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Cyclic Diguanylate in the Wild: Roles During Plant and Animal Colonization

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Abstract

Cyclic diguanylate (c-di-GMP) is a near-ubiquitous signaling molecule that regulates the motility-to-sessility transition in many bacterial species. Among the phenotypes influenced by c-di-GMP are biofilm formation, motility, cell cycle, and virulence. The hallmark phenotypes regulated by c-di-GMP—biofilm formation and motility—are key determinants of host–bacterial interactions. A large body of research has identified the roles of c-di-GMP in regulating phenotypes in culture. While numerous studies have investigated roles for c-di-GMP during the establishment and maintenance of pathogenic host–bacterial associations, considerably less attention has been devoted to defining the roles of c-di-GMP during beneficial and commensal associations. This review describes the known roles of c-di-GMP in regulating phenotypes that contribute to host colonization, with a focus on knowledge gaps and future prospects for examining c-di-GMP during beneficial colonization.

Contents

1. INTRODUCTION

Cyclic dimeric (3′ → 5′) GMP (cyclic diguanylate or c-di-GMP) is one of the most common and well-studied bacterial second messenger molecules. When they initially described it in terms of its activity as a cellulose synthase activator, Benziman and colleagues (122) determined that c-di-GMP is the active molecule that increases cellulose production in *Acetobacter xylinum* (now *Komagataeibacter xylinus*). In the 35-plus years since its identification, researchers have identified roles for c-di-GMP in significant bacterial phenotypes, most notably biofilm formation and motility. From culture-based studies, we have deep knowledge of individual signaling proteins and pathways. Many reviews (14, 32, 35, 51, 59, 61, 68, 91, 118, 119, 132, 142, 157) and a book (163) have provided valuable examinations of the large and ever-growing body of c-di-GMP literature.

In contrast to the abundant studies in culture, there have been fewer investigations of the role of c-di-GMP in plant and animal hosts (55, 79, 96, 125, 154). Despite obvious connections between bacterial biofilm, motility, and other behaviors regulated by c-di-GMP, studying the role of c-di-GMP in situ in the host presents significant challenges. It is not straightforward to manipulate c-di-GMP levels or to detect c-di-GMP in the host, and current host–bacterial model systems encompass only portions of the colonization process. Additionally, it is difficult to track the activities of individual c-di-GMP-modulating enzymes, especially in species that encode many such proteins. Nonetheless, early studies of c-di-GMP signaling in hosts revealed results that do not always match those from culture-based studies, emphasizing the value of extending research to the direct host environment to gain a fuller understanding of how this important metabolite influences bacterial behavior in relevant and complex environments.

Here, we argue for an additional focus on studies of c-di-GMP signaling in vivo, a term we use to mean research in plant or animal hosts. The rationale for this emphasis is twofold. First, given the relevance of c-di-GMP signaling to colonization behaviors in beneficial and pathogenic microorganisms, we argue that it is necessary to understand how signaling is regulated in host environments. The host incorporates spatial cues, innate immune signaling, and interactions with host cells that are difficult to capture in culture-based studies. Second, we articulate fundamental

questions in c-di-GMP signaling that may be advanced through studies in various host environments. Many laboratory model bacteria evolved in host contexts, and studies in those contexts are likely to yield relevant results on input signals and the evolution of c-di-GMP signaling networks that have been difficult to tease apart in culture.

2. c-di-GMP BACKGROUND: MECHANISTIC INSIGHTS FROM MODEL SYSTEMS

2.1. c-di-GMP Enzymes: Diguanylate Cyclases and Phosphodiesterases

Diguanylate cyclase (DGC) enzymes catalyze the formation of c-di-GMP from two GTP molecules in a two-step reaction with 5′-pppGpG as an intermediate (122). The GG(D/E)EF active-site amino acid motif specifically binds GTP via the first two glycine residues, and the fourth residue (glutamic acid) coordinates two metal cations (Mg^{2+} or Mn^{2+}) required for the reaction (22, 119, 158). Phosphodiesterase (PDE) enzymes degrade c-di-GMP. There are two different varieties of PDEs: those with EAL domains and those with HD-GYP domains. PDEs with EAL domains contain conserved ExLxR active-site motifs and hydrolyze c-di-GMP into 5′-pGpG, while HD-GYP domain proteins hydrolyze c-di-GMP to GMP (52, 121, 126, 138, 144, 148).

Functional studies in organisms such as *Vibrio cholerae*, *K. xylinus*, *Pseudomonas aeruginosa*, *Salmonella enterica*, and *Escherichia coli* revealed a broad landscape of DGC and PDE regulation of c-di-GMP levels. These studies were aided by protein domain databases (e.g., COG, Pfam, and SMART) that facilitated novel bioinformatic analyses linking GGDEF, EAL, and HD-GYP domains to c-di-GMP metabolism (12, 53, 134, 147).

Many c-di-GMP-modulating proteins contain both GGDEF and EAL domains (145). These dual-function proteins are not as well understood as single-function DGCs and PDEs because of the inherent complexity that is implied by their existence. On the surface, dual-function proteins seem contradictory: Why would a protein contain domains with opposing functions? Early in vitro (i.e., culture-based) studies suggested that proteins encoding both GGDEF and EAL domains had only one active domain (145). This finding holds true for some dual-function proteins, such as *Caulobacter crescentus* protein CC3396, which has one functional domain (EAL) and another domain (GGDEF) that is degenerate and coordinates activity of the functional domain (29). Other dual-function proteins, however, have two canonical, presumably functional active sites, and the activities of the GGDEF and EAL domains are regulated through various mechanisms to prevent both from operating at the same time (44, 152). The *P. aeruginosa* dual-function protein RmcA requires two-component response regulator CbrB to function predominantly as a PDE, and evidence suggests that CbrB may activate PDE activity by influencing subcellular localization of RmcA (78). In the plant-growth-promoting rhizobacterium *Azospirillum baldaniorum*, CdgB is a membrane-anchored dual-domain protein that has both DGC and PDE activities, depending on the conditions sensed, and a MHYT domain that may control localization of the protein in response to environmental signals (155). Therefore, environmental signals and interactions with other proteins regulate the activity of dual-function proteins. We discuss regulation of DGC and PDE enzymes broadly in the next section.

2.2. Signaling Inputs and Effectors of c-di-GMP Regulation

The earliest-identified DGCs and PDEs contain sensory domains, suggesting that c-di-GMP levels are controlled by environmental and cellular signaling inputs. Characterization of six DGC and PDE proteins in *K. xylinus* revealed N-terminal oxygen-sensing PAS domains in all six enzymes (145), and characterization of PleD revealed an N-terminal two-component signaling receiver (REC) domain (56). Now it is known that many sensory domains are associated with GGDEF,

EAL, and HD-GYP domain proteins. Cytoplasmic proteins commonly have REC, PAS, GAF, BLUF, and helix-turn-helix domains, while membrane-bound proteins commonly have periplasmic or extracellular sensory domains such as Cache, CHASE, MHYT, and MASE domains (50, 57, 119, 133). These domains perceive a variety of signals, including oxygen, redox conditions, light, and extracellular substances (57, 119). The activity of individual DGCs and PDEs in response to specific signals has been proposed to explain why some organisms encode a large number of DGCs and PDEs (34, 103, 159).

For DGCs and PDEs to regulate the activity of other proteins, those effector proteins must respond to c-di-GMP. Receptors for c-di-GMP include PilZ domains (of which there are three types); inhibition sites, which are found in about half of GGDEF proteins and consist of RxxD amino acid motifs; catalytically inactive EAL domains; c-di-GMP-specific riboswitches; anti-σ factors; deacetylases; the catalytic and ATP-binding domain of histidine kinases; eukaryotic STING (stimulator of IFN genes) proteins; and bacterial Toll/interleukin-1 receptor complexes as a part of cyclic dinucleotide–based antiphage signaling systems (CBASS) (20, 22, 23, 26, 28, 40, 49, 69, 104, 135, 136, 166). c-di-GMP also binds at protein interfaces and participates in protein–protein interactions (26). There is still much to learn about how c-di-GMP interacts with protein effectors, as many proteins have been observed to bind c-di-GMP through unknown mechanisms (26, 119).

2.3. Regulation of Bacterial Behavior by c-di-GMP

Biofilms are generally defined as surface-attached or surface-associated aggregate structures of microbes within a matrix of extracellular polymeric substances (EPSs), such as cellulose and/or other exopolysaccharides, proteins, and/or extracellular DNA (128). Biofilm formation was the first c-di-GMP-regulated phenotype to be characterized (122) and remains the most extensively studied (118). First observed in *K. xylinus*, c-di-GMP was subsequently shown to activate cellulose synthase activity in the model plant pathogen *Agrobacterium tumefaciens* (5), followed by parallel studies in many other species (7, 8, 120, 145, 161).

c-di-GMP also regulates noncellulose biofilms in diverse bacteria, including temperature-controlled Hms-dependent biofilm formation by *Yersinia pestis*, Psl and Pel exopolysaccharide production by *P. aeruginosa*, capsular polysaccharide production by *Vibrio parahaemolyticus*, *Vibrio* polysaccharide (Vps) and exopolysaccharide production by *V. cholerae*, and EPS production by *Vibrio vulnificus* and *Xanthomonas campestris* (13, 37, 39, 76, 77, 88, 106, 141, 177). Recent discoveries in *P. aeruginosa* have revealed that DGC SiaD posttranscriptionally regulates production of Psl exopolysaccharide (39), functional specialization of PDEs is required for Pel biofilm maintenance in the face of nutrient limitation (76), and c-di-GMP regulates transcription of the alginate operon (88). Factors other than exopolysaccharide production that contribute to biofilm formation are also regulated by c-di-GMP; these include the BpfAGD system in *Shewanella putrefaciens* (90), the Lap system in *Pseudomonas fluorescens* (31), and others that have been reviewed by Römling et al. (119). Enhanced community aggregative behaviors, and especially biofilm formation, are therefore strongly connected to elevated levels of c-di-GMP.

While elevated c-di-GMP promotes biofilm formation, it also leads to diminished motility, which is the second-best-studied output of c-di-GMP regulation, behind biofilm formation (118). c-di-GMP affects swimming motility in various organisms by employing diverse mechanisms, including regulation of flagellar gene expression and regulation of proteins that interact with the flagellar machinery (13, 17, 23, 24, 27, 43, 99, 111, 165, 175). Research in *V. cholerae* has revealed that flagellum assembly status regulates biofilm formation via the flagellum-dependent biofilm regulatory response that relies on c-di-GMP signaling to connect motility and biofilm formation (165) and that functional specialization of *V. cholerae* DGCs results in cooperative, rather than

additive, modulation of c-di-GMP levels to regulate motility (175). Bacteria also use other forms of motility, and evidence across organisms suggests that c-di-GMP influences swarming, twitching, and gliding motility (9, 18, 41, 60, 63, 80, 81, 100, 101, 140, 162). Biofilm formation and motility are typically thought of as opposing behaviors: One encourages bacteria to stick to one spot, while the other permits bacteria to move around the environment. By harmonizing biofilm and motility phenotypes, c-di-GMP provides a central junction point so that the resulting behaviors can respond in a coordinated fashion.

One of the best-studied developmental processes regulated by c-di-GMP is the swarmer-to-stalked-cell transition during *C. crescentus* cell division. *C. crescentus* has an asymmetric lifestyle in which every round of cell division yields a motile swarmer cell and a sessile stalked cell. c-di-GMP levels oscillate during the *C. crescentus* cell cycle, with levels highest during the swarmer-to-stalked-cell transition and lowest in swarmer cells just after cell division (1, 2, 42, 65, 74, 107, 127). The motile-to-sessile transition in *C. crescentus* is a prime example of c-di-GMP regulation coordinating bacterial behaviors in a temporal and spatial manner, incorporating motility control into a broader developmental program.

c-di-GMP contributes to resistance to environmental and host stressors (157). Biofilms protect bacterial cells from stressors by providing a physical barrier from adverse environments and by slowing metabolism and growth (47, 157, 172). Biofilm formation is not the only c-di-GMP-induced response to stress: Oxidative stress, temperature, and DNA repair are also influenced by c-di-GMP levels in several species independently of biofilm formation (157). In *V. cholerae*, c-di-GMP induces expression of Tag, a DNA glycosylase that repairs methylated DNA, via c-di-GMP-responsive regulators VpsR and VpsT, and it is hypothesized that DNA repair may be important for biofilm formation (45). c-di-GMP also contributes to *V. cholerae* hydrogen peroxide resistance by positively regulating catalase expression, which may promote persistence in aquatic and host environments (46). Temperature is another stressor that affects c-di-GMP regulation in *V. cholerae* as well as *Y. pestis* (72, 77, 150). Recent research in the cyanobacterium *Anabaena* revealed that c-di-GMP binding to the effector protein CdgR controls cell size through interaction with the transcription factor DevH (176).

Also relevant to studies in plant and animal hosts is the involvement of c-di-GMP in the regulation of various secretion systems that are responsible for delivering effector proteins to target cells or the extracellular space. Diverse bacterial species show evidence for c-di-GMP regulation of the type II secretion system (T2SS), the type III secretion system (T3SS), the type IV secretion system (T4SS), and the type VI secretion system (T6SS) (3, 4, 6, 23, 54, 70, 71, 73, 83, 85, 97, 102, 105, 112, 117, 151, 169). Generally, the T2SS, T3SS, and T4SS are negatively regulated by c-di-GMP, while the T6SS is positively regulated by c-di-GMP (119). Differential regulation of the T6SS by c-di-GMP in different organisms highlights the complexities of c-di-GMP regulation, as regulation is not universal and the overall lifestyle and goals of an organism must be considered when assessing the influence of c-di-GMP.

3. c-di-GMP IN THE CONTEXT OF HOST–BACTERIAL INTERACTIONS

Compared with the explosion of culture-based research, far fewer studies have been performed in plant and animal systems, and it is often more difficult to distill mechanisms from studies in a host organism. Here we examine how c-di-GMP signaling functions in the context of host colonization.

3.1. c-di-GMP in Pathogenic Interactions

Many of the phenotypes regulated by c-di-GMP—namely biofilm formation, motility, stress resistance, and secretion—are virulence/host colonization behaviors. The first indication that

c-di-GMP may contribute to host–bacterial associations was the demonstration that c-di-GMP activates cellulose synthase activity in the plant pathogen *A. tumefaciens*, which produces cellulose to facilitate the anchoring of bacteria to plant cells (5). c-di-GMP was first observed to influence bacterial virulence in *V. cholerae*, where low c-di-GMP levels activate cholera toxin while high c-di-GMP levels attenuate virulence (146). Since then, specific roles for c-di-GMP in the establishment and maintenance of host–bacterial interactions have been defined for many organisms, mainly pathogens.

Many c-di-GMP effectors that contribute to virulence are involved in surface attachment and biofilm formation (96, 119, 154). In both plant and animal pathogens, c-di-GMP activation of attachment and biofilm formation can either promote or attenuate virulence (55, 96) depending on the stage of infection. For example, in *P. aeruginosa* and *V. cholerae* c-di-GMP positively regulates production of polysaccharides and other adhesion factors that contribute to host infection, but in *E. coli* and *S. enterica* c-di-GMP-induced production of cellulose and curli attenuates virulence, as these factors may be recognized by the mammalian immune system for clearance (55). *V. cholerae* presents an example of how c-di-GMP needs to be tightly controlled to initially infect and subsequently disperse from the mammalian gut. c-di-GMP promotes expression of Vps EPS and mannose-sensitive hemagglutinin pili (MSHA) (13), both of which promote biofilm formation (160, 170, 171). Biofilm formation helps *V. cholerae* withstand hypotonic stress in the environment (75) as well as acidic pH in the stomach of human hosts (178), but MSHA acts as an antivirulence factor in that it is recognized by host IgA for clearance (55, 62). Therefore, *V. cholerae* c-di-GMP levels increase to promote survival in the environment and upper gastrointestinal tract, yet decrease in the lower gastrointestinal tract to avoid clearance by the host immune system (55).

c-di-GMP-regulated secretion systems, including the T2SS, T3SS, and T6SS, also contribute to virulence (68, 154). The *P. aeruginosa* T2SS ATPase is a c-di-GMP receptor (71, 117). Regulation of the *P. aeruginosa* and plant pathogen *Pseudomonas savastanoi* T3SS and T6SS is inversely controlled by c-di-GMP: c-di-GMP negatively regulates the T3SS, which is associated with acute infection and motility, and positively regulates the T6SS, which is associated with chronic infection and exopolysaccharide production (6, 105). The *P. aeruginosa* DGC DgcP promotes virulence by upregulating biofilm and the T6SS while downregulating the T3SS, and deletion of *dgcP* in *P. aeruginosa* and *P. savastanoi* reduces virulence in an acute mouse lung injury model and olive plants, respectively (6). The export ATPases of the *P. fluorescens* T3SS and T6SS bind and are regulated by c-di-GMP (151). In *A. tumefaciens*, c-di-GMP represses both the T4SS and T6SS; the T6SS result is the opposite of that in *P. aeruginosa* (97). However, downregulation of the T6SS in *A. tumefaciens* accords with its phases of infection. T4SS, T6SS, and motility are upregulated when *A. tumefaciens* arrives at a wound site and must outcompete other bacterial species. Then, once wound colonization begins, biofilm formation is upregulated while T4SS and T6SS are downregulated, perhaps to direct resources toward establishing infection (97). In *S. enterica* serovar Typhimurium, c-di-GMP proteins influence the invasion of epithelial cells, and the DGC YeaJ produces c-di-GMP upon binding of autoinducer-2 or the bile acid components taurocholate and taurodeoxycholate, thereby repressing expression of T3SS genes (3, 85). c-di-GMP regulation of the T3SS in *Dickeya dadantii*, *Bordetella bronchiseptica*, and *X. campestris* also contributes to virulence (23, 54, 70, 169), as do the T4SS in *Ehrlichia chaffeensis* and *Legionella pneumophila* (4, 83) and TfoX/TfoY-dependent regulation of the T6SS in *V. cholerae* (102).

c-di-GMP is also implicated in the biosynthesis and processing of virulence genes, such as inducing methyltransferase (33) and glycosyltransferase (143) activities in *P. aeruginosa* and *E. coli*, respectively. Gene products that help pathogens survive within the host environment are also regulated by c-di-GMP; c-di-GMP has been linked to resistance against stresses such as oxidative

stress, salt stress, iron stress, nutrient cues, and antibiotics (11, 48, 64, 87, 89, 115, 154). Pathogenic bacteria can also use c-di-GMP to evade the host innate immune system during infection as c-di-GMP directly binds to Siderocalin (LCN2), an antibacterial component of the innate immune system, to inhibit its activity (86).

One common theme apparent in the study of c-di-GMP regulation of bacterial pathogenesis is that it is difficult to determine the precise pathways regulated by c-di-GMP that contribute to host infection. One reason is that in vitro phenotypes are not always recapitulated in vivo, as pointed out by Römling et al. (119). For example, *fimX* and *wspR* mutants in *P. aeruginosa* have strong defects in cytotoxicity in vitro yet did not attenuate virulence in a murine thermal injury model (82), and a c-di-GMP-free strain of the plant pathogen *Dickeya zeae* has drastic biofilm and motility phenotypes in vitro but had little to no defect in virulence against rice seeds (25). Another reason it has historically been difficult to study c-di-GMP during bacterial pathogenesis is that appropriate animal models for certain infections have not yet been developed. For example, the c-di-GMP signaling network has been thoroughly studied in *P. aeruginosa*, but the lack of an animal model that encompasses all aspects of cystic fibrosis airway infection has made it difficult to assess the influence of c-di-GMP during infection (55). These reasons, combined with the inherent complexity and functional redundancy of c-di-GMP signaling networks, make the study of c-di-GMP during host associations particularly challenging. Additional details about the role of c-di-GMP during pathogenesis can be found in several excellent review articles (55, 68, 96, 119, 154, 168).

3.2. c-di-GMP in Beneficial and Commensal Interactions

Most studies aiming to elucidate the roles of c-di-GMP in host–bacterial interactions have been conducted in pathogens. Significantly less attention has been paid to beneficial and commensal associations, although these interactions are ubiquitous in nature and are key to plant and animal life (98). Additionally, many of the pathways and mechanisms used to establish beneficial interactions are similar to those used in pathogenic ones. Therefore, there is great value in studying beneficial interactions when the systems used to study them provide an advantage that enables the discovery of fundamental mechanisms. **Figure 1** summarizes the major themes of this review within the context of their study systems.

Members of the α-rhizobia produce various polysaccharides that contribute to symbiotic biofilm formation and resistance to environmental stresses (38, 139). Swimming and swarming motility are also required to establish interactions with the plant host (21). In *Sinorhizobium meliloti*, a model α-rhizobium that associates with the roots of legumes such as *Medicago truncatula* and *Medicago sativa*, elevated c-di-GMP enhances biofilm formation and inhibits motility, whereas a c-di-GMP-null strain of *S. meliloti* has motility similar to that of wild type and only slightly reduced biofilm formation (129). High c-di-GMP levels induce the production of arabinose-containing polysaccharide in *S. meliloti*, which is not required for symbiotic interaction with *M. sativa*, and induce production of EPS I and EPS II, polysaccharides that are required for nodulation on host roots (79, 129–131). Whether high c-di-GMP affects *S. meliloti* host interaction is unknown, but production of a mixed-linkage β-glucan, which is activated by high c-di-GMP levels, does not influence symbiotic performance (113). Furthermore, a c-di-GMP-null strain does not have a defect in host root colonization (129). In fact, a transcriptomics analysis revealed that expression of DGCs is repressed when associated with host roots compared with free-living cultures, indicating that c-di-GMP levels are maintained at a low level during the symbiosis (84). In *Rhizobium leguminosarum* and *Rhizobium etli*, elevated c-di-GMP levels increase cellulose production, which enhances attachment to plant roots, but inhibit the ability of the bacteria to promote plant growth (112). Additionally, a recent study of the extracellular proteome of a high c-di-GMP

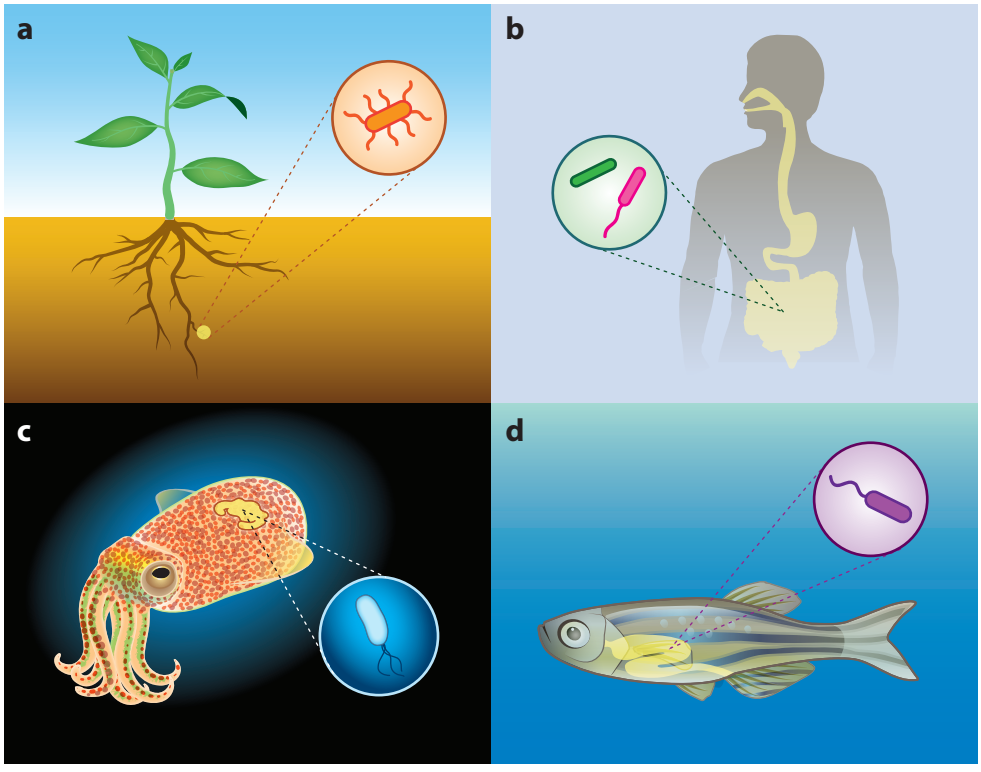

Figure 1

Roles of cyclic diguanylate (c-di-GMP) in model systems for beneficial host–bacterial interactions. (*a*) In α-rhizobial colonization of legume roots, high c-di-GMP interferes with colonization and low c-di-GMP levels either do not affect colonization or are beneficial for colonization (79). (*b*) Isolates from a healthy human gut microbiome, *Faecalibacterium prausnitzii* and *Eubacterium rectale*, encode moderate numbers of diguanylate cyclases (DGCs) and phosphodiesterases (PDEs), while pathogenic species with more diverse lifestyles encode large numbers, implicating c-di-GMP regulation in colonization of mammalian guts (124). (*c*) During *Vibrio fischeri* colonization of the Hawaiian bobtail squid light organ, high c-di-GMP interferes with colonization while low c-di-GMP does not negatively affect it (66). (*d*) Zebrafish beneficial gut isolate *Aeromonas veronii* uses the DGC SpdE to increase c-di-GMP to promote a motile lifestyle. SpdE is inactivated when in proximity to the host to promote biofilm formation and colonization (116).

strain of *R. etli* described posttranslational c-di-GMP activation of the export of extracellular proteins that are likely involved with biofilm formation (92), suggesting that c-di-GMP plays important roles as long as global levels are controlled. Overall, low c-di-GMP levels do not adversely affect rhizobia–legume interactions and may be preferred for host interaction, while high c-di-GMP levels adversely affect the interaction (**Figure 1*a***).

c-di-GMP has roles during bacterial–plant associations other than rhizobia–legume interactions. In the plant-growth-promoting rhizobacterium *Pseudomonas ogarae*, AmrZ regulates biofilm, motility, and c-di-GMP metabolism such that an *amrZ* mutant has decreased c-di-GMP, increased motility, decreased biofilm, and decreased rhizosphere colonization (16). However, while increasing c-di-GMP levels in the *amrZ* mutant restored motility and biofilm formation, it did not restore colonization, indicating that AmrZ regulates colonization independently of c-di-GMP (16). This observation serves as an example of how culture-based phenotypes do not necessarily recapitulate in vivo.

Turning to animal host colonization by nonpathogenic bacteria, a recent study on colonization of the *Caenorhabditis elegans* gut by *Pseudomonas lurida*, which provides protection from pathogens, demonstrated that c-di-GMP is upregulated in host-adapted *P. lurida* isolates (109). Furthermore, upregulation of c-di-GMP consistently increases host competitive fitness by other *Pseudomonas* species (109). Additional evidence from a comparative genomics analysis argues that c-di-GMP underlies host adaptation and is fundamental for the establishment of host–bacterial interactions (109). The results of this study suggest that c-di-GMP lies at the core of the evolution of host–bacterial associations and that our knowledge of the establishment of such associations is incomplete without an understanding of the role(s) of c-di-GMP during this process.

Rudlaff & Waters (124) assessed c-di-GMP signaling within the gut microbiome by identifying DGCs and PDEs in a survey of 17 fecal metagenomic sequence data sets. Overall, they found that c-di-GMP signaling is important for gut microbiome members that need to adapt to multiple environments, particularly pathogens such as *E. coli*, *V. cholerae*, and *Clostridioides difficile*, and that the number of DGCs and PDEs encoded by the gut microbiome as a whole were depleted by approximately two- to ninefold compared with some enteric pathogens (124) (**Figure 1*b***). The authors also found that the gut commensals *Faecalibacterium prausnitzii* and *Eubacterium rectale*, which are associated with healthy gut microbiomes, encode moderate numbers of DGCs and PDEs, suggesting that these species may use c-di-GMP signaling to colonize mammalian guts (124) (**Figure 1*b***). Therefore, c-di-GMP is likely to play roles during establishment of the gut microbiome, and further research is required to assess these roles.

The beneficial association between *Vibrio fischeri* and the Hawaiian bobtail squid (*Euprymna scolopes*) has long served as a biologically relevant and experimentally tractable model system to study mechanisms of animal colonization. In this association, *V. fischeri* is the sole symbiont of the squid light organ, providing a naturally occurring binary symbiosis that can be manipulated to dissect the bacterial factors that contribute to effective colonization (156). During the establishment of the symbiosis, *V. fischeri* must use swimming motility and biofilm formation to efficiently colonize the squid light organ (93, 95, 108, 123, 173, 174). Both motility and biofilm formation are regulated by c-di-GMP in *V. fischeri*, which encodes 50 proteins predicted to modulate c-di-GMP levels (10, 110, 137, 164). Several studies have described the in vitro functions of individual *V. fischeri* DGCs and PDEs in motility and/or biofilm formation, including the DGCs MifA, MifB, and CasA as well as the PDEs BinA and PdeV (10, 30, 67, 110, 149). Additional studies have shown that elevated *V. fischeri* c-di-GMP levels interfere with efficient host colonization (66) (**Figure 1*c***). Interestingly, while elevated c-di-GMP did influence swimming motility and biofilm formation, inhibition of squid light organ colonization was not due to either motility or biofilm, suggesting that c-di-GMP inhibits squid colonization via an unknown colonization factor (66). Similar to what has been observed in pathogenic species, in vivo c-di-GMP phenotypes in *V. fischeri* were not recapitulated in vitro: *V. fischeri* with elevated c-di-GMP levels had reduced symbiotic polysaccharide gene transcription within host-associated aggregates but not in culture-based assays when measured using the same fluorescent reporter system (66).

An elegant study of the zebrafish gut isolate *A. veronii* demonstrated that the DGC SpdE detects specific host-produced amino acids, which inactivate SpdE to promote gut colonization (116). In this association, the c-di-GMP produced by SpdE increases motility and reduces biofilm formation to promote a motile lifestyle, and inactivation of SpdE when in proximity to the zebrafish host decreases motility and increases biofilm formation to promote colonization (116) (**Figure 1*d***). This study not only elucidated a specific pathway through which a single DGC regulates host colonization but also identified host-emitted factors to which the DGC responds to transition from a motile state in the environment to a sessile host-associated state. Furthermore, the use of the *A. veronii*–zebrafish model, as well as the *V. fischeri*–squid model, to investigate

microbial factors that promote nonpathogenic colonization offers excellent examples of how a biologically relevant and experimentally tractable animal–bacterial model system can be a powerful tool to determine the mechanisms underlying the contributions of c-di-GMP to host–bacterial interactions.

4. CONCLUSIONS AND PERSPECTIVES

The past 40 years have seen a remarkable amount of progress in elucidating enzymes, receptors, and pathways at the heart of c-di-GMP signaling. Even with concerted efforts across multiple systems, there remain many exciting questions that are unanswered. We suggest that the host–microbe interface presents an opportunity to address many of those questions, including the role of c-di-GMP during colonization and fundamental aspects of c-di-GMP signaling in which the host environment provides a unique experimental venue and one that has coevolved with the microbe (especially for symbiotic interactions).

4.1. Understanding the Role of c-di-GMP Signaling in Key Colonization Behaviors

The study of c-di-GMP signaling networks and how they contribute to host colonization is incomplete, and likely misleading, without in vivo experiments. While it has long been suspected that c-di-GMP signaling contributes to the establishment and maintenance of host–bacterial relationships, only recently has the significance of this contribution begun to be unveiled. Studies of the roles of c-di-GMP in host–bacterial associations have made it clear that their effects are highly context dependent, a situation that is more complicated for bacteria that exist within multiple contexts. As a result, in vivo experiments are necessary to determine the functions of proteins within c-di-GMP signaling networks and their biological relevance.

At first glance, it may appear that, in multiple diverse study systems, low levels of c-di-GMP are tolerated, whereas elevated levels are detrimental. This has been observed in colonization studies during beneficial plant association (α-rhizobia), beneficial animal colonization (*V. fischeri*), and pathogenic animal infection (*V. cholerae*) (62, 66, 84, 112, 129, 146). However, this comparison has important limitations. Each study examined a single stage of the life cycle of its respective association, and given that c-di-GMP mediates transitions between stages, it is likely that key facets of its role were missed in these early studies. Future research should seek to cover a wider scope of the symbiotic life cycle, including environmental survival, transition into the host, growth and development within the host, and transmission back into the environment. It is clear that experimentally tractable model systems to study c-di-GMP networks will be particularly valuable as the field moves forward.

4.2. Use of In Vivo Systems to Probe Fundamental Aspects of c-di-GMP Signaling

Many open questions in c-di-GMP signaling might be able to be addressed by studies in plant and animal hosts, essentially with the use of these environments as relevant conditions in which to probe signaling mechanisms. As such, in vivo studies are likely to yield valuable insights into basic mechanisms, as they contain compartments, shear flow, and other physical and chemical parameters that are not reproduced in culture, in addition to specific biological signals that are exchanged with the colonizing microbe. Studies in the host also incorporate temporal progress in which molecular dialog between partners stimulates downstream processes in a stepwise back-and-forth process that is difficult to capture in a static assay. Here we pose questions in areas where mechanistic studies in vivo may expand our understanding of c-di-GMP signaling.

4.2.1. Why do many bacteria encode a large number of c-di-GMP enzymes? In contrast to cAMP, which is typically synthesized by a single regulator, it is common to have bacteria with multiple c-di-GMP enzymes. In fact, some of the best-studied c-di-GMP organisms include those with dozens of enzymes, including *E. coli* (29 enzymes) and *V. cholerae* (62 enzymes) (32, 114). A greater number of c-di-GMP proteins encoded in a genome may allow bacteria to adapt to diverse environments, and increasing evidence suggests that the high functional redundancy that exists within c-di-GMP signaling networks allows bacteria to fine-tune the expression of phenotypes in response to specific environments (34, 36, 58, 94, 103, 124, 150, 159, 167). The concept of local signaling suggests that key effects of c-di-GMP can occur through subcellular regions or protein complexes in which local or partitioned c-di-GMP levels are critical for its effects on a given pathway. A comprehensive investigation of the *P. fluorescens* c-di-GMP signaling network concluded that local changes in c-di-GMP play a primary role because ligand signaling, protein–protein interactions, and/or transcriptional regulation are all central to c-di-GMP signaling (34). This study highlights the importance of investigating c-di-GMP regulation in the context of different environments, in response to different signals, and within a network of other proteins (34, 159). We suggest that studies in vivo may provide relevant contexts in which to identify local signaling that is not apparent during culture-based studies. *V. fischeri* encodes 50 proteins with GGDEF and/or EAL domains; a culture-based analysis of individual deletions in the genes for these proteins revealed that fewer than half influence swimming motility, whereas overexpression of each protein revealed that most of the proteins are functionally capable of affecting c-di-GMP-relevant phenotypes (67, 137). This contrast suggests that there could be host-specific c-di-GMP signaling that cannot be observed in culture. In support of this hypothesis is the demonstration that c-di-GMP negatively affects squid colonization by *V. fischeri*, but not through known pathways that are apparent in culture (66). While none of the *V. fischeri* GGDEF and/or EAL proteins are known to individually influence host colonization, sophisticated in vivo studies have the potential to uncover host-specific c-di-GMP signaling pathways (66).

4.2.2. What environmental signals are sensed by c-di-GMP systems? Many GGDEF and/or EAL domain proteins also have sensory domains, indicating that the sensing of environmental signals is important for regulating intracellular c-di-GMP levels. However, our understanding of those specific inputs is rudimentary. Studying proteins in vivo has the potential to identify relevant signals that are not present in media. Study conditions that mimic the in vivo environment have been especially valuable in other systems and should be encouraged (15, 19, 70, 153). Nonetheless, unique host compounds, innate immune signaling, and biophysical constraints that are unique to host environments will require investigation within the host to reveal key aspects of c-di-GMP signaling.

4.2.3. How do dual-function proteins operate? A surprising number of proteins encode both GGDEF and EAL domains. While culture-based studies suggested that in most cases one of the domains is dominant, more recent investigations have revealed that the DGC versus PDE activities of at least some of these dual-domain proteins are context dependent. It would be informative to conduct similar studies across a symbiotic life cycle to query whether this theme is maintained when the organism is examined in a context that more closely resembles the full scope of its life cycle.

As microbiology expands to examine a wider range of organisms in a wider variety of contexts, plant and animal host environments will provide a rich arena in which to examine c-di-GMP networks and expand our knowledge of signaling inputs, pathways, and effectors across the full life cycle of organisms in nature.

DISCLOSURE STATEMENT

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