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Short Review

Local signaling enhances output specificity of bacterial c-di-GMP signaling networks

Eike H. Junkermeier[1] and Regine Hengge[1,2,*]

[1]Institut für Biologie/Mikrobiologie, Humboldt-Universität zu Berlin, Philippstr. 13 – Haus 22, 10115 Berlin, Germany
[2]Excellence Cluster *Matters of Activity*, Humboldt-Universität zu Berlin, Philippstr. 13 – Haus 22, 10115 Berlin, Germany
*Corresponding author. Institut für Biologie/Mikrobiologie, Humboldt-Universität zu Berlin, Philippstr. 13 – Haus 22, 10115 Berlin, Germany.
Tel: +49-30-2093-49686; Fax: +49-30-2093-49682; E-mail: regine.hengge@hu-berlin.de
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Abstract

For many years the surprising multiplicity, signal input diversity, and output specificity of c-di-GMP signaling proteins has intrigued researchers studying bacterial second messengers. How can several signaling pathways act in parallel to produce specific outputs despite relying on the same diffusible second messenger maintained at a certain global cellular concentration? Such high specificity and flexibility arise from combining modes of local and global c-di-GMP signaling in complex signaling networks. Local c-di-GMP signaling can be experimentally shown by three criteria being met: (i) highly specific knockout phenotypes for particular c-di-GMP-related enzymes, (ii) actual cellular c-di-GMP levels that remain unchanged by such mutations and/or below the K_d's of the relevant c-di-GMP-binding effectors, and (iii) direct interactions between the signaling proteins involved. Here, we discuss the rationale behind these criteria and present well-studied examples of local c-di-GMP signaling in *Escherichia coli* and *Pseudomonas*. Relatively simple systems just colocalize a local source and/or a local sink for c-di-GMP, i.e. a diguanylate cyclase (DGC) and/or a specific phosphodiesterase (PDE), respectively, with a c-di-GMP-binding effector/target system. More complex systems also make use of regulatory protein interactions, e.g. when a "trigger PDE" responds to locally provided c-di-GMP, and thereby serves as a c-di-GMP-sensing effector that directly controls a target's activity, or when a c-di-GMP-binding effector recruits and directly activates its own "private" DGC. Finally, we provide an outlook into how cells can combine local and global signaling modes of c-di-GMP and possibly integrate those into other signaling nucleotides networks.

Keywords: biofilm, exopolysaccharide, cellulose, second messenger, diguanylate cyclase, *Escherichia coli*, N4 phage

Introduction

Nucleotide-based second messengers are key players in signal transduction networks in all living cells. Bacteria make use of second messengers to transduce environmental or internal stimuli into physiological and behavioral outputs that include metabolic and developmental adaptations, stress responses, biofilm formation, defense against predators as well as virulence (Jenal et al. 2017, Hengge et al. 2019, Stülke and Krüger 2020, Zaver and Woodward 2020). A wide range of nucleotide-based second messengers, from mono- to di- and oligonucleotide-based molecules, are used by bacteria. The ever-growing list includes classics like cyclic (3',5')-adenosine monophosphate (cAMP), which is involved in regulating carbon metabolism, and guanosine-(penta)tetraphosphate ((p)ppGpp), which links metabolism and stress responses to growth rate (Busby and Ebright 1999, Hauryliuk et al. 2015). The currently most studied cyclic dinucleotide is bis-(3´-5´)-cyclic dimeric guanosine monophosphate (c-di-GMP). It was first identified as an allosteric activator of the bacterial cellulose secretion machinery (Ross et al. 1987), but it is now recognized as a ubiquitous signaling molecule, which is involved in cell adhesion, biofilm formation, cell cycle progression, development, and virulence (Jenal and Malone 2006, Hengge 2009, Bush et al. 2015, Jenal et al. 2017). As microorganisms never fail to amaze, they were recently found to also make use of cyclic uridine monophosphate (cUMP), cyclic cytidine monophosphate (cCMP), cyclic triadenylate (cAAA) as well as cyclic oligoadenylate (cOA) as signaling molecules involved in antiviral defenses (Lau et al. 2020, Tal et al. 2021, Athukoralage and White 2022).

Basic mechanisms of c-di-GMP signaling

c-di-GMP stands out among bacterial second messengers due to the remarkable numbers of enzymes that make and break it, which are encoded in the genomes of single organisms (Hengge 2009). A similar phenomenon has also been observed for adenylate cyclases in certain mycobacteria and alpha-proteobacteria (Baker and Kelly 2004, Shenoy and Visweswariah 2004), but implications, affordances, and consequences of such multiplicity have only been studied for c-di-GMP-related enzymes. C-di-GMP is synthesized by diguanylate cyclases (DGCs) with the catalytic activity residing in GGDEF domains. DGCs operate as dimers, in which each protomer coordinates a GTP molecule to enable their condensation to c-di-GMP. Many DGCs possess an additional c-di-GMP binding site (I-site), which allosterically inhibits the DGC activity (Schirmer and Jenal 2009). The degradation of c-di-GMP is performed by c-di-GMP-specific phosphodiesterases (PDEs). PDEs catalyze the hydrolysis reaction by either EAL or HD-GYP domains, which are two structurally and evolutionary distinct domains

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(Schirmer and Jenal 2009, Galperin and Chou 2022). EAL-type PDEs hydrolyze the dinucleotide to the linear 5′-phosphoguanylyl-(3′-5′)-guanosine (pGpG), which is further degraded to guanosine monophosphate (GMP) by the oligoribonuclease Orn (Orr et al. 2015) or enzymes with similar substrate preference. Some HD-GYP domain-containing PDEs are capable of hydrolyzing c-di-GMP to pGpG while others can break down c-di-GMP to two molecules of GMP in a one-step reaction (Galperin and Chou 2022). GGDEF and EAL domains can be found combined in a single hybrid protein in some cases, but in such composite proteins one domain is often degenerate for enzyme activity and plays a regulatory instead of a catalytic role (Christen et al. 2005). Bioinformatical analyses revealed that on average ten GGDEF domain proteins are encoded in a single bacterial genome, but numbers as high as 57 have been reported. Coding sequences of EAL and HD-GYP domain-containing proteins are present in bacterial genomes in comparable numbers. An overview is accessible at https://www.ncbi.nlm.nih.gov/Complete_Genomes/c-di-GMP.html (Römling et al. 2013).

Most DGCs and PDEs contain various N-terminal sensor domains, which control their enzymatic activities upon recognition of diverse input signals. These domains include several distinct MASE (membrane-associated sensor), PAS (Per-Arnt-Sim), CHASE (cyclases and histidine kinase-associated sensory extracellular), GAPES (gammaproteobacterial periplasmic sensor), or CSS domains (termed after a highly conserved functional amino acid motif) (Hengge et al. 2016). However, only few of the respective input signals have been identified so far. Examples include the sensing of oxygen by the DGC/PDE pair DgcO/PdeO (formerly DosC/DosP) (Tuckerman et al. 2009), of light by the PDE BlrP1 (Barends et al. 2009), of L-arginine by the DGC STM1987 (Mills et al. 2015), of the redox state of the periplasm by the PDE PdeC (Herbst et al. 2018), or of autoinducer-2 by the DGC DgcJ (Li et al. 2022). Many DGCs and PDEs that sense environmental cues are membrane-anchored. However, the perception of the environment is not limited to the DGCs/PDEs *per se*, but these can also be part of larger complexes in which they receive their input signals from interacting sensory proteins. Examples are the Wsp system, which detects surface contact via a chemosensor-like protein (O'Connor et al. 2012), the reaction to nitric oxide via the hemoproteins H-NOX or NosP (Nisbett et al. 2019) or DgcE, which is GTP-controlled via a dynamin-like GTPase system (Pfiffer et al. 2019).

A variety of intracellular effector components sense c-di-GMP to eventually trigger target components to generate certain cellular responses. Effectors and targets can be separate yet interacting components or domains of a single protein. c-di-GMP-binding effectors are highly diverse and include PilZ, MshEN, enzymatically inactive, or "degenerate" GGDEF domains, which bind c-di-GMP via an intact I-site, or degenerate EAL domains (Krasteva et al. 2012, Chou and Galperin 2016, Wang et al. 2016). In addition, c-di-GMP has been found in noncanonical modes of binding. In the case of the transcriptional regulator BldD of *Streptomyces*, an intercalated tetramer of the dinucleotide allows the formation of the functional BldD dimer by bridging the two protomers that do not even touch each other (Tschowri et al. 2014). Additionally, RNA riboswitches have been found to bind and respond to c-di-GMP (Sudarsan et al. 2008, Lee et al. 2010).

In a minimal c-di-GMP signaling model, the highly dynamic intracellular c-di-GMP level is antagonistically controlled by the activity of a DGC and a PDE. Upon reaching a certain concentration, c-di-GMP activates the respective effector/target systems. In principle, such a simple system could also operate in bacteria that possess multiple DGCs and PDEs, if these are highly selectively expressed and/or activated. However, systematic studies, e.g. with the model bacterium *Escherichia coli* K-12, have shown that most of its 12 DGCs and 13 PDEs are not only expressed but are also enzymatically active at the same time (Hengge 2009, Sommerfeldt et al. 2009, Sarenko et al. 2017). Moreover, single gene deletions of DGCs or PDEs have been reported to lead to distinct phenotypes in various model organisms, even though the intracellular c-di-GMP level remained unchanged in the mutants and/or under conditions of target activation (Newell et al. 2011a, Dahlström and O'Toole 2017, Sarenko et al. 2017). These findings raised the question of how a diffusible second messenger can control different output reactions in a highly specific manner via multiple DGCs and PDEs and their effectors that are present at the same time and apparently can act in parallel. This has led to the hypothesis that c-di-GMP signaling does not only occur at a global cellular level as in the minimal model described above, but also by more local mechanisms in multiprotein complexes (Jenal and Malone 2006, Hengge 2009). In recent years, paradigmatic examples of local c-di-GMP signaling have been detected and studied in detail and it has been recognized that cells can flexibly combine global and local c-di-GMP signaling (Hengge 2021).

In this review, we summarize the current theoretical framework of local second messenger signaling as well as the three criteria that have to be met in order to demonstrate experimentally that a particular system involves local c-di-GMP signaling. Modes of simple local signaling will be illustrated by c-di-GMP-dependent activation of the exopolysaccharide-producing Bcs and Nfr machineries of *E. coli*, in which a specific DGC teams up with a particular effector/target system to provide a local source of c-di-GMP in the direct vicinity of the respective c-di-GMP-binding effector domain. Furthermore, we show two cases of more complex local signaling, i.e. the PdeR–DgcM–MlrA complex in *E. coli* and the Lap system in pseudomonads, in which the direct interaction between DGCs and/or PDEs with their respective effector/target systems plays essential regulatory roles that go beyond just bringing the components closely together. Finally, we highlight that global and local signaling processes are integrated in complex dynamic signaling networks in bacteria.

Two principle concepts to achieve signaling specificity by either global or local c-di-GMP signaling

In principle, the controlled expression and activity of multiple DGCs with different K_i's at their respective I-sites provides a cell with the ability to fine-tune the intracellular c-di-GMP concentration in a gradual manner (Fig. 1A). Differential output reactions could then be achieved via discrete binding affinities (K_d's) of the respective effector components. Thus, when the intracellular c-di-GMP concentration is low, only effector/target systems with a high binding affinity would respond to c-di-GMP. By gradually "ramping up" the intracellular c-di-GMP concentration, effector systems with matching K_d's would then respond in a successive manner. Such a complex global signaling mode requires precise control of the activities of the DGCs via their I-sites as well as appropriate K_d's of the receptors (Pultz et al. 2012, Hengge 2021).

An alternative explanation for an observed high output specificity of a particular DGC and/or PDE can be local signaling (Fig. 1B). In its simplest form, a particular DGC and/or PDE directly teams up with a specific effector/target system in a multiprotein complex. In such a "local signaling module," c-di-GMP is produced

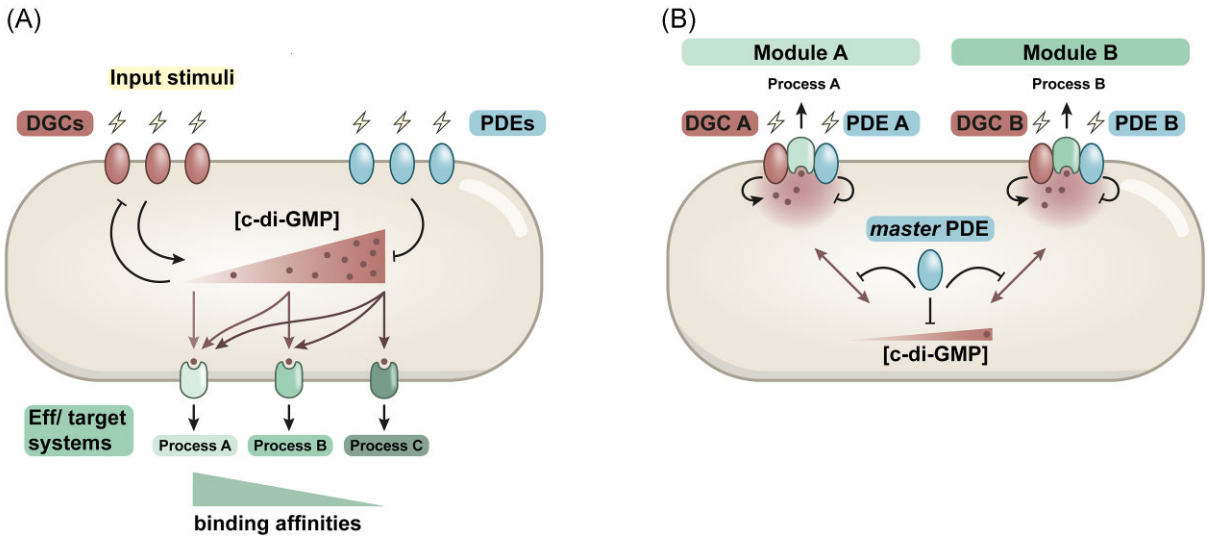

Figure 1. Global and local c-di-GMP signaling. **(A)** High input–output specificity can be achieved by global c-di-GMP when several DGCs (responding to different signals and with different K_i's) conditionally ramp up the cellular c-di-GMP concentration. Distinct effector/target systems can then respond successively based on their different K_d's for c-di-GMP binding. However, at high cellular c-di-GMP levels, specificity is lost as all systems respond. **(B)** Local c-di-GMP signaling can be obtained with a DGC, a PDE, and an effector/target system teaming up in a multiprotein complex or "c-di-GMP signaling module." Interactions may just serve to colocalize the local source (DGC) and/or the local sink (PDE) with the c-di-GMP-responding effector/target system. Alternatively, interactions can also assume regulatory roles, such as direct activation or inhibition of a partner protein. Specific activation of a particular colocalized effector/target systems by its local c-di-GMP source requires a strongly expressed master PDE to constantly drain the cellular c-di-GMP pool to avoid "cross-talk" from other DGCs (Hengge 2021). In order not to overload the figure, only membrane-associated effector/target systems (such as the exopolysaccharide-producing systems mentioned in the text) were included here, but c-di-GMP-responsive systems also occur in the cytoplasm. Details are provided in the text.

in close proximity to its effector binding site, enabling a direct control of the target generating the specific output (Hengge 2009). Three distinct criteria have to be met to experimentally define a local c-di-GMP signaling system. These are (i) a specific knockout phenotype for a distinct DGC and/or PDE, (ii) direct interactions between this DGC and/or PDEs with a particular effector/target system, and (iii) c-di-GMP concentrations, which either remain unchanged when the particular DGC and/or PDE are eliminated by mutations (which elicit a clear phenotype) or which remain significantly below the K_d of the cognate effector component even under conditions of activation of the regulatory output (Hengge 2021).

Particular DGCs control specific cellular processes

The first criterion for local signaling is a specific gene deletion phenotype for a single DGC or PDE. In particular, highly specific DGC knockout phenotypes were indeed reported quite early already for model bacteria such as *E. coli*: the biosynthesis, secretion, and modification of cellulose depends on the presence and catalytic activity of one of its 12 DGCs of *E. coli* K-12, DgcC (formerly YaiC or AdrA in *Salmonella*) (Zogaj et al. 2001, Brombacher et al. 2003, Thongsomboon et al. 2018). The same principle applies to the production of another exopolysaccharide, poly-β-1,6-*N*-acetylglucosamine (poly-GlcNAc), by the PgaABCD machinery, which depends on DgcZ (Boehm et al. 2009). More recently, the activation of the Nfr system, which synthesizes a yet uncharacterized exopolysaccharide that also serves as a phage N4 receptor, was found to require the presence and activity of DgcJ (Junkermeier and Hengge 2021, Sellner et al. 2021).

Besides this allosteric activation of several exopolysaccharide synthesis and secretion systems, another example for a specific DGC/PDE-dependent phenotype was discovered at the level of gene regulation in *E. coli*. The PdeR–DgcM–MlrA complex is a key player controlling transcription initiation of *csgD*, which encodes the biofilm regulator CsgD (Lindenberg et al. 2013, Hengge 2016). In this complex, which will be dissected in more detail below, the absence of the DGC DgcM leads to a significant reduction of cellular levels of the biofilm regulator CsgD, while a loss of the PDE PdeR produces the opposite effect (Weber et al. 2006, Lindenberg et al. 2013, Sarenko et al. 2017). Moreover, the complex itself receives a precise signal input specifically from yet another DGC, DgcE. Again, the absence of this particular DGC leads to a specific phenotype, which is the loss of the key trigger for this intricate signaling cascade initiating *csgD* expression (Pfiffer et al. 2019).

Comparable findings were also reported in other bacterial model organisms. In *Caulobacter crescentus*, specifically one of its four DGCs, PleD, is essential for the G1-to-S phase transition during cell cycle progression (Abel et al. 2013, Kaczmarczyk et al. 2020). In the predatory bacterium *Bdellovibrio bacteriovorus*, each of its three catalytically active DGCs were found to play a distinct physiological role: DgcB is strictly required for the invasion of its prey bacteria, DgcA controls its gliding motility, which is essential to exit the host, and DgcC controls the transition between its predatory lifestyle and growth without a host (Hobley et al. 2012). In *Pseudomonas fluorescens* only a small subset of its 30 DGCs was found to regulate biofilm formation and swimming motility, while the majority showed no effects under the conditions tested (Newell et al. 2011b).

Importantly, in most of the examples mentioned above, the respective phenotypes were observed while other DGCs and/or PDEs were present in the cells. Thus, together with the additional criteria described in the following, such highly specific knockout phenotypes are a strong indication for specific local actions of particular DGCs and/or PDEs.

DGCs and/or PDEs colocalize with specific effector/target systems via protein–protein interactions

The colocalization of a source and/or a sink of c-di-GMP with a specific effector/target system via direct protein–protein interactions serves as a second criterion for defining local signaling. Benziman and his colleagues, who initially identified c-di-GMP as an allosteric activator of the cellulose synthase of *Gluconoacetobacter xylinus* (Ross et al. 1987), already speculated that its c-di-GMP regulatory system (i.e. a DGC and PDE) could be situated in close proximity to the relevant c-di-GMP-binding site. In this proposed "discrete proteinaceous complex," the second messenger was thought to act as a local pacemaker to coordinate the activity of the single cellulose synthase subunits, which form a lateral array of cellulose extrusion pores on *G. xylinus* (Ross et al. 1991). Their concept of colocalization emerged from the question of how the cellulose synthase subunits could arrange in the intriguingly ordered structure on the cell surface (Zaar 1979). However, in view of the theoretical framework of local signaling, specific protein–protein interactions between DGCs/PDEs and effector/target systems play crucial yet different roles.

On the one hand, direct protein–protein interactions bring together all components of a c-di-GMP signaling complex. Such a protein complex allows the associated DGC and/or PDE to act as a local source and/or sink, respectively, for the colocalized effector/target system. Examples are the regulation of the bacterial cellulose synthase (Bcs) by DgcC and PdeK as well as the activation of the Nfr system by DgcJ in *E. coli* (Richter et al. 2020, Junkermeier and Hengge 2021, Sellner et al. 2021). In both cases, a specific DGC associates with the system and stimulates the activity of the glycosyltransferase (GT) domains of BcsA or NfrB by producing the second messenger in the vicinity of the respective c-di-GMP-binding site, namely the PilZ domain of BcsA and the MshEN domain of NfrB. Here, the protein–protein interaction has a scaffolding function in bringing the c-di-GMP regulatory system right next to the effector domain. Since the DGC and PDE act only as local c-di-GMP source and sink, respectively, substitutions of single crucial amino acids in the catalytically active centre of these enzymes fully phenocopy complete knockout mutations. Such a c-di-GMP control module forms a specific, yet open complex, in which the second messenger can either bind to the effector—the probability of which inversely correlates with the (short) distance between DGC and effector binding site—or diffuse away into the cytoplasm (Richter et al. 2020). The absence of any compartimentation of the effector binding site allows strong ectopic expression of some other active DGC or PDE to "bypass" locally acting DGCs or PDEs by either "flooding" the cells with c-di-GMP or providing a robust c-di-GMP drain, respectively, with corresponding effects on c-di-GMP binding to the effector (Richter et al. 2020, Sellner et al. 2021).

On the other hand, protein–protein interactions can also have a regulatory function. Within the PdeR–DgcM–MlrA complex of *E. coli*, the association of PdeR to the DgcM–MlrA module blocks the ability of the latter to act as an activating transcription factor complex (Lindenberg et al. 2013, Serra and Hengge 2019a). Here, PdeR acts as a trigger enzyme whose control via its macromolecular interaction is regulated by its substrate for enzymatic activity. Thus, once PdeR binds and degrades c-di-GMP, this loosens its "grip" on the DgcM–MlrA complex, which in turn allows the latter to stimulate transcription at the *csgD* promoter. Notably, the basic function of PdeR is its direct inhibition of DgcM and MlrA while its PDE-activity has a sensory function, i.e. responding to c-di-GMP produced by DgcE (Hengge 2016).

The trigger PDE PdeL operates in a similar but more minimalistic manner to stabilize subpopulations with either high or low intracellular c-di-GMP levels. At low intracellular c-di-GMP concentrations, the EAL-type PDE PdeL—which also features a LuxR-like DNA-binding domain—acts as a transcription factor that activates its own expression. Under these conditions, PdeL forms a tetramer formed by two canonical EAL domain dimers. Increasing c-di-GMP concentrations, e.g. upon strong induction of a DGC, lead to the dissociation of the tetramer. Although this stabilizes the catalytically active dimer, this also shuts off its own expression, which in the longer run will stabilize high c-di-GMP levels (Reinders et al. 2015). Maintaining global intracellular c-di-GMP levels stably low is essential for local signaling as it prevents cross-talk of specifically localized DGCs (Sarenko et al. 2017). On the other hand, generating and maintaining a high global c-di-GMP level enables a transition from local to global signaling regimes (Reinders et al. 2015).

Another example for regulatory protein interactions in a local c-di-GMP signaling module is the Lap system of *P. fluorescens*, whose full function is described in more detail below. Here, a c-di-GMP binding effector (LapD) physically interacts with the DGC GcbC. Only in this protein complex, GcbC shows DGC activity, which is strictly required for the activity of the system. Hence, the protein–protein interaction not only brings the DGC in close proximity of its specific target, but the latter also controls the catalytic activity of the former and thereby its own signal input (Dahlström et al. 2015).

A crucial role for the global cellular c-di-GMP level in local c-di-GMP signaling

As a third criterion for local signaling, the global intracellular concentrations of c-di-GMP have to be considered. Thus, intracellular c-di-GMP levels, which even under conditions of activation of a system under study remain considerably lower than the K_d's of the activation-triggering effectors, are a first indication for a locally acting DGC. For example, *E. coli* and *P. fluorescens* maintain remarkably low intracellular c-di-GMP concentrations (1–2.5 pmol/mg total protein, corresponding to about 60–150 nM in the cell), even though c-di-GMP-stimulated processes are active under these conditions (Dahlström et al. 2016, Sarenko et al. 2017). In *E. coli*, such a process is cellulose synthesis and secretion by cellulose synthase (BcsA), which is activated by a PilZ domain that binds c-di-GMP with a K_d of 6–8 μM (Chou and Galperin 2016). Similarly, the Nfr system, which requires c-di-GMP activation via its MshEN domain that binds c-di-GMP with K_d of about 1 μM, is active in vegetative cells that contain only approximately 80 nM of c-di-GMP (Junkermeier and Hengge 2021). In other words, the relevant effectors would not be expected to be in the c-di-GMP bound state—yet, the regulatory output can be observed. Notably, however, the K_d's mentioned above were determined *in vitro*, which does not necessarily reflect the complexity inside living cells and, therefore, the values obtained should be taken with care. Thus, in *Streptomyces* an accessory protein has been found that can change the K_d of a particular c-di-GMP binding effector 15-fold as compared to the effector component alone (Schumacher et al. 2021).

In addition to low intracellular c-di-GMP concentrations, unchanged intracellular c-di-GMP levels in single DGC or PDE knockout mutants (ideally with point mutations just affecting the active centre) that confer clear phenotypes, can also be a strong indication for local signaling (Sarenko et al. 2017). But again, measurements of intracellular c-di-GMP concentrations should be interpreted with care as these are population-wide averages, which may fail to take into account a potentially heterogeneous

distribution in the population. This does not seem relevant in cases where the overall c-di-GMP levels are already extremely low such as in *E. coli* or *P. fluorescens*, but should be considered in cases where these global levels are higher.

Overall, the global cellular c-di-GMP concentration thus matters crucially for local c-di-GMP signaling. The very low intracellular c-di-GMP concentration in *E. coli*—both in growing and in stationary phase cells in liquid culture—is maintained by the strongly expressed "master PDE" PdeH, which consists of an apparently not further regulated stand-alone EAL domain that constantly degrades c-di-GMP in the cytosol (Sarenko et al. 2017). This quenching of c-di-GMP by a strongly active nonlocalized PDE is a prerequisite for specificity of local signaling in an open system, since it limits the effect of c-di-GMP diffusion and thereby potential cross-talk among several specifically localized DGCs (Hengge 2021). This can be shown experimentally. Thus, knocking out PdeH leads to elevated intracellular c-di-GMP concentrations (up to 1 μM) and increased c-di-GMP signaling output (expression of *csg* genes), to which now several DGCs contribute, i.e. DGC specificity is lost (Sarenko et al. 2017). This also correlates with reduced motility, which in turn can be additively suppressed by combining mutations in these several DGCs (Girgis et al. 2007, Pesavento et al. 2008, Junkermeier and Hengge 2021).

In conclusion, low intracellular c-di-GMP concentrations are a prequisite for local signaling in "local c-di-GMP source" system, where a specific DGC teams up with an effector/target system. In principle, the theoretical framework of local signaling also applies to the inverse conditions, where a locally acting PDE associates with an effector/target system and constantly removes c-di-GMP close to the effector binding site. Establishing such a highly dynamic local c-di-GMP sink requires the maintenance of a high global c-di-GMP concentration by at least one strongly active DGC (Hengge 2021). Experimentally, this would show up as a system where a specific PDE would be found to play a crucial role—and its knockout or active site mutations to generate a clear phenotype—on a background of constantly high c-di-GMP levels.

Examples of simple local signaling via localized c-di-GMP sources and sinks

The well-studied enterobacterial cellulose synthesis and secretion machinery has emerged as a prime example of a locally c-di-GMP controlled effector system (Richter et al. 2020). Bacterial cellulose is a component of the extracellular biofilm matrix of multicellular bacterial communities (Serra and Hengge 2019b), which is an unbranched polysaccharide of β-1,4-linked D-glucose molecules. In *E. coli* and many other bacteria, every other glucosyl residue in cellulose is modified by the attachment of a phosphoethanolamine (pEtN) group (Thongsomboon et al. 2018). The bacterial cellulose synthesis (Bcs) machinery consists of a total of nine components (BcsRQABZCEFG) and spans from the cytosol throughout the cell envelope (Fig. 2A). As a transmembrane core part, a single BcsA subunit is bound to a multimeric "crown" of five or six BcsB subunits (Abidi et al. 2021). The processive polymerization of the glucose subunits is performed by the GT domain of BcsA, which is allosterically activated by the binding of c-di-GMP to its PilZ domain (Morgan et al. 2014). The nascent polysaccharide gets extruded through the Bcs macrocomplex (BcsRQABEF) into the periplasm, where the periplasmic domain of the membrane-anchored BcsG catalyzes the covalent attachment of the pEtN groups, which are transferred from the phosphatidylethanolamin in the phospholipid membrane (Thongsomboon et al. 2018). This modification process is controlled by the membrane-embedded BcsF and cytosolic BcsE subunits, with the latter being a second c-di-GMP sensing component besides the PilZ domain of BcsA. BcsE features a catalytically inactive GGDEF domain, which serves as its c-di-GMP binding effector domain (Fang et al. 2014). Thus, c-di-GMP stimulates synthesis, secretion and modification of the final product pEtN-cellulose.

For *E. coli*, the DGC DgcC (or its homologue AdrA in *Salmonella*), which consists of a membrane-intrinsic MASE2 domain and a canonical GGDEF domain, is indispensable for the production of cellulose (Zogaj et al. 2001, Brombacher et al. 2003). In line with the criteria for local signaling described above, the absence of DgcC in *E. coli* does not alter the global intracellular c-di-GMP concentration, although it is present in the cells and the purified enzyme showed DGC activity *in vitro* (Sarenko et al. 2017, Richter et al. 2020). A catalytically inactive DgcC variant failed to restore the cellulose-deficient phenotype of a Δ*dgcC* mutant, but an artificially increased c-di-GMP level obtained by plasmid-driven expression of diverse DGCs has been shown to do so. Thus, DgcC is not a critical structural component of the Bcs complex, but its role is to provide the stimulus for its activation. DgcC was found to interact both with the intramembrane parts of BcsB and the PDE PdeK, which is encoded right downstream of the *bcs* operon in *E. coli* (Richter et al. 2020). In contrast to the strong phenotype of a *dgcC* mutant, the deletion of *pdeK* shows only a minor phenotype of slightly enhanced cellulose production and, like the *dgcC* deletion, does not influence the global c-di-GMP pool. The cytoplasmic part of PdeK alone showed high PDE activity *in vitro*, indicating that its transmembrane and/or periplasmic domain may modulate its activity upon sensing of a so far unknown signal. Like DgcC, also PdeK is able to bind to BcsB. In summary, DgcC and PdeK form a locally acting c-di-GMP control module, which docks specifically onto the cellulose synthase complex (Fig. 2A) and fulfills all the criteria for acting as a target-specific local source and sink of c-di-GMP (Richter et al. 2020).

While the N4 resistance (Nfr) system of *E. coli* has long been know to be required for phage N4 infection (Kiino and Rothman-Denes 1989), it was recently discovered to actually produce a novel exopolysaccharide in a c-di-GMP stimulated manner (Junkermeier and Hengge 2021, Sellner et al. 2021). The system consists of three subunits, NfrB, NfrA, and YbcH, which are encoded in an operon. In principle, it shows the same key components of an exopolysaccharide synthesis and secretion system as the Bcs system, i.e. an inner membrane-embedded GT (NfrB), a periplasmic protein with a potential scaffolding role (YbcH), and an outer membrane pore (NfrA) (Fig. 2B). Just recently it was found, that phage N4 does not rely simply on the presence of the Nfr proteins for adsorption, but that in an initial step it binds to the secreted exopolysaccharide, enabling its interaction with its protein receptor (NfrA) on the bacterial cell envelope in a second step. Therefore, phage N4 and its ability to infect can serve as an excellent tool to study the function of the Nfr system. Thus, phage adsorption was found to depend on the catalytic activity of the GT domain of NfrB. This N-terminal GT domain is followed by a C-terminal MshEN domain, which tightly binds c-di-GMP, which in turn controls the activity of the GT domain in an allosteric manner (Junkermeier and Hengge 2021). Remarkably, the infection of *E. coli* K-12 with phage N4 requires the presence and catalytic activity of just one of its twelve DGCs: DgcJ (Mutalik et al. 2020, Junkermeier and Hengge 2021, Sellner et al. 2021). In line with the criteria for local signaling, infection could only be restored by complementation with enzymatically functional DgcJ. Even strong expression of *dgcZ*, encoding a highly active DGC, was able to restore the phenotype to some extent only, suggesting a specific role of DgcJ in the Nfr system. Moreover,

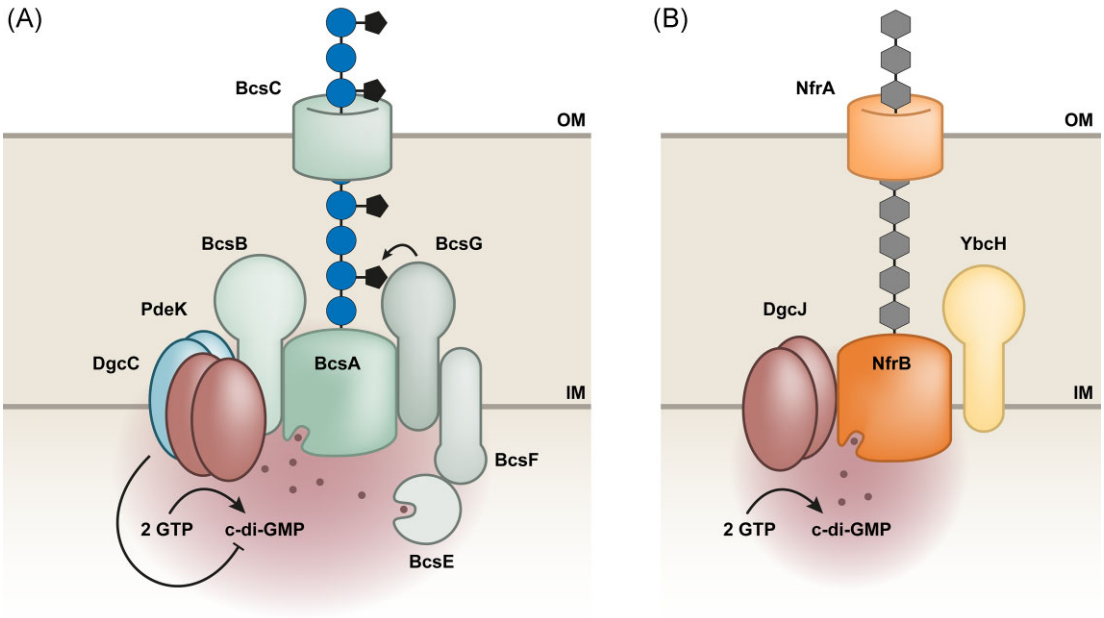

Figure 2. The Bcs and Nfr systems of *E. coli* operate with local c-di-GMP sources and/or sinks. **(A)** The different cell envelope-associated components of the Bcs system are shown that synthesize, transfer, and modify the exopolysaccharide pEtN-cellulose. **(B)** The equally cell envelope-located components of the Nfr system are shown that synthesize a yet uncharacterized exopolysaccharide. Both systems are typical representatives of targets controlled by local c-di-GMP sources (DgcC, DgcJ) and/or sinks (PdeK). c-di-GMP generated by the colocalized DgcC specifically activates the GT BcsA (via its PilZ domain) and the BcsE protein, which contributes to stability of the Bcs complex and controls modification of cellulose via the BcsF and BcsG components (Thongsomboon et al. 2018, Richter et al. 2020). c-di-GMP generated by the colocalized DgcJ specifically activates the GT NfrB (via its MshEN domain) (Junkermeier and Hengge 2021, Sellner et al. 2021). Note that in this minimal representation, proteins are not drawn to scale nor are protein stoichiometries taken into account. In cellulose, the -1,4-linked glucosyl residues are drawn as blue circles, the pEtN modification of cellulose is symbolized by black pentagons, and the glycosyl residues of the uncharacterized exopolysaccharide produced by NfrB are represented by gray hexagons. Details are provided in the text.

exclusively DgcJ, but not the other DGCs of *E. coli* K-12, were found to directly interact with NfrB under the conditions tested. Taken together, the Nfr system is a novel example of a c-di-GMP-activated system in *E. coli*, that depends on DgcJ as a local c-di-GMP source (Junkermeier and Hengge 2021, Sellner et al. 2021).

Besides being derived from thorough experimental analyses of the paradigmatic systems described above, the model of local c-di-GMP signaling via colocalized DGCs and PDEs acting as local c-di-GMP sources and sinks, respectively, is further supported by a theoretical modeling approach (Richter et al. 2020). A reaction–diffusion model was used to assess the question if a colocalized c-di-GMP source (in the model: DgcC) and sink (PdeK) right next to a c-di-GMP-binding effector (BcsA, the "target") are sufficient to explain the observed signaling specificity even in an open, i.e. non-compartmentalized system, in which c-di-GMP can freely diffuse. Its diffusion was modeled with three parameters: the diffusion coefficient D of c-di-GMP (assumed to be equal to or somewhat lower than the experimentally determined D for cGMP), the distance L between the source, sink, and target (modelled between 3 and 15 nm) and the reaction radius r, which defines the minimal distance of c-di-GMP to a target where a reaction takes place (i.e. binding to BcsA or degradation by PdeK). The simulations showed an exponential inverse relationship between the distance L and the reaction probability of c-di-GMP with BcsA, while D does not play a role and the reaction radius r only showed a scaling effect. Thus, the docking of a DGC to a specific effector alone can greatly increase signaling efficiency. Moreover, the reaction probabilities of the low cytosolic concentrations of c-di-GMP in *E. coli* were found to be negligable, while even a single locally produced c-di-GMP molecule increased the interaction probability for BcsA several 100-fold. This modeling approach further showed that colocalization alone can be sufficient to generate high signaling specificity, without the need of the formation of a closed microcompartment in the cells (Richter et al. 2020).

Examples of complex local signaling also involving regulatory protein–protein interactions

In addition to a scaffolding function in the formation of signaling complexes, protein–protein interactions can also exert a direct regulatory function. This is exemplified by the "trigger PDE" PdeR and its inhibitory role on the DgcM–MlrA complex in *E. coli* (Lindenberg et al. 2013). The DgcM–MlrA complex stimulates the expression of *csgD*, which encodes the biofilm master regulator CsgD. This transcriptional regulator plays a key role in biofilm formation of *E. coli*, as it controls the production of two main matrix components: amyloid curli fibres and pEtN-cellulose. The regulon of CsgD includes, among others, the *csgBA* operon, which specifies the nucleator protein and the major subunit of the amyloid curli fibres, as well as the *csgDEFG* operon, which also provides the components for the secretion of CsgA and CsgB through the cell envelope. Moreover, CsgD activates the transcription of *dgcC*, and thereby indirectly regulates synthesis and secretion of pEtN-cellulose via DgcC as described above.

As its primary activity, PdeR interacts with both DgcM and MlrA, and thereby inhibits the function of the DgcM–MlrA as a transcriptional activator of *csgD* (Fig. 3A) (Lindenberg et al. 2013). Within the PdeR–DgcM–MlrA complex, the enzymatic PDE activity of PdeR functions as a "trigger" to change its regulatory interaction with DcgM and MlrA. Thus, binding and hydrolysis of c-di-GMP

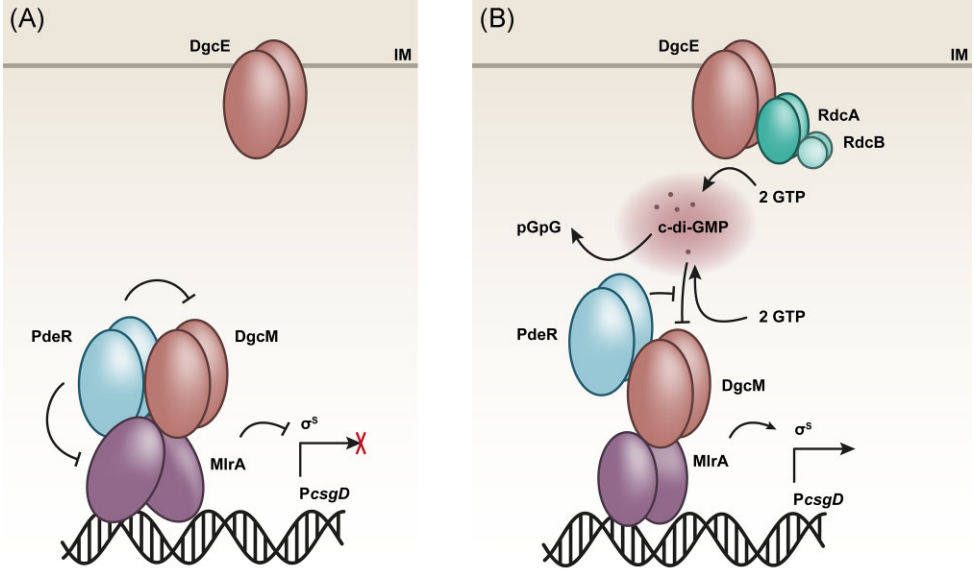

Figure 3. Local c-di-GMP signaling in the DgcE–PdeR–DgcM–MlrA cascade of *E. coli* involves regulatory protein–protein interactions. **(A)** If DgcE is inactive, PdeR inactivates both DgcM and the MerR-like transcription factor MlrA, which thereby form a transcriptionally inactive complex bound upstream of the csgD promoter. **(B)** Upon activation by RdcAB, DgcE produces c-di-GMP, which is bound and degraded by PdeR. This changes the interaction of PdeR with DgcM–MlrA in a manner that allows the latter complex to initiate transcription and DgcM to also synthesize c-di-GMP, which—in a positive feedback loop—contributes to keeping PdeR "busy" and, therefore, in a state where it does not inhibit DgcM–MlrA complex (Lindenberg et al. 2013, Hengge 2016). However, in a fine-tuning "internal" negative feedback loop, PdeR also prevents DcgM-produced c-di-GMP from binding to DgcM's own I-site, which would tune down DgcM's coactivation of MlrA as a transcription factor. Thus, PdeR combines major negative and fine-tuning positive functions in this complex system (Serra and Hengge 2019a). Further details are provided in the text.

via the EAL domain of PdeR relieves its inhibition of the DgcM–MlrA complex (Fig. 3B). This results in a dual output activity, i.e. the DNA-bound DgcM–MlrA complex stimulates *csgD* transcription, but DgcM also produces c-di-GMP, which provides a positive feedback by contributing to keeping PdeR in the "busy" state, in which it does not interfere with the function of the DgcM–MlrA complex in trancription initiation. This positive feedback is part of a bistable switch that plays a key role in the long-term committment to extracellular matrix production of a subpopulation of cells during transition into stationary phase (Yousef et al. 2015). In a growing macrocolony biofilm the formation of matrix-free and matrix-producing subpopulations is spatially controlled by both metabolic gradients and this bistable switch and leads to the formation of a complex matrix architecture (Serra et al. 2015, Klauck et al. 2018, Serra and Hengge 2019a).

Although being an active PDE, PdeR thus serves as the c-di-GMP-sensing effector in this signaling module. Moreover, it senses c-di-GMP generated specifically by DgcE. This happens in post-exponentially growing and/or early stationary phase cells, where DgcE indeed makes the largest contribution to the dynamic global pool of c-di-GMP (which is constantly drained by the master PDE PdeH), but several other DGCs are also active at the same time (Sarenko et al. 2017). Nevertheless, DgcE is the only one that controls how PdeR affects its target, i.e. the DgcM–MlrA complex, and thereby affects *csgD* transcription. The exact mechanism of how the membrane-attached DgcE can specifically control the cytosolic PdeR–DgcM–MlrA complex is not fully understood yet, but it requires the low global intracellular c-di-GMP concentration established by PdeH, thus indicating a local c-di-GMP source mode of action. DgcE seems able to bind to PdeR (Sarenko et al. 2017), which theoretically might allow a recruitment of PdeR or even the entire PdeR–DgcM–MlrA complex on the DNA to the membrane. The need for specific signal input by DgcE can be bypassed by a strong artificial increase in the cellular c-di-GMP level, for instance by a knock-out of PdeH or by ectopic overexpression of another DGC.

The PdeR–DgcM–MlrA system actually shows further regulatory intricacies with nested positive and negative feed-back loops (Fig. 3B). Besides providing for a positive feedback via its local DGC activity that prevents PdeR from inhibiting DgcM–MlrA as a transcription factor (see above), DgcM also acts as a direct co-transcriptional activator for MlrA and this activity does not depend on its DGC activity (Lindenberg et al. 2013). However, this cotranscription activity seems negatively controlled in an internal feedback loop by c-di-GMP produced by DgcM binding to DgcM's own I-site, which in turn is prevented by the presence of PdeR locally degrading this c-di-GMP (Serra and Hengge 2019b)—this seems an intriguing twist of the system, because it means that while PdeR is an overall strong inhibitor for the transcriptional activity of DgcM–MlrA, it also exerts a fine-tuning positive role in locally preventing DgcM-produced c-di-GMP from saturating DgcM's I-site and thereby tuning down the activation of *csgD* transcription by the DgcM–MlrA complex. Taken together, the complex regulation of the DgcE–PdeR–DgcM–MlrA circuit is another example of a locally controlled system, but instead of just colocalizing a local source and sink of c-di-GMP with an effector/target system, protein–protein interactions have crucial regulatory functions and allow the emergence of nested local feedback loops for a highly nonlinear fine-tuning of the system output.

The Lap system of *P. fluorescens* is another intriguing example of a c-di-GMP-regulated complex, in which the physical interaction of a specific DGC with the effector system has a regulatory impact (Fig. 4). LapA is an adhesive protein anchored in the outer membrane, which can attach to various biotic and abiotic surfaces, and thereby enables biofilm formation (Collins et al. 2020). It is secreted by a type I secretion system consisting of LapB, LapC, and

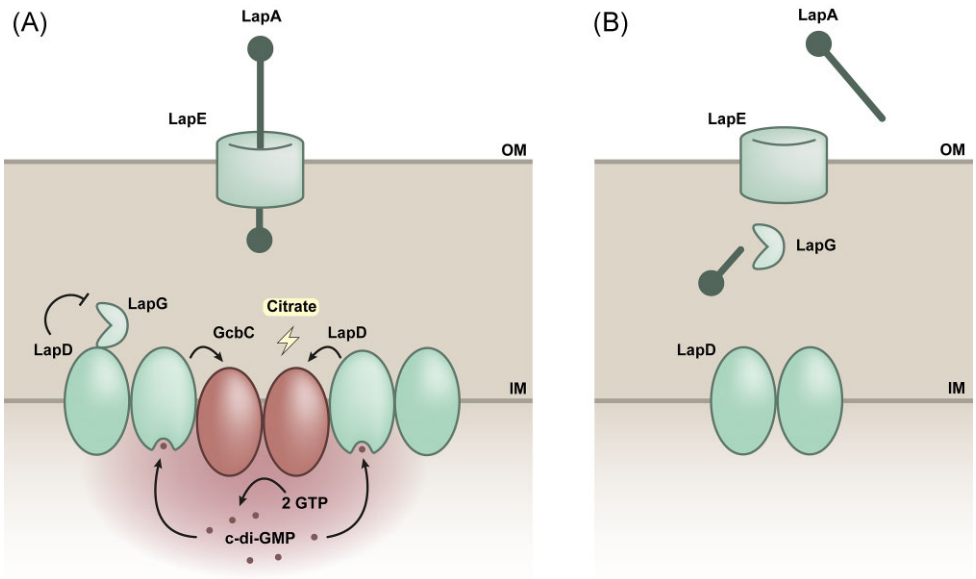

Figure 4. Regulatory protein–protein interactions in local c-di-GMP signaling in the biofilm-controlling Lap system of *P. fluorescens*. LapD is a membrane-associated bi-functional c-di-GMP-binding effector component. **(A)** In the c-di-GMP-bound state, LapD recruits the periplasmic protease LapG by direct interaction. This promotes biofilm formation by preventing proteolytic cleavage of the adhesine LapA, which thus remains cell surface-anchored via its periplasmic plug domain and the outer membrane pore protein LapE. The c-di-GMP binding to LapA is synthesized specifically by the DGC GcbC, which requires additive activation by its binding of citrate and its direct interaction with LapD. Thus, LapD recruits and activates a "private" DGC for itself. **(B)** If LapD is in the c-di-GMP-free state, LapG is released and can cleave the plug domain of LapA, which consequentially gets lost from the cell surface. Further details are provided in the text.

LapE (Hinsa et al. 2003). The N-terminal part of LapA folds into a globular plug or retention domain, which tethers it inside its secretion pore LapE, and thereby within the outer membrane, with its adhesive C-terminus facing outwards (Smith et al. 2018). The periplasmic protease LapG controls the release of LapA from the cell surface via the proteolytic cleavage of the retention domain of LapA (Boyd and O'Toole 2012, Chatterjee et al. 2014). LapG itself is regulated by the inner membrane protein LapD, which can sequester LapG away from its target (LapA), thereby preventing the proteolytic cleavage of its retention domain and promoting LapA-dependent biofilm formation (Newell et al. 2011a). LapD is a c-di-GMP-binding effector, in which a catalytically inactive EAL domain provides the binding site (Newell et al. 2009). Upon c-di-GMP binding, a conformational change of LapD, which is signaled via its HAMP domain, enables its periplasmic domain to sequester LapG, which in turn protects LapA from cleavage (Newell et al. 2011b).

The c-di-GMP that triggers this LapD-dependent cascade is produced locally by the DGC GcbC, which directly binds to LapD (Dahlström et al. 2015). But GcbC does not only colocalize with LapD—the intriguing twist here is that it is actually activated by LapD via this direct interaction. This means that LapD "privatizes" this DGC not only by recruiting it into its immediate vicinity but by making sure it is active nowhere else. This local activation further enhances the specificity between a DGC and a particular c-di-GMP-binding effector component. Furthermore, the interaction between GcbC and LapD shows a certain conditionality as it was found to be enhanced by the binding of citrate to the periplasmic CACHE domain of GcbC (Giacalone et al. 2018). In addition, among the 50 GGDEF and/or EAL domain-containing proteins of *P. fluorescens*, at least 15 were found to be able to interact with LapD (Dahlström and O'Toole 2017). While the exact input stimuli of most of these enzymes are not known yet, the system that appears to emerge here is that of a key locally c-di-GMP-controlled effector/target system, i.e. LapD, which recruits its highly specific "private" DGCs depending on a variety of input conditions. This multimodal strategy shows how local c-di-GMP signaling might also link a particularly important cellular output response—such as biofilm formation—to sensing a variety of environmental cues.

The integration of local and global c-di-GMP signaling in signaling networks

Maintaining very low intracellular c-di-GMP concentrations via the "master PDE" PdeH even when c-di-GMP-activated processes are turned on is a crucial trait of *E. coli*. A loss of PdeH and the resulting uncontrolled c-di-GMP cross-talk—all active DGCs contribute to the elevated c-di-GMP pool, which can now globally and indiscriminately activate effector/targeting systems—comes with a fitness cost: the flagellar break protein YcgR is activated and the Nfr system produces and secretes its exopolysaccharide product, which both lead to a motility defect (Girgis et al. 2007, Junkermeier and Hengge 2021). Analogous findings were made in *Salmonella*, which does not encode an Nfr homolog, but produces cellulose via the Bcs system, which, in a Δ*pdeH* mutant strain, was also found to inhibit bacterial motility (Zorraquino et al. 2013). Thus, both enteric bacteria strongly rely on local signaling to be able to fine-tune the activity of several concomitantly present c-di-GMP-controlled systems. Notably, this does not rule out the possibility that under certain conditions these bacteria might also ramp up their cellular c-di-GMP concentration by inducing and/or activating a strongly active DGC and thereby switch from a local to a global signaling mode.

Conversely, maintaining a high global intracellular c-di-GMP concentration opens up the possibility of using locally acting PDEs. Such PDEs associated with a specific effector/target system could work as local sinks, which could locally deprive an associated c-di-GMP-binding effector component from its ligand, hence

isolating it from the global high c-di-GMP pool. While such cases remain to be unequivocally identified in bacteria, this mechanism is reminiscent of signaling by cyclic nucleotide PDEs in eukaryotic systems (McCormick and Baillie 2014, Kokkonen and Kass 2017, Musheshe et al. 2018). On the other hand, a hyperinduced globally acting PDE may overrun local signaling by a localized DGC. Thus, the Lap system in *P. fluorescens* can also be controlled by the globally acting PDE RapA, which is induced at low phosphate levels. The PDE activity of RapA was found to strongly drain the overall cellular c-di-GMP pool, which may counteract local c-di-GMP production and activation of LapD and, therefore, result in the loss of LapA from the cell surface, which reduces initial attachment and can result in the dispersion of already established biofilms (Newell et al. 2009, Collins et al. 2020).

Further complexity may arise from cellular c-di-GMP levels being heterogeneous within a bacterial population. This has been demonstrated for *C. crescentus*, which modulates its c-di-GMP level along the cell-cycle (Christen et al. 2010, Lori et al. 2015, Kaczmarczyk et al. 2020) and *P. aeruginosa*, which maintains distinct c-di-GMP levels in subpopulations of progeny cells after the initial attachment to a surface (Armbruster et al. 2019). Novel methods that enable precise measurements of c-di-GMP at the single cell level are, therefore, a crucially needed tool to further study such cases of population heterogeneity (Zhou et al. 2016, Halte et al. 2022).

Taken together, local c-di-GMP signaling modes can explain how bacteria are able to regulate parallel output reactions in a highly specific manner. DGCs and/or PDEs can team up with specific effector/target systems to precisely control their output reactions. Modes of local and global signaling can be combined in complex networks, allowing bacteria to dynamically switch and adapt to changing conditions. Given the mechanistic versatility of c-di-GMP signaling and the multiplicity of the enzymes involved in many bacterial species, it is highly likely that more cases of locally controlled signaling modules as well as intersections with other nucleotide second messengers controlled circuits (Hengge et al. 2023) await to be discovered, especially beyond the currently well studied model organisms.

Authors' contributions

Both authors wrote and edited the text of this minireview.

Conflicts of interest statement. None declared.

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