HHS Public Access

Author manuscript

Annu Rev Cell Dev Biol. Author manuscript; available in PMC 2016 July 07.

Published in final edited form as:

Annu Rev Cell Dev Biol. 2012 ; 28: 439–462. doi:10.1146/annurev-cellbio-101011-155705.

Second Messenger Regulation of Biofilm Formation: Breakthroughs in Understanding c-di-GMP Effector Systems

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Abstract

The second messenger bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) has emerged as a broadly conserved intracellular signaling molecule. This soluble molecule is important for controlling biofilm formation, adhesion, motility, virulence, and cell morphogenesis in diverse bacterial species. But how is the typical bacterial cell able to coordinate the actions of upward of 50 proteins involved in synthesizing, degrading, and binding c-di-GMP? Understanding the specificity of c-di-GMP signaling in the context of so many enzymes involved in making, breaking, and binding the second messenger will be possible only through mechanistic studies of its output systems. Here we discuss three newly characterized c-di-GMP effector systems that are best understood in terms of molecular and structural detail. As they are conserved across many bacterial species, they likely will serve as central paradigms for c-di-GMP output systems and contribute to our understanding of how bacteria control critical aspects of their biology.

Keywords

motility; adhesion; exopolysaccharide; diguanylate cyclase; phosphodiesterase

INTRODUCTION

Finely tuned sensory systems enable bacteria to sense and respond to fluctuating environments and coordinate adaptive changes in metabolic pathways and physiological outputs. By integrating environmental cues, bacteria are able to make important decisions regarding how to respond to their constantly changing environs. Formation of a community of bacterial cells, or a biofilm, is one possible bacterial adaptation. These communities are often more advantageous than life as a planktonic cell, as biofilms have increased tolerance to antibiotics and the ability to survive periods of environmental stresses (Costerton et al. 1995, Hogan & Kolter 2002). Biofilms are found in natural, industrial, and clinical settings. Bacterial biofilms in nature play key roles in the production and degradation of organic matter, the degradation of many pollutants, and the cycling of nitrogen and sulfur. Industrial biofilms are utilized to process sewage and treat contaminated groundwater. Biofilms within the medical setting are quite problematic; they are much more antibiotic tolerant than their

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planktonic counterparts, and contamination of medical implants can occur owing to the bacteria's ability to form biofilms on abiotic surfaces (Davey & O'Toole 2000).

Bacteria transition from a planktonic to a sessile lifestyle in response to environmental cues such as osmolarity, pH, carbon availability, iron availability, oxygen tension, and temperature (Jackson et al. 2002a, 2002b; Lugtenberg et al. 1999; O'Toole & Kolter 1998b; O'Toole et al. 2000; Prigent-Combaret et al. 1999, 2001; Sauer et al. 2004; Singh et al. 2002; Thormann et al. 2006). Extensive research has revealed an array of cellular factors and diverse molecular mechanisms that are required for the transition from a planktonic to a sessile mode of life and the subsequent development of a biofilm. A unifying theme across bacterial species, however, is that biofilm formation coincides with the synthesis of the cellular signaling molecule bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP; Figure 1).

c-di-GMP has emerged as a broadly conserved bacterial intracellular second messenger. c-di-GMP was initially described as an activator of cellulose synthase in *Gluconacetobacter xylinus*, formerly *Acetobacter xylinum* (Ross et al. 1987). More recently this soluble molecule has been shown to also be important for controlling adhesion, motility, virulence, and cell morphogenesis in diverse bacterial species by exerting control at the level of transcription, translation, and posttranslation (Hengge 2009, Romling et al. 2005, Wolfe & Visick 2008). Over the past five to seven years, we have learned a great deal about how c-di-GMP is synthesized and degraded. Diguanylate cyclases (DGCs) containing the GGDEF domain, defined by this highly conserved set of amino acids, synthesize c-di-GMP from two molecules of GTP (Paul et al. 2004), whereas phosphodiesterases (PDEs) containing the EAL domain (Christen et al. 2005, Tischler & Camilli 2004) or HD-GYP domain (Ryan et al. 2006a), defined by these respective amino acids motifs, degrade c-di-GMP to 5′-phosphoguanylyl-(3′-5′)-guanosine (pGpG) or guanosine monophosphate (GMP), respectively (Figure 1). Many proteins contain both GGDEF and EAL domains but typically exhibit only DGC or PDE activity. GGDEF and EAL domains often occur in multidomain proteins in combination with diverse regulatory domains common to bacterial signaling proteins (Galperin et al. 2001). These regulatory domains, in response to the environment and environmental cues, can induce the synthesis or degradation of c-di-GMP in response to signals such as light (Barends et al. 2009, Tarutina et al. 2006) and oxygen (Chang et al. 2001, Qi et al. 2009, Sawai et al. 2010, Tuckerman et al. 2009, Wan et al. 2009), or through posttranslational modifications such as phosphorylation (Paul et al. 2007, Rao et al. 2009) or proteolysis (Tarutina et al. 2006).

c-di-GMP Signaling Pathways

Whole-genome sequencing has revealed that GGDEF and EAL domains are ubiquitous in bacteria; often dozens of these domain-containing proteins are found within a single bacterium (Hengge 2009). *Escherichia coli* contains 29 predicted c-di-GMP DGC or PDE proteins, whereas *Salmonella enterica* has 19 such proteins, *Pseudomonas aeruginosa* 41 proteins, *Vibrio cholerae* 72 proteins (Povolotsky & Hengge 2012), and *Pseudomonas fluorescens* 43 proteins (Newell et al. 2011b). Early studies focused on functionally analyzing GGDEF and EAL domain-containing proteins to biochemically demonstrate their

ability to synthesize and degrade c-di-GMP, respectively (Christen et al. 2005, Hickman et al. 2005, Paul et al. 2004, Schmidt et al. 2005, Tamayo et al. 2005).

Although the transition from a motile to a sessile lifestyle seems to generally require an increase in the concentration of c-di-GMP, until recently, the specific outputs impacted by c-di-GMP have been understudied. The surprisingly large number of predicted c-di-GMP signaling proteins in many bacterial species suggests that c-di-GMP signaling networks are extraordinarily complex. How microbes integrate multiple input signals to regulate critical biological processes and how the action of multiple enzymes involved in c-di-GMP metabolism can be integrated to control bacterial behaviors are critical questions to address if we hope to understand the mechanisms underlying how this second messenger exerts its affects.

Systematic analyses of DGCs and PDEs in *E. coli*, *Salmonella*, *V. cholerae*, and *P. fluorescens* have revealed that motility, virulence, and biofilm formation are regulated by c-di-GMP synthesized by a subset of DGCs. Boehm et al. (2010) identified a c-di-GMP network consisting of the DGCs YfiN, YegE, YedQ, and YddV as well as the PDE YhjH that synthesizes and degrades c-di-GMP to regulate motility in *E. coli*. In *Salmonella*, Ahmad et al. (2011) have revealed a complex c-di-GMP network, whereby distinct groups of GGDEF and EAL domain-containing proteins affect virulence phenotypes, including invasion of epithelial cells, induction of the proinflammatory cytokine interleukin-8, and biofilm formation. Systematic mutagenesis studies of c-di-GMP signaling proteins in *V. cholerae* conducted by the Yildiz laboratory (Beyhan & Yildiz 2007; Beyhan et al. 2006, 2007, 2008; Lim et al. 2006) identified several specific DGCs and PDEs that impact colony rugosity at transcriptional and posttranscriptional levels. Epistasis experiments suggest c-di-GMP signaling proteins may act in parallel to control rugosity. In *P. fluorescens*, Newell et al. (2011b) identified a c-di-GMP network consisting of four DGCs that synthesize c-di-GMP for biofilm formation. Newell and colleagues suggest that a particular subset of DGCs specifically control c-di-GMP-regulated outputs such as adhesin localization and motility. Thus, understanding how multiple c-di-GMP signaling pathways are isolated from each other, or integrated to produce coherent outputs, is a question of fundamental importance for understanding an array of c-di-GMP-mediated biological processes in bacteria.

c-di-GMP Effectors in a World of Complex Input Pathways

c-di-GMP networks must have mechanisms to both generate this signal and respond to its changing levels. Thus, to complement those enzymes involved in the synthesis and degradation of c-di-GMP, effector systems allow microbes to respond to changing levels of this second messenger. c-di-GMP receptors or effectors are c-di-GMP binding proteins or RNA; c-di-GMP binds to and exerts its control by altering the structure and output function of the effector (Hengge 2009).

Amikam & Galperin (2006) were the first to implicate the PilZ domain as a specific c-di-GMP binding motif. PilZ domains are defined by an amino acid motif of extensively conserved residues (Amikam & Galperin 2006). Their bioinformatic study suggested that the PilZ domain is present in numerous bacterial cellulose synthases, including Alg44, an alginate biosynthesis protein found in *P. aeruginosa*, and the YcgR protein of *E. coli*.

Functional studies later confirmed that the PilZ domain of Alg44 and YcgR binds c-di-GMP (Merighi et al. 2007, Ryjenkov et al. 2006). Importantly, the bioinformatic study of Amikam & Galperin (2006) revealed that for some bacterial genomes that encode GGDEF-containing proteins, PilZ domains are not always present, which suggests that protein domains other than PilZ may be able to act as c-di-GMP effectors.

Additional protein domains and motifs shown to bind c-di-GMP (summarized in Table 1) include the EAL domain of FimX from *P. aeruginosa* and of LapD from *P. fluorescens* (Navarro et al. 2009, Newell et al. 2009), the RxxD motif of PopA from *Caulobacter crescentus* and of PelD from *P. aeruginosa* (Duerig et al. 2009, Lee et al. 2007), the W[F/L/M][T/S]R motif of CpsQ from *Vibrio parahaemolyticus* and of VpsT from *V. cholerae* (Ferreira et al. 2011, Krasteva et al. 2010), the interface between a cyclic nucleotide-binding and DNA-binding domain of Clp from *Xanthomonas campestris* (Chin et al. 2010, Tao et al. 2010), the GEMM motif of c-di-GMP riboswitch class I (Sudarsan et al. 2008), and the pseudoknot motif of c-di-GMP riboswitch class II (Smith et al. 2011). Although Bcam1349 from *Burkholderia cenocepacia*, FleQ from *P. aeruginosa*, Clp from *Xanthomonas axonopodis*, and polynucleotide phosphorylase (PNPase) from *E. coli* (Fazli et al. 2011, Hickman & Harwood 2008, Leduc & Roberts 2009, Tuckerman et al. 2011) bind c-di-GMP, which domain or motif is necessary for binding is unclear.

Effectors bound to c-di-GMP utilize a range of mechanisms to relay signals to cellular processes and impact exopolysaccharide synthesis (Lee et al. 2007, Merighi et al. 2007, Weinhouse et al. 1997), motility (Boehm et al. 2010, Christen et al. 2007, Fang & Gomelsky 2010, Guzzo et al. 2009, Paul et al. 2010, Pratt et al. 2007, Ryjenkov et al. 2006), transcription (Hickman & Harwood 2008, Krasteva et al. 2010, Leduc & Roberts 2009), and subcellular (Duerig et al. 2009) or cell-surface protein localization (Monds et al. 2007, Newell et al. 2011a). Clearly, c-di-GMP modulates multiple outputs through discrete mechanisms. Even though many c-di-GMP metabolizing enzymes and effectors are expressed simultaneously, subsets of enzymes impact discrete phenotypic outputs, and bacterial cells are able to coordinate the actions of complex c-di-GMP networks. This coordination is likely achieved through both temporal and spatial regulation (Hengge 2009). Some DGCs, PDEs, and effectors may be expressed only under particular growth conditions or in response to environmental changes (Hengge 2009). For example, *rapA*, which encodes a PDE in *P. fluorescens*, is transcribed in response to low levels of inorganic phosphate (P_i) (Monds et al. 2007), whereas YcgR, an effector protein in *E. coli*, is coregulated with the genes required for the synthesis of the flagellum (Girgis et al. 2007). c-di-GMP signaling components may associate with one another in multiprotein complexes or microcompartments within the cell to influence the local concentration of c-di-GMP (Hengge 2009). In support of this idea, fluorescent c-di-GMP sensors have demonstrated both temporal and spatial separation of c-di-GMP pools (Christen et al. 2010), whereas work in *P. aeruginosa* has shown a lack of correlation between total c-di-GMP pools and particular phenotypic outputs (Merritt et al. 2010). Additionally, DGCs and PDEs in the same pathway can interact with one another (Andrade et al. 2006, Bobrov et al. 2008), and direct communication between an effector protein and a downstream target has been suggested (Oglesby et al. 2008). The diverse ways in which effector proteins bind to c-di-GMP and interact with DGCs and PDEs allow bacteria to confer the specificity needed for the

regulation of various outputs, a notion first suggested by early work characterizing this signal (Ross et al. 1987).

In this review we first focus our discussion on characterized c-di-GMP effector systems for which the mechanism has been explored in some detail. Specifically, we provide an overview of three systems that illustrate, in detail, distinct mechanisms that serve to sense and respond to c-di-GMP. These three systems highlight the diversity of c-di-GMP effector mechanisms, which likely only scratch the surface in regard to how cells sense and respond to this dinucleotide. We focus our discussion on the LapD protein of *P. fluorescens*, the YcgR protein of *E. coli*, and the VpsT protein of *V. cholerae*, which function in the transition from a motile to a sessile mode of life. LapD, YcgR, and VpsT bind c-di-GMP via distinct domains and impact discrete outputs through cell-surface protein localization, protein-protein interaction, and transcription, respectively. We discuss the role of these effector systems in the complexity of c-di-GMP signaling in the context of where the field needs to go next. The review concludes by highlighting some newly discovered or poorly understood effectors that warrant additional studies, as these effectors play a variety of roles in bacterial biology.

c-di-GMP EFFECTOR SYSTEMS

LapD Binds c-di-GMP to Regulate Cell Surface Protein Localization

Studies of *P. fluorescens* have identified LapD as an inner membrane effector protein that binds c-di-GMP via a degenerate EAL domain and, through an inside-out signaling mechanism, impacts cell-surface levels of the biofilm adhesin LapA (Newell et al. 2009, 2011a). To initiate the transition from a planktonic to a sessile mode of life, and thus formation of a biofilm, the large adhesin protein LapA must be present at the cell surface (Hinsa et al. 2003). Presumably, such radical changes in lifestyle occur only in response to defined signals. For example, in *P. fluorescens*, restriction of P_i availability to low micromolar levels results in loss of biofilm formation with little or no impact on planktonic growth, which indicates a biofilm-specific regulatory signal. Growth in low-P_i medium resulted in loss of the critical LapA adhesin from the cell surface (Monds et al. 2007).

What mechanism was employed to regulate cell-surface localization of LapA, and thus biofilm formation, in response to changing P_i availability? Initial genetic and biochemical analyses indicated that when cells are grown in a low-P_i medium, the cytoplasmic PDE RapA is expressed and degrades c-di-GMP to pGpG. Genetic studies supported the conclusion that reduced cellular levels of c-di-GMP resulted in the loss of LapA from the cell surface and thus eliminated biofilm formation (Monds et al. 2007). These results demonstrated that the modulation of intracellular levels of c-di-GMP regulates cell-surface protein localization and prompted us to investigate the mechanisms through which c-di-GMP levels were being sensed and monitored within the cell. Importantly, these findings suggested that changing levels of c-di-GMP in the cytoplasm impacted the localization of an adhesin on the cell surface, that is, two membranes away. These data suggested the possibility of a novel mechanism to monitor and regulate this c-di-GMP-dependent output.

A transposon mutagenesis screen identified the *lapA* gene as required for biofilm formation (O'Toole & Kolter 1998b), and subsequent work demonstrated that maintenance of the LapA adhesin at the cell surface required LapD (Hinsa & O'Toole 2006). That is, growth in low-P_i medium yielded a phenotype quite similar to that of loss of LapD function, which indicates that P_i may exert its effects through this protein. LapD is an inner membrane protein (Hinsa & O'Toole 2006) that contains degenerate and inactive GGDEF and EAL domains (Newell et al. 2009), which have been proposed to function as regulatory domains that bind c-di-GMP (Christen et al. 2006, Ryan et al. 2006b). Therefore, given that c-di-GMP and LapD are both required for biofilm formation and maintenance of LapA at the cell surface, we hypothesized that LapD may bind c-di-GMP through its GGDEF or EAL domain. Binding assays and mutagenesis revealed that the EAL domain of LapD is necessary and sufficient to bind c-di-GMP (Newell et al. 2009).

LapD contains a HAMP domain, a motif often found in transmembrane signaling proteins such as the sensor histidine kinases of two-component regulatory systems and chemotaxis receptors (Taylor 2007, Williams & Stewart 1999). Therefore, we hypothesized that upon sensing cytoplasmic levels of c-di-GMP, the HAMP domain relays this signal to the cell surface through activation of the periplasmic domain. In support of this model, deletion of a portion of the HAMP domain known to be required for signaling resulted in a hyperbiofilm phenotype, which was interpreted to result from the constitutive activation of the LapD output. The HAMP deletion mutant bound c-di-GMP at levels comparable with the wild type, whereas a HAMP deletion mutation combined with the c-di-GMP binding mutation $LapD^{E617A}$ could not bind c-di-GMP but still formed a hyperbiofilm. This intramolecular epistasis analysis suggested that a HAMP domain deletion locks LapD in an active state regardless of whether or not c-di-GMP is bound (Newell et al. 2009).

Predictions of LapD membrane topology, confirmed experimentally, indicated that this protein has two transmembrane domains separated by a periplasmic loop near its N terminus (Newell et al. 2009). To assess the role of the LapD periplasmic domain in mediating biofilm formation, a mutation was made within the periplasmic loop, $LapD^{L152P}$. This mutation reduced the extent of biofilm formation (i.e., the phenotype was similar to the *lapD* null mutation and the opposite of that observed for the HAMP locked-on mutation), thus suggesting the necessity of the periplasmic domain for the LapD output. Combination of the $LapD^{L152P}$ mutation and the HAMP deletion resulted in loss of the HAMP deletion-mediated hyperbiofilm phenotype, which suggests that the c-di-GMP signal is propagated from the inside out.

These mutational analyses are consistent with a model wherein LapD senses the cytoplasmic levels of c-di-GMP, and through an inside-out signaling mechanism, the periplasmic domain mediates the LapD output (Newell et al. 2009). However, the mechanism by which an inner membrane effector protein bound to c-di-GMP impacts a cell-surface output remained unclear. We hypothesized that a gene encoding a periplasmic protein may regulate LapA at the cell surface. The *lapG* gene located directly upstream of *lapD* presented itself as a good candidate. Genetic analyses indicated that *lapG* is epistatic to *lapD*, whereas *lapA* is epistatic to *lapG*, thus placing *lapG* in the LapA-dependent biofilm pathway. Biochemical and enzyme activity assays showed that LapG removes LapA from the cell surface through

proteolytic cleavage of the N terminus of LapA (Newell et al. 2011a). Furthermore, LapD and LapG interact in a c-di-GMP-dependent manner (Newell et al. 2011a). We proposed a model whereby LapD, when bound to c-di-GMP, interacts with LapG such that it prevents LapG from cleaving and releasing LapA, thus promoting biofilm formation. In conditions unfavorable for biofilm formation and in which c-di-GMP is low, LapD is no longer able to interact with LapG, and LapG through its cysteine protease activity cleaves LapA from the cell surface (Newell et al. 2009, 2011a).

X-ray crystallographic studies have elucidated the mechanistic basis for c-di-GMP-dependent regulation of LapA (Navarro et al. 2011). Three crystal structures of LapD were solved, including the c-di-GMP-unbound cytoplasmic portion of the protein (excluding the HAMP domain), the c-di-GMP-bound EAL domain dimer, and the periplasmic domain. The overall GGDEF and EAL domain folds of LapD are very similar to those of both catalytically active and inactive DGCs and PDEs, but nonconservative amino acid substitutions in the LapD GGDEF and EAL domains affect catalytic activity, thus rendering the domains nonfunctional (Navarro et al. 2011).

Studies of the c-di-GMP-unbound cytoplasmic LapD domain suggested that the protein attains an autoinhibited state. That is, the GGDEF domain likely restricts access of c-di-GMP to the EAL domain, whereas the signaling helix motif (S-helix), a helical extension of the HAMP domain, buttresses the EAL domain (Navarro et al. 2011). X-ray crystallographic studies of the c-di-GMP-bound EAL domain revealed that the EAL domain dimerizes at the surface occupied by the S-helix. Thus, upon c-di-GMP binding and EAL domain dimerization, the S-helix and GGDEF domain undergo a conformational change and displacement (Navarro et al. 2011). On the basis of these results, a model was proposed in which intramolecular interactions between the EAL domain, the GGDEF domain, and the S-helix prevent c-di-GMP from binding to the EAL domain. Autoinhibited LapD would then undergo conformational changes upon c-di-GMP binding (Figure 2). Structure-guided mutagenesis studies to test c-di-GMP binding and biofilm formation in both sufficient and insufficient P_i conditions confirmed that c-di-GMP binding and dimerization of the EAL domain are likely interdependent events and suggested that the S-helix stabilizes the autoinhibited state of c-di-GMP, whereas the positioning of the GGDEF domain blocks c-di-GMP from accessing the binding pocket within the EAL domain (Navarro et al. 2011).

The cytoplasmic conformation of LapD, which is indicative of c-di-GMP levels, must be relayed to the cell surface to regulate cell-surface adhesion through the modulation of LapG activity. To better understand how the cytoplasmic level of c-di-GMP is sensed in the periplasm, the structure of the periplasmic output domain of LapD was determined. It forms a domain-swapped dimer and is connected to the HAMP domain (Navarro et al. 2011). A sequence alignment of the LapD periplasmic domain with LapD homologs revealed a conserved tryptophan residue located at the most distal point of the periplasmic domain. Mutagenesis of this residue prevented biofilm formation and disrupted the interaction between LapG and LapD, which suggests its requirement for interaction with and modulation of LapG activity (Navarro et al. 2011).

From these structure-based mechanistic studies the following model (Figure 3) was proposed: in the absence of c-di-GMP, LapD exists in an autoinhibited state, as the S-helix and the GGDEF domain block c-di-GMP from accessing the EAL domain (Navarro et al. 2011). In this autoinhibited state, the periplasmic domain of LapD attains a LapG-binding-incompetent state. LapG through its cysteine protease function cleaves LapA from the cell surface and thereby prevents biofilm formation. It is proposed that the HAMP domain relays the cytoplasmic state of LapD to the periplasmic domain of LapD. Upon an increase in cytoplasmic c-di-GMP, a large conformational change within the GGDEF and S-helix occurs that allows for EAL domain dimerization. This signal is relayed to the periplasm, which allows this domain to attain a LapG-binding competent state. LapG is bound by LapD and no longer able to cleave LapA from the cell surface, which results in biofilm formation (Navarro et al. 2011).

Navarro et al. (2011) reported that the LapD-LapG c-di-GMP signaling system is conserved in many diverse environmental and pathogenic bacterial species including those in the *Pseudomonas*, *Legionella*, and *Vibrio* genera. Although studies of the effector protein LapD detail the mechanisms of c-di-GMP binding and regulation of biofilm formation in *P. fluorescens*, the types of behaviors and outputs these conserved LapD-LapG signaling systems regulate in other bacteria are largely unknown. As these LapD-LapG systems are studied in more detail, we expect similar mechanistic details will be elucidated, potentially unraveling the regulation of novel outputs.

Motile-to-Sessile Transition in *E. coli* Is Induced Via YcgR

Flagellar motility is critical to biofilm formation in many microbes (Deflaun et al. 1990; Genevaux et al. 1996; O'Toole & Kolter 1998a, 1998b; Pratt & Kolter 1998; Watnick et al. 1999). Thus, understanding how the cell controls flagellar motility is integral to understanding the planktonic-to-biofilm transition. Studies in *E. coli* have demonstrated that c-di-GMP binding to the PilZ domain of the effector protein YcgR stimulates its interaction with the flagellar motor and/or switch complex to positively affect the motile-to-sessile transition (Boehm et al. 2010, Fang & Gomelsky 2010, Paul et al. 2010). However, as outlined in detail below, these three studies identified different components of the flagellar motor as the downstream target.

The motor is comprised of the stator proteins MotA and MotB and the rotor proteins FliG, FliM, and FliN, which together function to drive and regulate flagellar rotation. The current model for motor function predicts that protons flow through an ion channel within the stator and bind to an Asp residue in MotB. A subsequent conformational change in MotA stimulates electrostatic interactions between MotA and FliG to generate torque and turning of the rotor.

Although ion flux and torque generation power flagellar rotation, in the case of swimming motility, a phosphorelay signaling cascade and the switch complex composed of FliG, FliM, and FliN control the direction of rotation. Chemotaxis proteins sense and respond to environmental stimuli and transmit signals to the motor. The phosphorylated state of the response regulator, CheY, impacts its ability to interact with the switch complex. The current model of flagellum switching suggests that phosphorylated CheY-P interacts with FliM,

which leads to an alteration in the interaction between FliM and the C terminus of FliG, which induces a modification of the rotor-stator interface between FliG-MotA to result in a switch in rotational direction (Terashima et al. 2008).

Studies by Ko & Park (2000) first identified the *ycgR* gene in a suppressor mutagenesis screen. The nucleoid protein H-NS is a positive regulator of the flagellar regulon (Bertin et al. 1994) and interacts with FliG to modulate the rotational speed of the flagellum (Donato & Kawula 1998). To examine H-NS involvement in flagellum function, a suppressor mutagenesis screen was performed in *hns*-deficient cells constitutively expressing *flhDC* in an H-NS-independent manner (to allow for flagellum production). Cells lacking H-NS but constitutively expressing *flhDC* contain paralyzed flagella and are nonmotile (Ko & Park 2000). Mutations in the *ycgR* gene were able to suppress the motility defect, whereas the *yhjH* gene was identified in a search for multicopy suppressors of the motility defect (Ko & Park 2000).

An analysis of the *ycgR* and *yhjH* promoters suggested that both are part of the flagellar regulon (Ko & Park 2000). Phenotypic assays showed that deletion of *ycgR* or overexpression of *yhjH* in the *flhDC*-constitutive *hns* mutant background resulted in an increase in swimming speeds (Ko & Park 2000). The percentage of rotating cells in *flhDC*-constitutive *hns* mutants was drastically less than that of the *ycgR* mutant or the *yhjH* overexpression strain, but the rotational speed was similar in all strains (Ko & Park 2000). Overexpression of genes encoding the motor components, namely, *motB*, *motAB*, and *fliG*, in the *flhDC*-constitutive *hns* mutant did not restore motility; however, overexpression of *motA* increased motility slightly (Ko & Park 2000). The study suggested that H-NS, YcgR, and YhjH participate in motor function, but how was unclear. Ko & Park (2000) hypothesized that the stator proteins were not able to associate properly with the components of the rotor in the *hns* mutant, which resulted in a defective motor complex.

Later studies identified YcgR as a PilZ domain-containing protein (Amikam & Galperin 2006) and YhjH as a PDE (Schmidt et al. 2005). Ryjenkov et al. (2006) tested the hypothesis that YcgR binds c-di-GMP through its PilZ domain. Using equilibrium dialysis and size exclusion chromatography, YcgR was shown to bind c-di-GMP, and results suggested that YcgR binds one c-di-GMP dimer at a single binding site (Ryjenkov et al. 2006). The $YcgR^{R118D}$ mutant was unable to bind c-di-GMP, whereas the PilZ domain alone binds c-di-GMP but at a decreased efficiency when compared with the full-length protein (Ryjenkov et al. 2006). These data suggested that other regions within the protein are important in c-di-GMP binding or that c-di-GMP binding induces conformational changes within YcgR (Ryjenkov et al. 2006). Ryjenkov et al. also tested whether c-di-GMP binding induced oligomerization. Size exclusion chromatography results suggested that YcgR, the PilZ domain of YcgR, and the PilZ domain of BcsA from *G. xylinus* exist as monomers in the presence and absence of c-di-GMP (Ryjenkov et al. 2006). This biochemical study established YcgR as a c-di-GMP-binding protein in *E. coli*.

Structures of three PilZ domain-containing proteins bound to c-di-GMP have revealed differences in binding stoichiometries and oligomeric states. PlzD from *V. cholerae* exists as a dimer in the unbound state and when bound to c-di-GMP. One molecule of c-di-GMP

binds to the C-terminal PilZ domain, which brings this domain in closer proximity to the N terminus (Benach et al. 2007). PP4397 from *Pseudomonas putida* binds two molecules of c-di-GMP and undergoes a dimer-to-monomer transition (Ko et al. 2010). PA4608 from *P. aeruginosa* is a single-domain-containing protein that binds to one molecule of c-di-GMP and undergoes rearrangement to expose a negative surface of the protein that is predicted to function in downstream processes (Habazettl et al. 2011, Shin et al. 2011). These differences in stoichiometry and oligomeric states are predicted to allow for diverse forms of c-di-GMP-dependent regulation (Habazettl et al. 2011, Ko et al. 2010, Shin et al. 2011).

In *E. coli* and the *Salmonella* rdar morphotype (rough, dry, and rugose colony), adherence to an abiotic surface, biofilm formation, and swimming and swarming motility are dependent on c-di-GMP level (Ryjenkov et al. 2006). To assess the effects of YcgR on outputs regulated by c-di-GMP, *ycgR* was deleted in the *yhjH* mutant background (in which c-di-GMP levels were elevated, as the strain lacks the YhjH PDE). The *yhjH ycgR* double mutant showed an increase in both swimming and swarming motility when compared with the *yhjH* deletion mutant. The $YcgR^{R118D}$ point mutant could not complement the swimming and swarming motilities of the *yhjH ycgR* double mutant, which suggests that YcgR regulates motility in a c-di-GMP-dependent manner (Ryjenkov et al. 2006). Phenotypes for rdar morphotype, adherence to an abiotic surface, and biofilm formation were similar in the *ycgR* mutant and *yhjH ycgR* double mutant. Taken together, these results suggested that YcgR specifically regulates flagellum-based motility in a c-di-GMP-dependent manner (Ryjenkov et al. 2006).

Ko & Park (2000) established that YcgR participates in motor function, whereas Ryjenkov et al. (2006) demonstrated that YcgR regulates flagellum-based motility in a c-di-GMP-dependent manner. In reviewing c-di-GMP regulation of flagellum-based motility, Wolfe & Visick (2008, p. 469) put forth the following model: “YhjH and an unknown DGC set the levels of c-di-GMP, which binds to YcgR. In a mechanism yet to be determined, this complex interferes with the proper association of the Mot proteins with FliG and the rest of the switching device. The result is a paralyzed flagellum.” They suggested that H-NS could enhance the interaction of the Mot proteins with FliG, whereas the YcgR-c-di-GMP complex could inhibit this interaction.

Within the same month three groups published studies exploring the posttranslational mechanism whereby c-di-GMP-bound YcgR interacts with components of the flagellar motor to facilitate the transition from a motile to a sessile lifestyle, but each identified different components of the flagellar motor as the downstream target (Boehm et al. 2010, Fang & Gomelsky 2010, Paul et al. 2010). Fang & Gomelsky (2010) hypothesized that YcgR binding to FliG in a c-di-GMP-dependent manner disrupts FliG-FliM interactions to result in a counterclockwise flagellum rotation. Cells unable to switch from counterclockwise to clockwise flagellum rotation migrate poorly in semisolid agar, thus facilitating the motile-to-sessile transition. In liquid, these cells are unable to change the direction of their movement and are set on a crash course with a surface. To test the hypothesis that YcgR interacts with components of the switch complex, pull-down assays and a bacterial two-hybrid system were used to determine binding partners of YcgR. YcgR pulled down the soluble FliGMN switch complex expressed in the nonflagellated *E. coli*

strain. FliG interacted with YcgR in the bacterial two-hybrid system. Furthermore, when c-di-GMP levels were manipulated in the bacterial two-hybrid system, YcgR still interacted with FliG in the absence of c-di-GMP, but the interaction was enhanced in the presence of c-di-GMP. Interestingly, $YcgR^{R118D}$, which has impaired c-di-GMP binding, interacted weakly with FliM in the bacterial two-hybrid system (Fang & Gomelsky 2010). Interaction between YcgR and FliG was predicted to disrupt the energy transfer to the flagellum rotor, impair flagellum assembly or stability, or disrupt the FliG-FliM interaction to impede flagellum rotational behavior and the switch from counterclockwise to clockwise rotation (Fang & Gomelsky 2010). When analyzed under the microscope, the wild type, *yhjH* mutant, and *yhjH ycgR* double mutant were similarly motile, which suggests that energy transfer is not hindered. Levels of the flagellin subunit, FliC, were also similar in the three strains, which suggests that the stability and assembly of the flagellum is not impaired. Tethering assays to measure flagellum rotation revealed that the *yhjH* mutant rotated counterclockwise and reversed to clockwise much less frequently than did the wild type. The strong counterclockwise rotational bias of the *yhjH* mutant was first reported by Girgis et al. (2007). The flagellum was effectively locked in the counterclockwise direction. Deletion of *ycgR* in the *yhjH* mutant background reversed the counterclockwise rotation bias, restoring a bias similar to that of the wild type (Fang & Gomelsky 2010). On the basis of these results, Fang & Gomelsky (2010) proposed the following model (Figure 4*a*): In response to c-di-GMP levels, YcgR binds to FliG and increases flagellum counterclockwise rotational bias by disrupting the FliG-FliM interaction, which leads to poor migration, and thus facilitates the motile-to-sessile transition.

Paul et al. (2010) similarly hypothesized that YcgR interacts with FliG and FliM in a c-di-GMP-dependent manner to reduce the efficiency of torque generation and induce a counterclockwise flagellum rotation. To more closely examine the effects of the *yhjH* deletion and the overexpression of *ycgR* on motor performance, tethered cell assays were used to assess motor speed as an indicator of motor torque and rotation bias. Although Fang & Gomelsky (2010) did not visually observe differences in the rotational speeds of tethered cells, Paul et al. (2010) noted that motor torque is reduced by 30%, but only in the presence of nonphysiologically increased c-di-GMP levels and overexpression of *ycgR*. As in the Fang & Gomelsky study, Paul et al. observed wild type cells rotating counterclockwise and clockwise, whereas *yhjH* mutants overexpressing *ycgR* predominately rotated counterclockwise. Chemotaxis mutants, wherein the level of CheY-P is elevated, are biased for clockwise rotation. Deletion of *yhjH* or overexpression of *ycgR* in such a chemotaxis mutant background resulted in many cells switching from a clockwise bias to a counterclockwise bias (Paul et al. 2010). As in the Fang & Gomelsky study, Paul et al. hypothesized that YcgR targeted components of the switch complex. Pull-down assays showed that FliG and FliM, but not FliN or MotA, interact with YcgR in both the presence and absence of c-di-GMP (Paul et al. 2010). As observed in *Salmonella* by fluorescence microscopy, YcgR-GFP (green fluorescent protein) displayed punctate fluorescence in cells, which became more apparent in the *yhjH* deletion background. Deletion of *fliM* or *fliG*, but not *motA*, eliminated the punctate fluorescence, which suggests that YcgR localizes to the rotor (Paul et al. 2010).

To identify regions in FliM and FliG that interact with YcgR, mutations were made in *fliM* and *fliG* and tested for their ability to interact with YcgR using pull-down assays, their effects on YcgR-GFP localization by fluorescence microscopy, and their ability to disrupt the in vivo interaction with YcgR and improve motility. Mutations in *fliM* were constructed at residues encoding surface regions. $FliM^{N155E}$ and $FliM^{L160E}$ mutants weakened and eliminated binding to YcgR, respectively, and YcgR-GFP puncta were eliminated in the $FliM^{N155E}$ and $FliM^{L160E}$ mutants (Paul et al. 2010). Mutations in *fliG* were also constructed at residues encoding various surface regions (Brown et al. 2007) near the charged ridge that interacts with the stator (Lloyd & Blair 1997, Zhou et al. 1998). $FliG^{Q252W}$, $FliG^{N292W}$, and $FliG^{P295W}$ caused a significant reduction in YcgR binding, whereas $FliG^{D248W}$ resulted in a slight reduction. YcgR-GFP puncta were not eliminated in the $FliG^{P295W}$ mutant. Because YcgR interacts with FliG and FliM, it was predicted that motility may improve if this interaction was disrupted by mutating residues in FliG and FliM shown to be involved in interacting with YcgR. Indeed, in *Salmonella*, overexpression of *fliM* or *fliG* mutants improved motility when compared with strains expressing wild type copies of *fliM* or *fliG* (Paul et al. 2010).

To identify residues in YcgR important for interacting with FliM and FliG, a series of amino acid substitutions in the C-terminal α-helix of YcgR were constructed on the basis of the crystal structure of PlzD, the YcgR-like protein in *V. cholerae* (Benach et al. 2007), and residues of DgrA of *C. crescentus* predicted to interact with downstream targets (Christen et al. 2007). $YcgR^{Q223W}$ and $YcgR^{I227W}$ could not bind to FliM, even in the presence of c-di-GMP, and the $YcgR^{Q223W}$-GFP mutant displayed no punctate localization. When overexpressed in the *yhjH* mutant, $YcgR^{Q223W}$ improved the motility defect and motor speed as well as corrected the rotational bias of the *yhjH* mutant. YcgR α-helix mutants were able to bind FliG, which suggests that the C-terminal α-helical region of YcgR is important for binding FliM. The N terminus of YcgR was hypothesized to be important for binding to FliG. When $YcgR^{K42D}$, $YcgR^{N62W}$, or $YcgR^{K81D}$ was overexpressed in the *yhjH* mutant, it showed improved motility in comparison with the *yhjH* mutant, whereas the interactions between FliG and $YcgR^{N62W}$ or $YcgR^{K81D}$ were weakened. Binding of N-terminal YcgR point mutants to FliM was unchanged. These results suggested that the N terminus of YcgR is important for binding to FliG.

To establish whether the motility defect in the *yhjH* deletion mutant is only due to a counterclockwise motor bias, the gene encoding the clockwise-signaling protein CheY was overexpressed in the *yhjH* mutant background. Motility was not improved, which suggested that another factor was contributing to the motor defect. Therefore, Paul et al. (2010) overexpressed components of the stator, as Ko & Park (2000) had done previously. Overexpression of *motA* or *motAB* in the *yhjH* mutant background improved the motility defect. Motility was further enhanced upon overexpression of *motA* and *cheY* in the *yhjH* mutant background. These results suggested that not only does YcgR induce a rotational bias, but it may also impact torque generation (Paul et al. 2010).

Paul et al. (2010) hypothesized that YcgR may disrupt the organization of the C-terminal portion of FliG within the motor. To test this hypothesis, cross-linking experiments to detect FliG dimers and multimers in the *yhjH* mutant and the *yhjH* mutant overexpressing *ycgR*

were performed. A FliG Cys replacement at residue 297 cross-linked into dimers in the wild type background and in the *yhjH* mutant overexpressing the gene encoding YcgR^{R118D}, but in the *yhjH* mutant strain and the *yhjH* mutant strain overexpressing *ycgR*, cross-linking was reduced. This result suggested that YcgR bound to c-di-GMP may disrupt the ability of FliG to properly organize within the motor (Paul et al. 2010).

On the basis of the above experiments, Paul et al. (2010) suggested a backstop break model whereby c-di-GMP-bound YcgR reduces torque generation and biases counterclockwise flagellum rotation to promote a motile-to-sessile transition. c-di-GMP-bound YcgR binds to FliM, which disrupts the FliG-FliM interaction and promotes a counterclockwise rotational bias (Figure 4*b*). For YcgR to interact with FliG, FliG must reorient its charged ridge within proximity to YcgR. This reorientation disrupts the FliG-MotA interactions and reduces torque generation. Paul et al. (2010) also suggest that YcgR interaction with one FliG may disrupt orientation of neighboring FliG. Therefore, "YcgR could thus function as a `backstop brake,' both slowing the motor and inhibiting preferentially its rotation in one direction" (Paul et al. 2010, p. 136). Interestingly, using the bacterial two-hybrid system, Fang & Gomelsky (2010) showed that YcgR^{R118D}, which is unable to bind c-di-GMP, binds to FliM, whereas Paul et al. (2010) found that YcgR^{R118D} interacts with FliM and this interaction is stimulated in the presence of c-di-GMP. YcgR^{R118D} retains the ability to bind to FliG, but binding is reduced when compared with wild type YcgR (Paul et al. 2010). Fang & Gomelsky (2010) did not detect an interaction between FliG and YcgR^{R118D}. Overexpression of YcgR^{R118D} in the *yhjH* mutant background improved the motility defect. The experiments with YcgR^{R118D} indicate that interaction with FliM occurs in both the presence and absence of c-di-GMP. As stated above, YcgR^{R118D} is unable to disrupt FliG organization within the motor. Therefore, YcgR may be positioned to associate with the flagellar switch complex to allow for rapid c-di-GMP-dependent regulation of flagellar motility. In the absence of c-di-GMP, YcgR associates with FliM, but once levels of c-di-GMP increase within the cell, YcgR binds c-di-GMP and impacts torque generation and rotational bias through interactions with FliG and FliM (Paul et al. 2010).

In a third study, Boehm et al. (2010) suggest that YcgR, in a c-di-GMP-dependent manner, interacts with the stator protein MotA to reduce flagellar motor function by inactivating individual stator units in a brake-like fashion. To establish that the *yhjH* mutant motility defect is due to a chemotaxis or velocity defect, the trajectories of individual cells were measured in conditions that eliminated chemical gradients. Decreased swimming speeds for *yhjH* mutant cells were observed when compared with wild type cells or cells harboring a *yhjH ycgR* double mutant (Boehm et al. 2010). Deletion of four DGCs shown to regulate cell motility in the *yhjH* mutant background restored swimming velocity to wild type levels (Boehm et al. 2010). To establish that reduced swimming speeds are not due to a chemotaxis defect, in vivo fluorescence resonance energy transfer (FRET) was used to assess the interaction between CheY and FliM in the wild type and *yhjH* mutant background. FliM and CheY interacted similarly in both backgrounds, which suggests that c-di-GMP regulates motility through adjustment of swimming speeds (Boehm et al. 2010). The analysis of this result implied that the interaction of c-di-GMP and YcgR at the motor does not induce a rotational defect, although Fang & Gomelsky (2010) and Paul et al. (2010) observed such

rotational defects. Rather, Boehm et al. (2010) suggested that YcgR and c-di-GMP decrease swimming speed.

As YcgR binds c-di-GMP (Ryjenkov et al. 2006), a screen to isolate spontaneous motile suppressors of the *yhjH* mutant was performed. It was predicted that mutations would be identified in YcgR-binding partners, as a mutation would render the binding partner insensitive to elevated levels of c-di-GMP. Four point mutants were identified in *motA*, which led Boehm et al. (2010) to hypothesize that YcgR and MotA interact. YcgR colocalizes with FliM to the cell envelope, as observed by fluorescence microscopy. YcgR localization was disrupted upon decreases in c-di-GMP through overexpression of *yhjH*, upon deletion of *motA*, or when a *motA* suppressor allele was overexpressed in a *motA* deletion background. Acceptor photobleaching FRET experiments were used to measure an interaction between MotA and YcgR in vivo. The FRET signal increased in the presence of elevated levels of c-di-GMP, whereas the FRET signal decreased in the absence of c-di-GMP or when a *motA* suppressor allele was overexpressed in a *motA* deletion background (Boehm et al. 2010). The identification of *motA* suppressor alleles and the results of the colocalization and acceptor photobleaching FRET experiments suggested that MotA and YcgR interact, and this interaction is predicted to interfere with the stator-rotor interaction (Boehm et al. 2010).

Boehm et al. (2010) predicted that YcgR may regulate flagellar motility by disengaging rotor and stator units or by decreasing and preventing flagellar rotation. Tethering assays were employed to evaluate whether the wild type, the *yhjH* mutant, and the *yhjH ycgR* double mutant displayed actively rotating behavior, indicative of a functional flagellar motor, passively rotating behavior, indicative of disengaged rotors and stators, or static cell behavior, indicative of a locked motor. Populations of the wild type, *yhjH* mutant, and *yhjH ycgR* double mutant contained similar proportions of passively rotating cells, whereas *yhjH* mutant*s* displayed more static cells and fewer actively rotating cells than did the wild type or *yhjH ycgR* double mutants. From this analysis, Boehm et al. (2010) concluded that YcgR regulates flagellar motors by decreasing torque generation. The *yhjH* mutant cells exhibit some rotating behavior; therefore, a subset of flagellar motors apparently were functional. To test whether YcgR inactivation of some stator units is sufficient to completely inhibit flagellar motility or if flagellar motility gradually decreases as more stator units are inactivated, combinations of chromosomal and plasmid wild type *motA* and $motA^{G93E}$ (a mutant *motA* gene that encodes $MotA^{G93E}$) suppressor alleles were expressed in the *yhjH* deletion background under varying inducing conditions and motility was quantified. In suboptimal inducing conditions, *yhjH* mutants expressing only the wild type *motA* gene had the slowest swimming velocity, followed by the *yhjH* mutant expressing chromosomal $motA^{G93E}$ and the wild type *motA* gene on a plasmid, followed by *yhjH* mutants expressing chromosomal wild type *motA* and $motA^{G93E}$ on a plasmid; *yhjH* mutants expressing only the protein encoded by the $motA^{G93E}$ allele had the greatest swimming velocity. This analysis suggested that both the wild type and mutant MotA comprise the flagellum motor and that inactivation of one stator unit by YcgR does not inhibit all stator units but rather leads to a gradual decrease in the motility output (Boehm et al. 2010). Boehm et al. propose the following model to explain the mechanism of c-di-GMP-dependent YcgR regulation of motility: c-di-GMP-bound YcgR interacts with MotA to reduce flagellar motor function by

inactivating individual stator units in a brake-like fashion to facilitate the transition from a motile to a sessile lifestyle (Figure 4*c*).

Clearly, YcgR and c-di-GMP function posttranslationally to regulate flagellum motility by interacting with motor components. The YcgR- and c-di-GMP-regulated motor components identified by Paul et al. and Fang & Gomelsky are more consistent with one another than with those described by Boehm et al. It is difficult to compare the studies directly, as different assays were used to measure outputs or similar assays conferred different results. Additionally, some assays were performed in *E. coli* or *Salmonella*, and the strains used in each study were not consistent. Reanalysis of mutants in the same strains and with the same assays would be useful. Through extensive experimentation, amazingly detailed models of flagellum torque generation and switching have been developed, but without more detailed structural studies of the motor and stator, it is not yet possible to understand the complete molecular mechanism (Sowa & Berry 2008). As these mechanistic details are unknown, it is rather challenging to assert confidently how and where YcgR affects the flagellar motor. Thus, further structure-function analyses of the flagellum motor will provide much insight into the specific mechanisms of the YcgR-c-di-GMP-regulated motile-to-sessile transition.

Transcriptional Regulation of the *Vibrio* Polysaccharide Genes Proceeds Via VpsT Bound to c-di-GMP

Unlike YcgR in *E. coli*, the PilZ domain-containing proteins in the *V. cholerae* biotype El Tor are not essential for biofilm formation, which indicates that non-PilZ domain-containing effector proteins function to bind c-di-GMP and regulate biofilm formation (Beyhan et al. 2008). Studies of *V. cholerae* have identified that c-di-GMP binding to the effector protein VpsT, a transcriptional regulator, induces oligomerization, which is required for DNA recognition and transcriptional regulation of the *Vibrio* polysaccharide (*vps*) genes (Krasteva et al. 2010).

Biofilm formation in *V. cholerae* depends upon the production of VPS, a major component of the extracellular matrix (Yildiz & Schoolnik 1999). *V. cholerae* undergoes phase variation to produce either smooth or rugose variants (White 1938); rugose variants produce more VPS than do smooth variants (Yildiz & Schoolnik 1999). Through whole-genome expression profiling it became apparent that the expression of VpsT is elevated in the *V. cholerae* rugose variant when compared with the smooth variant (Casper-Lindley & Yildiz 2004). VpsT is homologous to the transcriptional regulators found in *E. coli* and *Salmonella* that are required for the production of biofilm-associated products (Casper-Lindley & Yildiz 2004). Therefore, mutagenesis studies of *vpsT* were carried out to assess its role in regulating VPS production and biofilm formation. VpsT is required for maintaining the rugose variant colony phenotype, contributes to rugose variant biofilm formation, and induces *vps* gene expression (Casper-Lindley & Yildiz 2004). These results suggested that VpsT regulates biofilm formation through positively regulating the expression of *vps* genes (Casper-Lindley & Yildiz 2004).

Rugose variants contain higher levels of c-di-GMP than do smooth variants (Lim et al. 2006). When compared with a wild type rugose variant, deletion of the gene *vpvC*, which is predicted to encode a DGC, resulted in a decrease in intracellular concentrations of c-di-

GMP, a decrease in the expression of *vps* genes and *vpsT*, and alterations in the thickness and heterogeneity of the biofilm (Beyhan & Yildiz 2007). These studies suggested that levels of c-di-GMP may impact phenotypes associated with the rugose variant (Beyhan & Yildiz 2007, Lim et al. 2006). Given that VpsT may regulate biofilm formation through the positive regulation of the *vps* genes (Casper-Lindley & Yildiz 2004), it was suggested that VpsT integrates c-di-GMP signals to induce transcription of the *vps* genes (Krasteva et al. 2010).

To elucidate the mechanism by which VpsT integrates c-di-GMP levels to positively regulate *vps* expression and biofilm formation, X-ray crystallographic analyses of VpsT were undertaken. The structure of VpsT was solved in both the presence and the absence of c-di-GMP. VpsT contains an N-terminal receiver (REC) domain and a C-terminal helix-turn-helix domain that mediates DNA binding. An additional helix (helix α6) extends the C-terminal portion of the REC domain. Two nonoverlapping dimerization interfaces are present in VpsT. The first mediates c-di-GMP-independent dimerization of VpsT through a methionine residue at amino acid position 17. The second mediates c-di-GMP-dependent dimerization of VpsT, as c-di-GMP binds to helix α6 and stabilizes dimerization. The c-di-GMP binding motif in VpsT was determined to be W[F/L/M][T/S]R (Krasteva et al. 2010). Isothermal titration calorimetry (ITC) analyses suggested that a dimer of c-di-GMP is bound to a dimer of VpsT. Mutations within the c-di-GMP binding motif, namely, $VpsT^{W131F}$, $VpsT^{T133V}$, and $VpsT^{R134A}$, abolished c-di-GMP binding as assessed by ITC. Additionally, a mutation in helix α6, $VpsT^{I141E}$, also disrupted c-di-GMP binding, which suggests that c-di-GMP binding is dependent upon dimerization of VpsT. A $VpsT^{M17D}$ mutation at the c-di-GMP-independent dimerization interface did not disrupt c-di-GMP binding (Krasteva et al. 2010). Dimerization of VpsT at both c-di-GMP-dependent and c-di-GMP-independent interfaces was further assessed by static multiangle light scattering of various VpsT mutants. $VpsT^{M17D}$ remained monomeric in the absence of c-di-GMP, whereas $VpsT^{R134A}$ and $VpsT^{I141E}$ formed both monomeric and dimeric species. Addition of c-di-GMP to $VpsT^{M17D}$ resulted in the formation of dimeric species (Krasteva et al. 2010).

The c-di-GMP-binding mutant $VpsT^{R134A}$ and dimerization interface mutants $VpsT^{I141E}$ and $VpsT^{M17D}$ were used in electrophoretic mobility shift assays to assess the effects of c-di-GMP binding and VpsT dimerization on VpsT regulation of *vps* genes. $VpsT^{R134A}$ and $VpsT^{I141E}$ were unable to bind to the promoter of *vpsL*, a gene positively regulated by VpsT (Krasteva et al. 2010). The three mutants were then used to assess the requirements of c-di-GMP binding and VpsT dimerization for transcription of *vps* genes. Results from *vpsL*p-*lacZ* transcriptional fusion assays demonstrated that $VpsT^{R134A}$ and $VpsT^{I141E}$ were unable to increase the expression of *vpsL* to the same extent observed for the wild type VpsT and the $VpsT^{M17D}$ mutant protein. These results suggested that both c-di-GMP binding and c-di-GMP-dependent dimerization of VpsT are critical for VpsT transcriptional regulation of the *vps* genes (Krasteva et al. 2010).

Whole-genome expression profiling of the *vpsT* deletion mutant overexpressing $VpsT^{I141E}$, $VpsT^{M17D}$, or $VpsT^{R134A}$ revealed that c-di-GMP binding of VpsT and c-di-GMP-dependent dimerization of VpsT are required for induction of *vps* genes. *vps* genes were induced at significantly lower levels when $VpsT^{I141E}$ or $VpsT^{R134A}$ was overexpressed as compared with the overexpression of $VpsT^{M17D}$ or wild type VpsT. Whole-genome

expression profiling also revealed that overexpression of VpsT^{I141E} or VpsT^{R134A} did not reduce flagellar gene expression, as did overexpression of VpsT^{M17D} or wild type VpsT. Motility assays confirmed that c-di-GMP binding to VpsT and c-di-GMP-dependent dimerization of VpsT are required to reduce motility (Krasteva et al. 2010). Lastly, the conversion from smooth to rugose colony morphology requires c-di-GMP binding and VpsT dimerization (Krasteva et al. 2010). The above studies demonstrate that c-di-GMP binding to and c-di-GMP-dependent oligomerization of VpsT is necessary and sufficient for DNA regulation and transcription of *vps* genes. Thus, VpsT integrates c-di-GMP to inversely regulate biofilm formation and motility in *V. cholerae* (Krasteva et al. 2010) (Figure 5).

c-di-GMP levels affect the biofilm-associated curli fimbriae through the transcriptional regulator CsgD found in *Salmonella* and *E. coli* (Kader et al. 2006, Weber et al. 2006). CsgD is a VpsT homolog (Casper-Lindley & Yildiz 2004), but the binding pocket of CsgD, YF[T/S]Q, differs from that of the VpsT c-di-GMP binding pocket and is unlikely to be able to bind c-di-GMP in this pocket (Krasteva et al. 2010). Furthermore, whereas VpsT does not seem to be regulated by phosphorylation (Krasteva et al. 2010), CsgD DNA binding is (Zakikhany et al. 2010). Krasteva et al. suggest that whereas dimerization to induce DNA binding may be a general mechanism in these homologous transcriptional regulators, the mechanisms of c-di-GMP binding and dimerization in VpsT may be conserved only in similar proteins.

In *V. parahaemolyticus* the *cps* genes encode proteins required for the production of a sticky capsular polysaccharide required for biofilm formation (Boles & McCarter 2002) in response to c-di-GMP levels (Ferreira et al. 2008). Ferreira et al. (2011) suggest that CpsQ, a homolog of VpsT that contains a strongly conserved c-di-GMP binding pocket in comparison to that of VpsT, binds c-di-GMP and is a direct transcriptional regulator of the *cps* genes. Whether the mechanism of c-di-GMP binding to CpsQ is similar to that of VpsT and whether binding of c-di-GMP induces oligomerization of CpsQ remain to be determined.

Other Potential Effector Mechanisms

In recent years several other c-di-GMP effector systems have been identified, but the mechanisms by which these effectors function are less well understood. However, the study of such systems is an exciting future avenue of research that will likely unveil new secrets regarding mechanisms by which c-di-GMP regulates bacterial biology. An overview of other identified c-di-GMP effector systems is summarized in Table 1.

CONCLUSION

The diversity of c-di-GMP control mechanisms and their varied targets highlights the scope and intricacy of signaling by this second messenger. c-di-GMP binding induces effectors to undergo conformational changes, transcriptional activation, DNA binding, protein-protein interactions, derepression of genes, localization, and enhanced enzymatic activity. The diversity of mechanisms through which c-di-GMP impacts effector function suggests that effectors are able to impact a variety of outputs, such as motility, biofilm formation,

virulence, cell cycle regulation, RNA processing, gene expression, and likely others that are yet to be uncovered.

In the context of c-di-GMP signaling, bacteria possess a complex network of enzymes and effectors that sense and respond to environmental signals, adjust cellular levels of c-di-GMP, and regulate phenotypic outputs. Understanding how multiple c-di-GMP signaling pathways are isolated from each other, or integrated to produce coherent outputs, is a question of fundamental importance for understanding an array of biological outputs in bacteria. Assigning molecular functions to DGCs and PDEs as well as determining which specific outputs are regulated by the coordinated action of these enzymes will aid in that understanding. Additionally, studies must focus on identifying new effectors and deciphering the mechanisms by which c-di-GMP effectors appropriately coordinate inputs. Going forward, much progress needs to be made in understanding interactions between specific DGCs, PDEs, and effectors. Such studies will provide a better understanding of c-di-GMP signaling mechanisms that are critical for bacterial biology.

ACKNOWLEDGMENTS

This work was supported by a National Institutes of Health T32 AI007519 predoctoral fellowship to C.D.B. and by National Science Foundation grant MCB-9984521 to G.A.O. We also thank M. Gomelsky and H. Sondermann for critical reading of the manuscript.

DISCLOSURE STATEMENT The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

Glossary

Biofilm
a community of bacterial cells attached to a surface

Bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP)
bacterial intracellular second messenger that stimulates biofilm formation and inhibits motility

Diguanylate cyclase (DGC)
enzyme that synthesizes c-di-GMP

GGDEF
core amino acid motif conserved in DGCs for synthesizing c-di-GMP

Phosphodiesterase (PDE)
enzyme that degrades c-di-GMP

EAL
core amino acid motif conserved in PDEs for degrading c-di-GMP to pGpG

HD-GYP
core amino acid motif conserved in PDEs for degrading c-di-GMP to GMP

Effector

c-di-GMP receptor; protein or RNA that binds c-di-GMP

PilZ

c-di-GMP binding domain found in some effector proteins

HAMP

domain of approximately 50 amino acids found in transmembrane signaling proteins; functions to mediate signal transduction

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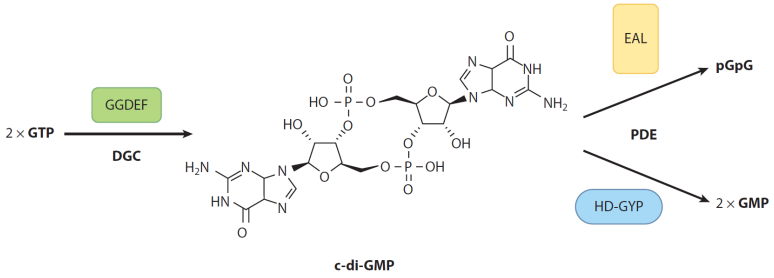

Figure 1.

c-di-GMP synthesis and degradation. Diguanylate cyclases (DGCs) containing the GGDEF domain synthesize c-di-GMP (*shown center*) from two molecules of GTP. Phosphodiesterases (PDEs) containing the EAL domain or HD-GYP domain degrade c-di-GMP to pGpG or GMP, respectively. Illustration courtesy of William Scavone, MA, CMI, Kestrel Illustration Studio, LLC. Abbreviations: GMP, guanosine monophosphate; pGpG, 5′-phosphoguanylyl-(3′-5′)-guanosine.

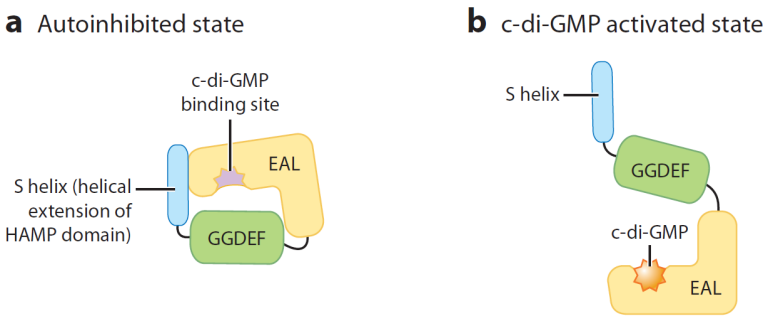

Figure 2.

LapD binds c-di-GMP. c-di-GMP binding to LapD induces a conformational change in the protein. (*a*) Autoinhibited state. Intramolecular interactions among the EAL domain, the GGDEF domain, and the S-helix prevent c-di-GMP from binding to the EAL domain. Structure-guided mutagenesis studies suggest that the S-helix stabilizes the autoinhibited state of LapD, whereas the positioning of the GGDEF domain blocks c-di-GMP from accessing the c-di-GMP binding pocket with the EAL domain (Navarro et al. 2011). (*b*) c-di-GMP activated state. Upon c-di-GMP binding to the EAL domain, LapD undergoes a conformational change as the S-helix and GGDEF domain are displaced (Navarro et al. 2011; Illustration courtesy of William Scavone, MA, CMI, Kestrel Illustration Studio, LLC). Abbreviations: GMP, guanosine monophosphate.

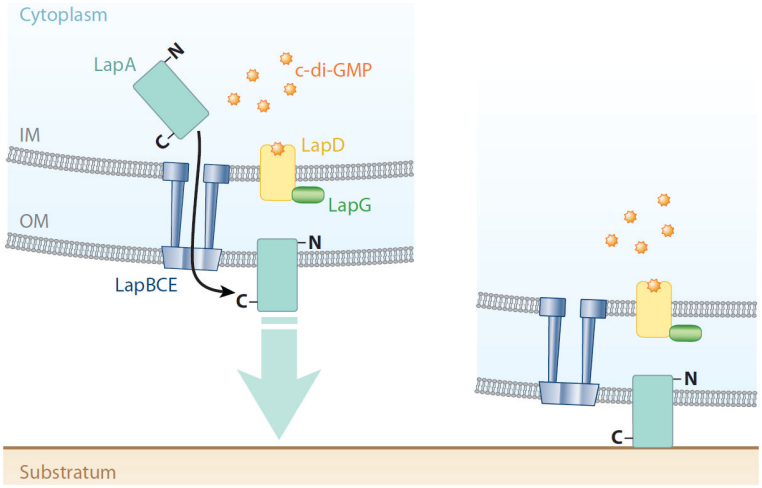

Figure 3.

c-di-GMP effector system in *Pseudomonas fluorescens*. This diagram depicts a summary of the current model for the c-di-GMP effector system in *P. fluorescens*, which impacts the motile-to-sessile transition. The LapA protein, a predicted cell-surface adhesion, is transported to the cell surface through the ABC transporter, comprised of the LapBCE proteins. LapD binds c-di-GMP, and through an inside-out signaling mechanism, the periplasmic domain of LapD binds LapG. Thus, LapG is prevented from cleaving and releasing LapA from the cell surface, thereby promoting biofilm formation (Newell et al. 2011a; Illustration courtesy of William Scavone, MA, CMI, Kestrel Illustration Studio, LLC). Abbreviations: GMP, guanosine monophosphate; IM, inner membrane; OM, outer membrane.

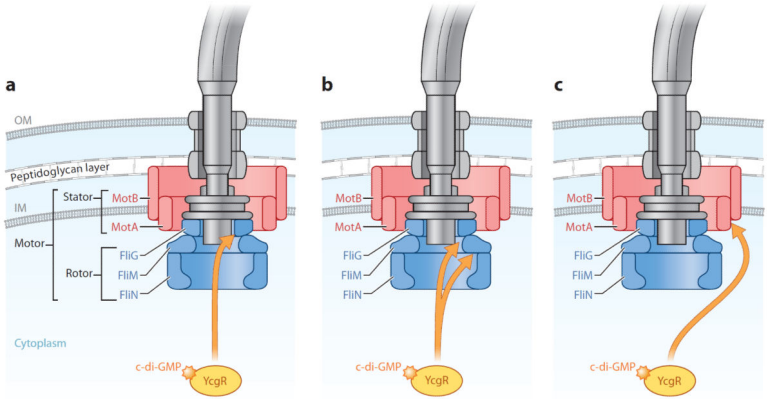

Figure 4.

c-di-GMP effector system in *Escherichia coli*. This diagram depicts a summary of the current models of the c-di-GMP effector system in *E. coli*, which impacts the motile-to-sessile transition. YcgR bound to c-di-GMP stimulates YcgR to interact with one or more components of the flagellar motor, reducing or altering motor function and thus promoting the motile-to-sessile transition. (*a*) Fang & Gomelsky (2010) suggest that c-di-GMP-bound YcgR interacts with FliG and disrupts the FliG-FliM interaction. An increased bias in counterclockwise flagellar rotation ensues, which leads to poor migration and thus facilitates the motile-to-sessile transition. (*b*) Paul et al. (2010) suggest that c-di-GMP-bound YcgR binds to FliM, disrupting the FliG-FliM interaction and thereby promoting a counterclockwise rotational bias. They also propose that YcgR also interacts with FliG, which leads to a disruption in the FliG-MotA interactions and a reduction in torque generation. (*c*) Boehm et al. (2010) suggest that c-di-GMP-bound YcgR interacts with MotA to reduce flagellar motor function. Illustration courtesy of William Scavone, MA, CMI, Kestrel Illustration Studio, LLC. Abbreviations: GMP, guanosine monophosphate; IM, inner membrane; OM, outer membrane.

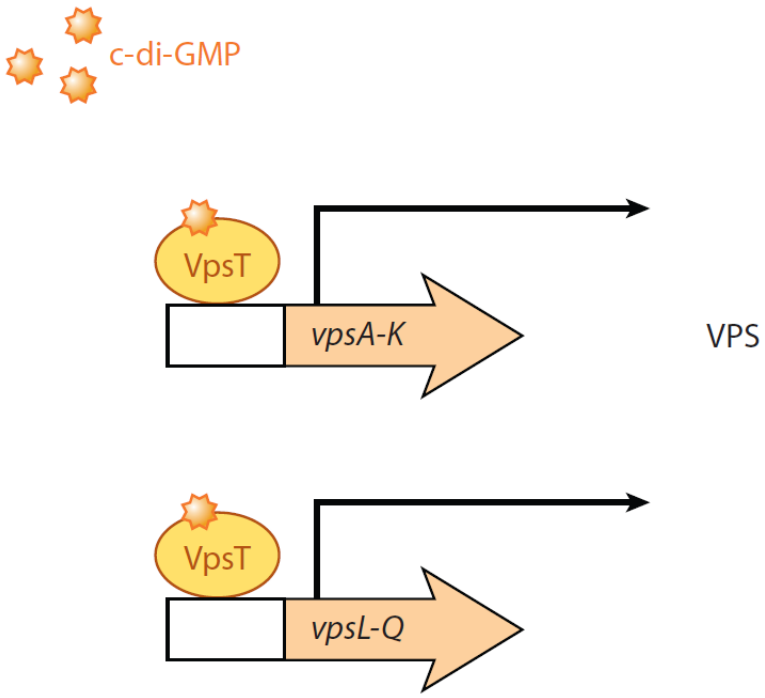

Figure 5.

c-di-GMP effector system in *Vibrio cholerae*. This diagram depicts a summary of the current model of the c-di-GMP effector system in *V. cholerae*, which impacts the motile-to-sessile transition. The transcriptional regulator VpsT binds c-di-GMP to positively regulate the transcription of the *vps* genes encoding the proteins needed for VPS production. The VPS polysaccharide is required for biofilm formation (Krasteva et al. 2010; Illustration courtesy of William Scavone, MA, CMI, Kestrel Illustration Studio, LLC).

Table 1

c-di-GMP effector systems

Effector	Motif/domain	Organism	Mechanism	Regulated output	Reference (s)
Alg44	PilZ	*Pseudomonas aeruginosa*	Protein-protein interaction	Production of alginate	Merighi et al. (2007), Oglesby et al. (2008)
MrkH	PilZ	*Klebsiella pneumoniae*	Transcriptional activation, DNA binding	Expression of type 3 flmbriae and biofllm formation	Wilksch et al. (2011)
PlzA	PilZ	*Borrelia burgdorferi*	Unknown	Motility, infectivity	Freedman et al. (2010), Pitzer et al. (2011)
DgrA	PilZ	*Caulobacter crescentus*	Protein-protein interaction	Motility	Christen et al. (2007)
BcsA	PilZ	*Gluconacetobacter xylinus*	Unknown	Cellulose synthase	Ryjenkov et al. (2006), Weinhouse et al. (1997)
PlzD	PilZ	*Vibrio cholerae*	Unknown	Motility, biofllm formation, virulence	Pratt et al. (2007)
FleQ	Unknown	*Pseudomonas aeruginosa*	Derepression of *pel* operon	Biofllm formation	Hickman & Harwood (2008)
Clp	Unknown	*Xanthomonas axonopodis*	Inhibition of Clp-DNA binding	Likely virulence	(Leduc & Roberts 2009)
Clp	Interface between cyclic nucleotide binding and DNA binding domains	*Xanthomonas campestris*	Inhibition of Clp-DNA binding	Virulence	Chin et al. (2010), Tao et al. (2010)
Bcaml349	Unknown	*Burkholderia cenocepacia*	Transcriptional regulation, c-di-GMP enhances DNA binding	Biofllm formation, virulence	Fazli et al. (2011)
FimX	EAL	*Pseudomonas aeruginosa*	Conformational change	Twitching motility	Huang et al. (2003), Kazmierczak et al. (2006), Navarro et al. (2009), Qi et al. (2011)
PelD	RxxD	*Pseudomonas aeruginosa*	Unknown	Exopolysaccharide production	Lee et al. (2007)
PopA	RxxD	*Caulobacter crescentus*	Localization to the pole	Cell cycle progression	Abel et al. (2011), Duerig et al. (2009)
PNPase	Unknown	*Escherichia coli*	Enhances PNPase activity	RNA processing	Tuckerman et al. (2011)
Vc2 Riboswitch Class I	GEMM	*Vibrio cholerae*	Conformational change	Gene expression	Kulshina et al. (2009), Smith et al. (2009), Sudarsan et al. (2008)

Effector	Motif/domain	Organism	Mechanism	Regulated output	Reference (s)
84Cd Riboswitch Class II	Pseudoknot	*Clostridium difficile*	Triggers alternative splicing	Altered transcripts	Lee et al. (2010), Smith et al. (2011)