent preponderance of phosphorelay-type signaling systems involved in controlling GGDEF and EAL and HD-GYP proteins (additional hybrid sensors with demonstrated or implicated links to c-di-GMP control that were not discussed because of space limitations include RetS/RtsM, GacS and LadS of P. aeruginosa [42–44] and RpfC of X. campestris [15,45]). An important question that is currently unanswered with regard to two-component signal transduction relates to what advantage, if any, the four-step phosphorelay provides over the simpler two-step phosphotransfer mechanism. On the basis of the BvgAS phosphorelay that controls virulence gene expression in Bordetella species, we have hypothesized that phosphorelays containing polydomain sensors enable rheostat-like responses to a range of signal intensities [46]. The Bvg domain structure of BvgS is identical to those of VieS and RocS1/SadS and, as demonstrated for VieS, BvgS is also involved in activation of virulence gene expression in vivo [47–51]. The bcrASR locus is also similar to vieSAB and rocSRA1/sadSRA, including that it contains a gene encoding an EAL domain protein, bcrR (bcrR was the first identified member of the EAL domain family) [52]. Whether VieS, RocS1/SadS, or any of the other hybrid sensors involved in controlling GGDEF and EAL domain proteins function in a rheostat manner remains to be explored. Involvement of the chemosensory-like Wsp system in P. aeruginosa however, suggests a need for controlling GGDEF and EAL domain proteins over a broad range of signal intensities. Alternatively, or additionally, the inclusion of polydomain sensors in these systems might reflect the fact that, except in the case of BvgS, the sensors appear to interact with at least two response regulator
proteins. It might be that conformational constraints limit the ability of histidine kinase domains to interact specifically and discretely with multiple receiver domains. The lack of the ATPase subdomain might render Hpt functionally and discriminately with multiple receiver domains.

Acknowledgements
We thank Steve Lory, Andrew Camilli, Jeff F Miller, Corinne Williams and Allison Jones for insightful comments on the manuscript and Andrew Camilli for sharing data prior to publication. We apologize to many investigators who have contributed to this field but whose work we could not highlight or cite due to space limitations. Work in the authors’ laboratories is supported by grants from the National Institutes of Health to PAC.

References and recommended reading
Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- **of outstanding interest


This paper describes the discovery of the V. cholerae acgA and acgB genes and, through their characterization, the demonstration of a link between regulation of virulence genes and genes encoding GGDEF and EAL domain proteins that are likely to be involved in regulation of biofilm phenotypes.


This paper demonstrates a role for c-di-GMP in control of cholera toxin gene expression in V. cholerae.


This paper shows biochemically that the EAL domain of V. cholerae VirA is a cyclic diguanylate phosphodiesterase.


Here, Hickman et al. show that the P. aeruginosa Wsp system, which is analogous to chemotaxis-controlling systems, controls biofilm formation by controlling c-di-GMP levels with the EAL domain protein WspR.


See annotation for [37**].


These papers [36**] and [37**] identify the RocS1RA1/SadSRA regulatory system and demonstrate its role in controlling the regulation of genes involved in virulence and biofilm formation.


This bioinformatic analysis provided compelling evidence for the potential c-di-GMP-binding ability of the PiZ domain.


This paper demonstrates that PiZ domain proteins are able to bind c-di-GMP and that PiZ domain proteins, and their c-di-GMP binding ability, are important for controlling biofilm and virulence phenotypes.


