Cyclic Di-GMP: Second Messenger Extraordinaire

Urs Jenal*, Alberto Reinders±, and Christian Lori*

Biozentrum of the University of Basel
Klingelbergstrasse 50/70, 4054 Basel, Switzerland
*for correspondence: urs.jenal@unibas.ch
±equal contribution
Abstract

Cyclic dinucleotides are highly versatile signaling molecules in both prokaryotes and eukaryotes involved in the control of various important biological processes. The best-studied example is bis-(3′-5′)-cyclic dimeric GMP (c-di-GMP). Known since the late 1980’s it is now recognized as near-ubiquitous second messenger in bacteria that coordinates diverse aspects of bacterial growth and behavior including motility, virulence, biofilm formation or cell cycle progression. In this Review, we discuss important new insights into the molecular principles of c-di-GMP synthesis and degradation, and its function in cellular control and give a short overview on the signaling versatility of other CDNs including c-di-AMP, cAMP-GMP and cGAMP.

Introduction

The roles of the prototypical second messengers cAMP and cGMP have been studied for over 50 years, whereas recognition of their larger relatives, the cyclic dinucleotides (CDNs), lagged behind. The first CDN was discovered in 1987, when Moshe Benziman reported on “an unusual cyclic nucleotide activator” that was able to stimulate cellulose synthase from Acinetobacter xylinum and identified this compound as bis-(3′-5′)-cyclic diguanylic acid (c-di-GMP)\(^1\). More than 20 years later, c-di-AMP was discovered as a factor involved in DNA repair in Bacillus subtilis\(^2\). Moreover, different versions of c-GMP-AMP (cGAMP) were first discovered in bacteria\(^3\) and later in mammalian cells\(^4\) and were shown to have prominent roles in bacterial virulence and the innate immune response. Despite of their chemical similarities, different CDNs seem to have distinct evolutionary origins with enzymes involved in their synthesis and breakdown being structurally unrelated\(^2,5,6\). The idea that different CDNs evolved in parallel emphasizes the potency and versatility of this macrocyclic ring with two purine moieties as key carrier of cellular information.

The discovery of CDNs has provided novel entry points into studying important biological processes and cell behavior, including how bacteria coordinate their own growth and replication cycle, how they adapt to surfaces by forming multicellular consortia called biofilms, or how pathogenic bacteria control their virulence and persistence. This was possible by first identifying the enzymes involved in CDN synthesis and degradation, followed by the characterization of specific effectors and target molecules. The CDN field is now rapidly expanding into different directions exploring signaling aspects at the atomic, molecular and cellular lev-
els. In the past years, we have learned that CDNs are widespread and immensely versatile signaling molecules that regulate cellular processes at multiple levels of control and that are well integrated with other global regulatory pathways in bacteria like phosphorylation networks, quorum sensing or with other small signaling molecules like cGMP, cAMP or ppGpp. In this Review, we discuss some of the recent advances in CDN biology without the claim to be comprehensive and with a primary focus on aspects of c-di-GMP signaling. C-di-GMP is not only the most widespread CDN in bacteria but so far is also the most intensely studied and best understood member of this family of second messengers. We first summarize properties of the core components of c-di-GMP signaling, enzymes involved in its synthesis and breakdown as well as effector proteins that convert dynamic changes of c-di-GMP concentrations into specific cellular responses. We then highlight recent progress in understanding the most prominent cellular processes regulated by c-di-GMP. While we draw some parallels between c-di-GMP and other CDNs, c-di-AMP signaling in bacteria and the role of cGAMP in the mammalian innate immune response will not be discussed in detail. Instead we refer readers to some excellent and comprehensive recent reviews on CDNs and their prominent role in bacterial physiology.

Makers and breakers

The c-di-GMP monomer shows 2-fold symmetry with two GMP moieties fused by a 5’-3’ macrocyclic ring (Fig. 1a). High-resolution structures of c-di-GMP, in solution or bound to protein, indicated that the ligand exists either as elongated monomer or as condensed intercalated dimer. At physiological concentrations c-di-GMP is a monomer in solution arguing that intercalated dimers form by successive binding of two molecules to specific effector proteins. Cellular levels of c-di-GMP are regulated in response to environmental and internal cues. This is achieved through the activity of two antagonistic enzyme families, diguanylate cyclases (DGCs) and c-di-GMP specific phosphodiesterases (PDEs) (Figure 1a) with equivalent enzyme activities being responsible for c-di-AMP metabolism (Box 2). DGCs and PDEs are found in members of all major bacterial phyla, representing two of the largest known families of signaling proteins in the bacterial kingdom. The synthesis of c-di-GMP is catalyzed by DGCs through the cooperative action of two catalytic GGDEF domains that arrange in an antiparallel fashion with one GTP molecule bound by each protomer. Pioneering structural and mechanistic studies with PleD, a DGC from Caulobacter crescentus, proposed modes of substrate binding, catalytic mechanism, enzyme activation and product inhibition...
for this class of enzymes\textsuperscript{5,7,19-21}. A metal-catalyzed mechanism was proposed, whereby two GTP molecules are positioned in an antiparallel manner to enable the formation of two intermolecular phosphodiester bonds\textsuperscript{8,22} (Figure 1b). The requirement for dimerization conveys a simple mechanism to control DGC activity by using an accessory domain that forms homodimers in a signal-dependent manner. In the case of PleD or its \textit{Pseudomonas aeruginosa} homolog WspR this is facilitated by an N-terminal receiver domain, which dimerizes upon phosphorylation\textsuperscript{5,9-11,19,23} (Figure 1b). An alternative mechanism for the activation of DGCs was proposed recently for DgcZ from \textit{Escherichia coli}, an enzyme with a catalytic GGDEF domain fused to an N-terminal zinc-binding (CZB) domain. DgcZ is a constitutive dimer with its activity being allosterically regulated by the CZB domain\textsuperscript{22} (Figure 1c). When zinc is present, the GGDEF domains of DgcZ, although facing each other, are not positioned in a catalytically competent conformation. DgcZ activation in the absence of zinc may occur via repositioning of the GGDEF domains to enable the productive encounter of bound substrates molecules (Figure 1b).

The arrangement of the catalytic GGDEF domains was also implicated in feedback inhibition. Many of these enzymes are subject to non-competitive product inhibition by binding of c-di-GMP to the allosteric I-site on the surface of the GGDEF domain\textsuperscript{5,21}. In PleD or WspR, an intercalated c-di-GMP dimer binds to this primary site and to a secondary binding site thereby immobilizing the GGDEF domains in a non-productive state\textsuperscript{19,23} (Figure 1b). Product inhibition of DGCs may establish precise cellular threshold concentrations of c-di-GMP or contribute to the reduction of stochastic perturbations and increased stability of c-di-GMP networks by maintaining c-di-GMP levels in defined concentration windows\textsuperscript{21}. While a functional connection between the I-site and product inhibition is clearly established, c-di-GMP binding to some GGDEF domains may also serve other purposes like protein-protein interaction\textsuperscript{24} (see chapter on receptors, below).

Structurally and mechanistically distinct c-di-GMP specific PDEs have been described that are based on EAL and HD-GYP domains, respectively. EAL-type PDEs hydrolyze c-di-GMP in the presence of Mg\textsuperscript{2+} or Mn\textsuperscript{2+} to yield the linear pGpG dinucleotide\textsuperscript{25}. EAL domain-containing proteins are active as dimers\textsuperscript{26,27}. But in contrast to DGCs, where the fusion of two GTP molecules requires a dimer arrangement of the enzyme, the necessity of this quaternary arrangement for PDE catalysis is not intuitive. Recent structural studies implied a regulatory role for EAL dimerization. Based on distinct structural arrangements of EAL dimers, a clamshell-like opening and closing of the EAL dimer was proposed to regulate PDE activity\textsuperscript{27,28}. The evolutionary conserved dimerization interface is formed by two helices, α5 and α6, with
α5 directly connecting via the β5-α5 loop (loop 6) to two central Asp residues that coordinate the metal ions in the active site
(Figure 1d). Structural and biophysical studies revealed that the α5-loop6 region undergoes substantial rearrangements during the clam-like movements of the EAL dimer, indicating that this part of the protein may play a hinge-joint-like role to couple EAL conformation to catalytic activity via the positioning of metal ions in the active site
. Consistent with this, accessory domains known to control PDE activity directly communicate with the EAL dimerization region
. The observation that substrate binding can induce EAL dimerization and also determines the conformation of the α5-loop6 region proposed a bidirectional allosteric communication between EAL domains and regulatory domains with the α5-loop6 region serving as central communication platform
. Interestingly, EAL domain-containing proteins that have adopted roles as c-di-GMP effectors seem to exploit similar c-di-GMP–mediated dimerization and α5-loop6 remodeling to regulate cellular processes
 (see below).

A second, unrelated family of c-di-GMP-specific phosphodiesterase harbors conserved HD-GYP domains
. Recently, the first structure of an active HD-GYP PDE was solved implicating a novel trinuclear iron-binding site in catalysis
. While EAL-based enzymes convert c-di-GMP into the linear product pGpG, HD-GYP hydrolyzes c-di-GMP in a one-step reaction to yield two molecules of GMP
. Thus, for bacteria that lack HD-GYP-domain proteins it remained unclear how pGpG is further catabolized into GMP. This puzzle was solved recently by demonstrating that the oligoribonuclease Orn, a ribonuclease hydrolyzing two- to five-nucleotide-long RNAs, is the primary enzyme capable of removing pGpG
.

Despite detailed knowledge on structure and function of DGCs and PDEs, it has remained challenging to assign physiological roles to individual enzymes under laboratory conditions. Genetic studies often fail to disclose clear phenotypes. Since only few specific input signals have been identified for these enzymes so far, this may be due to the limited physiological conditions that are assayed in the laboratory. Evidence for this was provided by a recent study of PDEs in E. coli. Despite of a total of 13 PDEs being encoded in the genome of this organism, only PdeH is able to reduce c-di-GMP levels and license motility in growing E. coli cells
 (see below). The observations that most PDEs are readily expressed and that a large fraction of these enzymes can be genetically activated to substitute for PdeH in motility control implied that most of these enzymes simply lack the appropriate stimuli under laboratory conditions
. DGCs and PDEs also engage in downstream signaling through direct interactions with their target molecules thereby providing a platform for “spatially localized” control of cellular processes
. Within such supra-molecular complexes, these proteins not
only regulate the synthesis and degradation of c-di-GMP, but can also act as “c-di-GMP sensors” to control neighboring partner proteins.\(^{39}\)

**C-di-GMP receptors**

While "makers and breakers" explain how c-di-GMP levels are controlled in time and space, c-di-GMP pathways ultimately hinge on the respective effectors that bind c-di-GMP and on their downstream targets, cellular components that are regulated by specific c-di-GMP effectors. Given the global influence of c-di-GMP on bacterial cell physiology and given the sheer abundance of DGCs and PDEs in some bacteria, it must be assumed that a large number of such effectors and cellular targets exist. Several families of effector proteins and RNAs have been identified and are now well characterized structurally and functionally.\(^{40}\) This includes mRNA riboswitches,\(^{41}\) transcription regulators,\(^{42}\) proteins containing PilZ domains, a small prototypical c-di-GMP binding unit\(^{36,43,44}\) and proteins harboring degenerate GGDEF and EAL domains.\(^{45}\) With one of these examples the field has recently come full circle. The discovery of c-di-GMP goes back to the observation that c-di-GMP activates the membrane-bound BcsAB cellulose synthase complex in *Glucanacetobacter xylinus* thereby boosting the production of this exopolysaccharide matrix component.\(^{1}\) The availability of the structure of the BcsAB complex now revealed an elegant mechanism, whereby c-di-GMP binding to the C-terminal PilZ domain of BcsA releases autoinhibition of the glycosyltransferase activity to activate the complex (Figure 1e). This example illustrates how c-di-GMP effectors such as PilZ or the newly discovered YajQ protein family can act as versatile adaptors that link c-di-GMP signal input to the activity of enzymes complexes or transcription factors.

The discovery that c-di-GMP binds to a range of transcription factors such as members of the response regulator or CRP/FNR families in a way that was not predictable from protein sequence, argued for a more versatile nature of effector-ligand interactions.\(^{48-50}\) This is supported by the identification and characterization of a range of novel c-di-GMP effectors, an endeavor that was greatly aided by the introduction of innovative high-throughput methods and biochemical techniques (Box 1). One of the most exciting recent discoveries is the emergence of ATPases as molecular targets of c-di-GMP. The first example is FleQ, a bacterial enhancer-binding protein from *P. aeruginosa*. While members of this family of transcription factors are normally activated by phosphorylation, FleQ activity is controlled by c-di-GMP.\(^{51}\) Structural studies revealed that the second messenger interacts with the AAA+ ATPase domain of FleQ at a site distinct from the ATP binding pocket. Binding of c-di-GMP obstructs
FleQ ATPase activity, thereby altering its quaternary structure and transcriptional activity\textsuperscript{50}. Similarly, c-di-GMP specifically binds to MshE, an AAA\textsuperscript{+} ATPase involved in the assembly of mannose sensitive hemagglutinin (MSHA) pili in \textit{Vibrio cholerae}\textsuperscript{52,53}. The observation that HxrA, a MshE homolog and type 2 secretion (T2S) ATPase from \textit{P. aeruginosa} also specifically binds c-di-GMP opened up the exciting possibility that this general protein secretion pathway that employs a pilus-like extrusion mechanism might also be controlled directly by c-di-GMP\textsuperscript{52}. The idea that c-di-GMP might have taken a more global control over bacterial protein secretion is reinforced by some recent observations indicating that this second messenger also controls Type 6 (T6S)\textsuperscript{54} as well as Type 3 secretion (T3S)\textsuperscript{55}. While the exact role of c-di-GMP in T6S is yet unclear, its influence on T3S seems to be direct and again mediated via a central ATPase. It was shown that the flagellar export ATPase FliI from a range of distantly related bacteria specifically bind c-di-GMP\textsuperscript{55}. Binding of c-di-GMP to FliI and to its homolog HrcN from the virulence related T3SS inhibits ATPase activity arguing that it directly interferes with flagellar export and T3S. The authors of this study proposed that the c-di-GMP binding arrangement might be widely conserved among the rotary export ATPases, making the second messenger central to the function of many of these secretion proteins. It will be interesting to compare the c-di-GMP binding mode of the individual members of this family once structural information is available. Finally, sensor histidine kinases, the central components of phosphorylation pathways in bacteria, have also been identified as c-di-GMP targets. The histidine kinase CckA from \textit{C. crescentus} was shown to bind c-di-GMP via its catalytic and ATPase domain, thereby shifting the kinase/phosphatase balance of this bifunctional enzyme\textsuperscript{7} (see below). The discovery that ATPases serve as regulatory hubs for c-di-GMP may reflect on the global role of c-di-GMP in monitoring bacterial cell physiology. ATPases often function as central regulatory switches governing key cellular processes. Apparently, c-di-GMP leverages part of its global influence by seizing control over these essential cellular players.

**Physiological roles of c-di-GMP**

**Development and morphogenesis**

Several bacteria make use of c-di-GMP to control morphogenesis and developmental transitions. This includes \textit{Caulobacter crescentus}, an aqueous organism with an inherently asymmetric life cycle. \textit{C. crescentus} produced two specialized progeny cells during each division cycle, a motile swarmer (SW) and a sessile stalked cell (ST). Predivisional cells of \textit{C. cres...
*crescentus* are highly polarized with a stalk and adhesive holdfast exposed at one cell pole and a flagellum, pili and a chemotaxis apparatus assembled at the opposite pole. While the surface attached ST progeny re-initiates chromosome replication (S-phase) and cell division (G2-phase) immediately following division, the newborn SW cell is motile but blocks replication throughout an extended period (G1-phase). Replication and division resume when the SW cell differentiates into a ST cell, a process during which it ejects its flagellum, retracts its pili and replaces them with a holdfast and a stalk. Recent studies identified c-di-GMP as a major driver of *C. crescentus* pole morphogenesis and cell cycle control. Mutants unable to synthesize c-di-GMP lost all polar appendages and showed striking cell morphology aberrations. Levels of c-di-GMP oscillate during the *C. crescentus* cell cycle with trough values in the motile SW, a peak during the swarmer-to-stalked cell transition and intermediate concentrations during division (Figure 2). The increase in c-di-GMP concentration during the SW-to-ST transition is produced primarily by PleD, a DGC that is activated when cells enter S-phase. PleD activity is confined to the ST cell by two antagonistic histidine kinases, PleC and DivJ, which position to opposite poles of dividing cells and differentially segregate into SW and ST progenies (Figure 2). While PleC acts as phosphatase keeping PleD–P levels low in SW cells, DivJ acts as kinase to impel PleD phosphorylation in ST cells. Counteracting PDEs are thought to keep c-di-GMP levels low in the motile SW cell. One of these, PdeA, localizes to the flagellated pole before division and later partitions into the newborn SW cell where it authorizes motility by keeping c-di-GMP levels low. PdeA is removed by specific proteolysis during the SW-to-ST transition coincident with PleD activation, thereby contributing to the sharp upsurge of c-di-GMP at this stage of the cell cycle.

But how does c-di-GMP oscillation instigate the exact timing of *C. crescentus* cell cycle events? The TipF-TipN pathway regulating flagellar polarity illustrates such an example. Upon binding of c-di-GMP, TipF localizes to the pole opposite of the stalk where it connects with its polar receptor, the birth scar protein TipN. TipF then recruits flagellar proteins to this subcellular site to initiate flagellar assembly in the predivisional cell. TipF is stable when bound to c-di-GMP but is rapidly degraded when c-di-GMP levels drop in the SW cell. Removal of TipF was proposed to reset the flagellar polarization state and to avoid mispositioning of the flagellar motor at the incipient stalked cell pole (Figure 2). Recent studies also linked c-di-GMP oscillations to the G1-S cell cycle transition and chromosome replication control. The transcription factor CtrA acts as inhibitor of replication initiation in *C. crescentus*. CtrA is phosphorylated and active in swarmer cells (G1) where it binds to the origin of replication (Cori) to block replication initiation. During differentiation into ST cells, CtrA is
inactivated to license replication start. CtrA activity is controlled by the bifunctional cell cycle histidine kinase CckA, which phosphorylates CtrA through the phosphotransfer protein ChpT. CckA exhibits kinase activity in the SW cell but adopts strong phosphatase activity during the G1-S transition, thereby reversing the phosphate flux through the CckA-ChpT-CtrA cascade and inactivating CtrA. Concurrent with its dephosphorylation, CtrA is degraded by the ClpXP protease\textsuperscript{56}. Both dephosphorylation and degradation of CtrA are controlled by the c-di-GMP upshift during G1-S. While degradation is mediated by the ClpXP protease adaptor PopA, which binds to c-di-GMP and delivers CtrA to the protease\textsuperscript{45,62,63}, CtrA inactivation results from c-di-GMP directly interfering with the CckA kinase-phosphatase switch. Biochemical and structural studies demonstrated that c-di-GMP binds to CA domain of CckA, thereby inhibiting its default kinase activity and stimulating phosphatase activity (Figure 2)\textsuperscript{7}. In addition to adopting a cyclin-like role to drive G1-S, c-di-GMP imposes spatial control on CckA during division to install asymmetric replication of future daughter cells. In predivisional cells CckA positions to opposite cell poles, adopting kinase and phosphatase activity at the flagellated and stalked pole, respectively. This leads to a gradient of CtrA~P in the cell and to asymmetric replication initiation with the Cori at the ST pole being activated before cell division is completed, while the Cori at the flagellated pole remains inactive\textsuperscript{64,65}. The unequal distribution of c-di-GMP was proposed to control differential activity of CckA at opposite poles. While the bulk of dividing cells experiences high levels of c-di-GMP, a microenvironment with low levels of c-di-GMP was proposed to promote CckA kinase activity at the flagellated pole (Figure 2). The authors of this study proposed that CckA sequestration to the flagellated pole could shield the protein from the cellular pool of c-di-GMP. How such a low c-di-GMP microenvironment is organized and which PDEs are involved in this spatial control remains to be shown.

Asymmetric distribution of c-di-GMP during cell division was also observed in other bacteria arguing that this might represent a general principle controlling cell behavior and/or reproduction\textsuperscript{59}. For example, during the \textit{P. aeruginosa} cell cycle, c-di-GMP levels drop during a short period after cell division in the daughter cell that inherits the polar flagellum. This pattern is caused by the asymmetric distribution of Pch, a PDE that during division localizes to the chemotaxis machinery at the flagellated cell pole\textsuperscript{56}. Akin to the G1 period of the \textit{Cab{u}lobacter} cell cycle, reduction of c-di-GMP at this stage of the \textit{P. aeruginosa} division cycle may promote diversity in the swimming behavior, which in turn could help to adapt to new environments.
Streptomyces undergo a complex life cycle with two distinct filamentous cell forms. Germinating spores develop into vegetative hyphae, which grow into the substrate to scavenge nutrients. Upon nutrient depletion aerial hyphae are formed, which eventually differentiate into long chains of spores. Recently, c-di-GMP was found to have a key role in the transition from vegetative mycelial growth to the formation of a reproductive aerial mycelium. Deletion of genes encoding proteins involved in c-di-GMP metabolism had a notable effect on colony morphology and development. Moreover, increasing internal levels of c-di-GMP blocked development, while depleting c-di-GMP caused premature spore production bypassing the formation of aerial hyphae. Premature sporulation is also observed in mutants lacking BldD, the master regulator of Streptomyces development that represses a global regulon of ~170 sporulation genes. Recently, a direct connection between these two key components of developmental control was identified when BldD was shown to be a c-di-GMP effector protein that represses its target genes in a manner that depends on its binding to c-di-GMP. A drop in cytoplasmic c-di-GMP levels, which causes the BldD dimer to fall apart and dissociate from the DNA, may then trigger BldD inactivation and sporulation. Other examples illustrating the broad impact of c-di-GMP on development and morphogenesis in bacteria include Myxococcus xanthus, Bdellovibrio bacteriovorus or cyanobacteria.

**Motile-sessile transition and biofilm formation.**

Controlling the motile-sessile transition of bacteria is a universal feature of c-di-GMP. Generally, low levels of c-di-GMP are associated with motility of individual cells, while increased c-di-GMP concentrations direct bacteria into surface attached communities and biofilms. But rather than being a simple on/off switch, complex regulatory steps seem to be involved in a multi-stage process leading to surface colonization. In line with motility being a primary target of c-di-GMP, building and operating the bacterial flagellar motor is highly regulated. This includes regulation of flagellar gene expression, motor assembly, or motor function. While controlling flagellar gene expression is likely to be part of a long-term adaptation strategy, tuning motor activity might be important for rapid decisions during bacterial surface encounter. For example, in E. coli and Salmonella enterica increased c-di-GMP levels result in flagellar obstruction by the c-di-GMP effector protein YcgR, which in its c-di-GMP-bound form interacts with the flagellar rotor/stator interface (Figure 3a). To block YcgR activity and to authorize swimming these bacteria co-express the PDE PdeH together with their flagellar genes. A similar mechanisms was proposed to tune motility in Bacillus subtilis, where PdeH controls motility by preventing flagellar obstruction by the YcgR homolog.
DgrA\textsuperscript{74}. YcgR has high ligand affinity arguing that the flagellar motor may respond to small spikes of c-di-GMP that are required to initiate surface attachment. Consecutive steps of surface colonization may involve incremental steps of c-di-GMP increase and the sequential activation of distinct cellular processes. This could be accomplished by a successive intervention of DGCs harboring distinct levels of feedback inhibition\textsuperscript{21} and by the activation of c-di-GMP receptors with gradually reduced affinities\textsuperscript{75}. For example, in \textit{P. aeruginosa} different DGCs, PDEs and receptor proteins are required at discrete stages of biofilm formation\textsuperscript{71}.

Upon surface contact, bacteria rapidly change their program, expose adhesins, activate surface motility organelles and produce an extracellular matrix to protect the developing microcolonies. This adaptation is coordinated by c-di-GMP at the transcriptional (e.g.\textsuperscript{76}), translational (e.g.\textsuperscript{77}) and posttranslational level (e.g.\textsuperscript{78}). For example, c-di-GMP regulates Type IV pili (T4P), the prototypical surface adherence and motility organelles, in various bacteria including \textit{M. xanthus}\textsuperscript{79}, \textit{V. cholerae}\textsuperscript{53}, \textit{P. aeruginosa}\textsuperscript{80}, \textit{C. crescentus}\textsuperscript{58} or \textit{Clostridium difficile}\textsuperscript{81} (Figure 4). Likewise, in \textit{E. coli} the production of the two principle biofilm matrix components, curli fibers and cellulose, is regulated by c-di-GMP\textsuperscript{82}. During the motile-sessile switch, c-di-GMP levels increase as a result of $\sigma^S$-induced expression of DgcE and other DGCs and the consecutive downregulation of the PDE PdeH, which acts as gatekeeper for motility and is part of the large flagellar regulon\textsuperscript{36,83} (formerly: YegE and YhhH\textsuperscript{35}). Increased global c-di-GMP levels then set in motion a local control module consisting of DgcM and PdeR, a DGC/PDE pair that directly interacts with and stimulates the transcription factor MlrA, which in turn activates the expression of the central curli regulator CsgD. Interestingly, the role of PdeR and DgcM is not primarily a catalytic one but rather to sense the global increase in c-di-GMP and in response serve as co-activators for MlrA\textsuperscript{39}. CsgD then mediates transcription of curli genes and at the same time induces the expression of DgcC, the primary DGC to allosterically activate cellulose production via the cellulose synthase complex (Figures 1e, 3b)\textsuperscript{46}. This is a prime example of how different levels of the c-di-GMP network are interconnected to generate highly flexible and expanding responses, which in this case enable for differential tuning of individual matrix components. An alternative exopolysaccharide, poly-beta-1,6-N-acetyl-glucosamine (PGA) can promote \textit{E. coli} surface adherence and biofilm formation. PGA biogenesis and secretion requires the Pga complex (PgaA-D) and its allosteric activation by c-di-GMP. Both the pga\textit{ABCD} operon and two DGCs, DgcT and DgcZ, are controlled by Csr, a global regulatory system that mediates \textit{E. coli} virulence and biofilm formation\textsuperscript{84}. Recent findings indicated that c-di-GMP activates the Pga machinery by binding directly to both
PgaC and PgaD, the two inner membrane components of the Pga complex to stimulate their glycosyltransferase activity.\(^7\) While the processes driving biofilm formation are relatively well understood, mechanisms underlying biofilm dispersal have remained understudied. Given the prominent role of c-di-GMP in biofilm formation, careful control of the second messenger must also be linked to active biofilm dispersal.\(^8\) Such an escape mechanism was identified in *Pseudomonas fluorescens*, where the LapA surface protein mediates surface adhesion and stabilization of biofilms.\(^8\) At high c-di-GMP levels, c-di-GMP binds to LapD to help sequester the LapG protease in the periplasm. When c-di-GMP levels drop upon induction of the PDE RapA, LapD is inactivated thereby releasing the protease to cleave the LapA adhesin and to weaken the biofilm. (Figure 3d).

As biofilms contribute to acute and chronic infections, it is not surprising that the c-di-GMP network is under selective pressure in human patients. Slow growing, autoaggregative *P. aeruginosa* isolates from airways of patients with cystic fibrosis were shown to harbor mutations leading to strong activation of some of the major DGCs.\(^8\) The observation that such variants effectively persisted in animal models and in the presence of sub-inhibitory concentration of antibiotics, despite of reduced growth rates in vitro, indicated that they may have an important role in persistence during antimicrobial chemotherapy.\(^8\)

**Role of c-di-GMP in bacterial virulence**

C-di-GMP modulates virulence of animal and plant pathogens.\(^1\) Processes controlled by c-di-GMP include host cell adherence, secretion of virulence factors, cytotoxicity, invasion, resistance to oxidative stress, and modulation of the immune response. Importantly, recent findings have linked c-di-GMP to the most prominent secretion systems for virulence factors including T2SS, T3SS and T6SS.\(^5\) This opens up the possibility that c-di-GMP interferes with these processes on a more global scale. An emerging example for the importance of c-di-GMP in virulence is *Clostridium difficile*. In contrast to most Gram-positive bacteria *C. difficile* encodes a large number of enzymes involved in c-di-GMP turnover.\(^8\) In the course of infections, *C. difficile* undergoes a c-di-GMP-mediated switch from a motile to a surface adherent state with cells adhering to the intestinal mucosa via T4P and other adhesins.\(^9\) This transition is mediated by a total of 16 c-di-GMP-responsive riboswitches, 12 of which being OFF switches (Type I) and four being ON switches (type II).\(^9\) Through these regulatory elements, c-di-GMP controls the expression of flagella, pili, adhesion factors and other virulence
factors including toxins TcdA and TcdB, the main virulence factors of *C. difficile* (Figure 4). For example, a collagen binding protein (CBP) and its specific protease are inversely controlled by Type I and II riboswitches, respectively. Expression of the protease at low c-di-GMP concentrations effectively prevents host cell adherence, while expression of the CBP at high c-di-GMP concentrations promotes attachment to host tissue. Thus, c-di-GMP-mediated riboswitches control *C. difficile* host colonization by coordinating motility, toxin production, surface adhesion and biofilm formation.

Another example for the prominent role of c-di-GMP in virulence is the 2011 German outbreak of *E. coli* O104:H4, which caused a unusually high incidence of haemolytic uraemic syndrome (HUS). The genome of the causative strain showed characteristics of both enter-haemorrhagic *E. coli* (EHEC) and enteroaggregative *E. coli* (EAEC) and revealed the presence of a highly expressed diguanylate cyclase (*dgcX*), which is prevalent in EAEC O104:H4 strains. This indicated that the outbreak strain and EAEC in general produce high levels of c-di-GMP and likely form biofilms in the host. The observation that the *dgcX* gene is inserted at the *attB* locus, the integration site for phage lambda, and is flanked by prophage elements, suggested acquisition by horizontal gene transfer. The analysis of *E. coli* O104:H4 also emphasized the key importance of adaptation and regulatory flexibility of the c-di-GMP network. While strong adherence, together with Shiga-toxin expression, is a key virulence factor of *E. coli* O104:H4, this strain produces curli but is cellulose negative. The authors of this study speculated that the strong pro-inflammatory effect of curli together with the absence of cellulose (which normally counteracts this effect) may facilitate entry into the bloodstream and kidneys where this pathogen can cause life-threatening hemolytic uremic syndrome.

Given their unique distribution in bacteria and their importance in bacterial virulence it is not surprising that bacterial CDNs did not go unnoticed by the host’s immune system. Recent evidence points to a prominent role for c-di-GMP and c-di-AMP as PAMPs, pathogen-associated molecular patterns that are specifically recognized by the innate immune system of the host (Box 2).

**Conclusion and outlook**

Above we have summarized some of the recent findings describing mechanistic and functional aspects of c-di-GMP signaling in bacteria. Although c-di-AMP was discovered more recently, the field is picking up rapidly exposing comparable physiological complexity (Box 2). It is possible that additional CDNs still await their discovery offering even greater signal-
ing diversity by varying either the nucleotide composition or linkage chemistry. But why are
CDNs so prevalent in controlling important biological processes in bacteria? One major ad-
27
vantage of second messenger-based networks over other information transfer systems based
on protein-protein interaction might be the ease with which they are able to evolve. For ex-
ample, recruiting additional cellular processes into an existing c-di-GMP network seems rela-
tively straightforward, considering that c-di-GMP often binds on the surface of pre-existing
protein domains with only a few amino acids contributing to ligand affinity and specificity
(Figure 5a). Simple recruitment of additional effectors together with the rapid expansion of
makers and breakers by gene duplication might thus have predisposed CDN-based regulatory
networks for the coordination of global metabolic and behavioral transitions in bacteria.

CDN based second messengers also offer various advantages in signal transduction. Their
rapid cellular diffusion stages an instantaneous and global internal response. At the same time
CDNs may act in a highly specific manner either through temporal or spatial control. For ex-
ample, the combination of DGCs or PDEs with distinct inhibition constants and substrate af-
finities, respectively, together with effector proteins or RNAs of matching c-di-GMP affinities
would permit cells to regulate different processes in a highly specific manner (Figure 5b). Al-
ternatively, spatial organization with DGCs and/or PDEs interacting directly with their re-
spective targets in combination with effective mechanisms isolating individual signaling
modules from each other would permit parallel CDN signaling modules with highly specific
readouts (Figure 5c). CDNs like c-di-GMP control the expression, activity, stability, localization or interaction of specific proteins (e.g.\textsuperscript{42,61,78,97}). Moreover, c-di-GMP can control the
same biological process at different levels including transcription, translation or allosteric
control (Figure 5d) (e.g.\textsuperscript{52}). Such a multi-layered signaling architecture can impose tight con-
trol and continuous evaluation power over strictly unidirectional cellular processes like cell
cycle progression or processes with considerable metabolic cost like the motile-sessile switch.
It can also provide bacteria with the ability to rapidly sample the environment and to adjust
their behavior without the need for \textit{de novo} protein synthesis. Or it could serve to integrate
two distinct processes but at the same time uncouple them if necessary by the use of distinct
DGC/PDE modules (Figure 5d). An example of such a process is illustrated by the production
of \textit{E. coli} curli and cellulose (see above). Finally, it could be used to define activity windows
for specific cellular processes, for example by sequential expression control (module 1) and
inactivation of a downstream effector (module 2), which is either turned off by c-di-GMP or
subject to c-di-GMP mediated degradation.
Despite the advances in the CDN field, many important questions remain to be addressed in the future. For example, are there additional CDNs to be included in this emerging signaling paradigm? Which cellular activities do specific CDN networks control and how extensively do these compounds interfere with basic cellular processes in bacteria? What is the exact architecture of CDN networks and how do they contribute to the highly dynamic behavior of bacterial cells? And how do CDN-based networks integrate with other signaling networks like quorum sensing, phosphorylation cascades or regulation by ppGpp? It is safe to predict that this field of research will continue to provide exciting novel insights into bacterial signaling, growth and behavior.
Several novel approaches were developed in the past years to identify and characterize CDN effector proteins on a global scale. This included affinity pull-downs followed by subsequent mass spectrometry analysis. Trivalent chemical scaffolds with a CDN binding, a biotin sorting, and a crosslinking moiety were used as capture compounds in combination with strepavidin coated magnetic beads\(^{98,99}\). A similar approach used c-di-GMP coated sepharose beads for affinity pull-down\(^{100}\). The advantage of these methods is that potential binding proteins can directly be isolated from cell extracts without the need for time consuming fractionation or biochemical purification. Moreover, once specific binding proteins have been identified, such pull-down methods can also be employed for diagnostic purposes in combination with specific antibodies\(^{78}\). Both methods were successfully applied with different bacteria including *Pseudomonas, Caulobacter, Listeria, Streptomyces* or *Bdellovibrio*\(^{42,98,101,102}\). A more indirect approach made use of the complete ORFeome and subsequent testing of cell lysates with a high-throughput binding assay\(^{52,103}\). Differential radial capillary action of ligand assay (DRaCALA) was developed to directly mix proteins with (radio)-labeled nucleotide(s) on a nitrocellulose membrane. Upon washing the filters free ligand will diffuse away, while ligand specifically bound by proteins will be immobilized at contact site\(^{103}\). Application of these techniques as well as more conventional approaches like Isothermal Titration Calorimetry (ITC) or Microscale Thermophoresis (MST) have led to the identification of a plethora of novel effector proteins\(^{52,55,100,104}\).

Several tools and biomarkers were established for *in vitro* and *in vivo* analysis of c-di-GMP. This includes sensitive high performance liquid chromatography-coupled tandem mass spectrometry (HPLC-MS/MS) to accurately determine the concentration of second messengers in cell extracts\(^{105,106}\), fluorescence-based reporters fused to c-di-GMP dependent promoters or riboswitches\(^{107-110}\), and a c-di-GMP specific FRET probe that allows direct measurements of c-di-GMP concentrations in individual live cells\(^{59,66}\).
Box 2: CDNs beyond c-di-GMP

The CDN c-di-AMP was discovered as a ligand bound to the N-terminal domain of the DNA damage-sensing protein DisA of *B. subtilis*[^2]. Biochemical studies identified this domain as diadenylate cyclase (DAC), the founding member of a family of enzymes that converts ATP to c-di-AMP. Specific PDEs associated with DHH-DHHA1 or HD domains hydrolyze c-di-AMP into pApA or AMP[^111-113]. C-di-AMP is essential in a variety of different bacteria and any dysregulation causes abnormal phenotypes[^114,115]. A recent report suggested that in *Listeria monocytogenes* this effect is due to overshooting levels of (p)ppGpp, a global second messenger linked to carbon metabolism and nutrient starvation[^116]. C-di-AMP is associated with a growing list of cellular functions primarily in gram-positive bacteria. These include cell wall homeostasis[^115,117-120], DNA integrity[^2,121-123], potassium homeostasis[^104,124-126] and osmoprotection[^127,128], gene expression[^129,130], biofilm formation[^131,132], sporulation[^133], metabolism[^102], resistance to antibiotics[^134], and, similar to c-di-GMP, cell-mediated adaptive immune response (see below).

C-GMP-AMP is of special interest because it is produced by bacteria and metazoans[^3,135]. Bacterial c-GMP-AMP shows 3’-3’ linkage and is produced by the dinucleotide synthase DncV originally identified in *V. cholera*[^3]. Structural studies revealed that in the first nucleotidyl transfer reaction DncV preferably recognizes ATP and GTP as acceptor and donor nucleotides, respectively[^136]. c-GMP-AMP is required for host colonization by *V. cholera* and for exoelectrogenesis in different delta-proteobacteria[^137,138]. Mammalian c-GMP-AMP (2’-3’) (cGAMP) has adopted a prominent role in a vertebrate innate immunity pathway responsible for surveillance of cytoplasmic DNA[^139]. cGAMP is synthesized by the cGAMP synthase (cGAS), which is activated in response to binding cytoplasmic DNA[^140,141]. cGAMP binds to and activates the host receptor STING which in turn recruits TANK-binding kinase 1 (TBK1) to phosphorylate IFN regulatory factor 3 (IRF3), ultimately leading to type I interferon (IFN) production. Evolutionary studies recently revealed that the cGAS-STING function is conserved in anemone, which diverged from the human lineage more than 500 million years ago. Because Anemone cGAS produces a bacteria-like 3’-3’ linked CDN that is recognized by Anemone STING, it was proposed that cGAMP (2’-3’) is a recent vertebrate innovation and that during evolution the protein components of this pathway remained structurally conserved, while chemical changes in the second messenger were driving functional innovation[^142].

Recent evidence suggests that c-di-GMP and c-di-AMP, secreted or released outside bacteria, are also sensed by STING (stimulator of interferon genes) thereby converging with the cGAS-cGAMP cytosolic DNA surveillance pathway[^143-145]. Interestingly, bacteria seem to
have evolved strategies to dampen IFN production by avoiding STING activation. Group B Streptococcus was recently shown to express an ectonucleotidase, CdnP, which hydrolyzes extracellular bacterial c-di-AMP to attenuate the cGAS-STING axis.¹⁴⁶
**Figure legends**

**Figure 1: Components of the c-di-GMP signaling network.** (a) Principles of c-di-GMP signaling. Enzymatic reactions are depicted as grey arrows. GGDEF, EAL and HD-GYP represent conserved catalytic domains of diguanylate cyclases and phosphodiesterases, respectively. (b) Schematic of DGC activation. Upper panel: phosphorylation-dependent activation of PleD from *C. crescentus*. Receiver-domains (Rec) are shown in green and GGDEF-domains in orange. Phosphorylation-induced dimerization of Rec-domain stem leads to dimerization and activation of GGDEF-domains. Lower panel: metal-dependent activation of *E. coli* DgcZ. DgcZ is a constitutive dimer. Zinc-depletion from the CZB-domain leads to competent positioning of the GGDEF-domains. (c) Structure of the zinc-binding diguanylate cyclase DgcZ from *E. coli* (PDB: 4H54)\(^2\). GGDEF-domains (orange) and zinc-binding CZB-domain (grey) are highlighted. Zinc metal ions are depicted as red spheres. C-di-GMP (magenta) binding to antipodal inhibitory I-sites (IP & IP') and GTPαS (green) binding to active sites (A & A') are shown. (d) Overlay of the EAL domains of the phosphodiesterase PdeL in the tight, substrate-bound (blue; PDB: 4LJ3) and relaxed (apo) conformation (grey, PDB: 4LYK)\(^2\). Inlet: zoom-in of the active site and conserved loop 6 region. The loop 6 conformations in the relaxed, apo (yellow) and tight, c-di-GMP-bound (orange) dimer are indicated. Yellow and magenta spheres indicate the positions of Mg\(^{2+}\)-ions in the relaxed and tight protein conformations, respectively. The conserved double-aspartic acid motif (D262, D263) and anchoring glutamate (E235), which determine the structural arrangement of loop 6 are highlighted. (e) Structure of *Rhodobacter sphaeroides* cellulose synthase complex with the BcsA subunit (green), its C-terminal PilZ-domain (magenta) and the BscB subunit (grey) (PDB: 4P02)\(^46\). The cytoplasmic membrane is outlined in grey. A dimer of c-di-GMP bound to the PilZ domain is marked.

**Figure 2: Role of c-di-GMP in *C. crescentus* pole morphogenesis and cell cycle progression.** A schematic of the *Caulobacter* cell cycle is shown with flagellated swarmer cells (SW, G1-phase), stalked cells (ST, S-phase) and predivisional cells (division) indicated. Polar organelles (flagellum, pili, stalk and holdfast) of individual cell types are marked. The replication status of the circular chromosome is indicated schematically with SW cells being replication silent while chromosome replication initiates in ST cells. Cell type-specific levels of c-di-GMP are as indicated. The subcellular localization of the DGC PleD, the PDE PdeA, the flagellar placement protein TipF and the sensor histidine kinases PleC, DivJ and CckA are
Figure 3: Role of c-di-GMP in biofilm formation and dispersal. Bacterial surface attachment, biofilm formation and dispersal are indicated schematically in the central panel. (a) c-di-GMP-mediated control of flagellar motility in *E. coli*. DGCs (orange), PDE (blue) and the c-di-GMP effector YcgR (purple) are highlighted. YcgR interacts with and curbs the flagellar motor upon binding of c-di-GMP. PdeH adopts a key role to inactivate YcgR by keeping c-di-GMP levels low thereby enabling motor function. (b) c-di-GMP-dependent production of amyloid curli fibers and cellulose in *E. coli*. The global (DgcE/PdeH) and local (DgcM/PdeR) modules of DGCs and PDEs controlling csgD transcription are indicated. DgcM/PdeR sense the global concentration of c-di-GMP and, in response, activate the transcription factor MlrA. The global transcription factor CsgD then activates the expression of curli components and of DgcC, the main activator of the cellulose synthase complex. (c) c-di-GMP-mediated synthesis of poly-beta-1,6-N-acetyl-glucosamine (GlcNAc) in *E. coli*. The Csr global regulatory system co-regulates the *pga* genes encoding components of the GlcNAc synthesis machinery (PgaA-D) and *dgcT* and *dgcZ* encoding two DGCs (orange) responsible for the allosteric activation of PgaCD. The histidine kinase BarA is stimulated by short-chain fatty acids and through the phosphorylation of the response regulator UvrY, activates the expression of two small RNAs, 

marked at individual stages of the cell cycle. Individual panels highlight stage-specific processes at the stalked and flagellated poles. Autophosphorylation of histidine kinases (DivJ, CckA) and phosphotransfer to response regulators (PleD, CtrA) are indicated. a) Flagellar assembly. TipF binds to c-di-GMP to localize to the flagellated pole, where it recruits flagellar components PflI and FliG to initiate flagellar assembly. b) Low c-di-GMP levels at the flagellated pole of dividing cells and in SW cells promote TipF degradation by the ClpXP protease and CckA kinase activity. The CckA kinase activates the CtrA replication initiation inhibitor by phosphorylation via the P-transfer protein ChpT. PdeA and as yet unidentified PDE(s) contribute to the reduction of the c-di-GMP concentration at this cell cycle stage. c) Degradation of PdeA and CtrA by the ClpXP protease during the SW-to-ST transition and at the stalked pole of the dividing cell. PleD and as yet unidentified DGC(s) contribute to the upshift of c-di-GMP upon entry into S-phase and in the predivisional cell. Activation of the protease adaptor PopA by c-di-GMP leads to the degradation of CtrA. d) Inactivation of CtrA by the CckA phosphatase during the SW-to-ST transition and at the stalked pole of the dividing cell. PleD and as yet unidentified DGC(s) contribute to the upshift of c-di-GMP upon entry into S-phase and in the predivisional cell. Binding of c-di-GMP causes the switch of the CckA histidine kinase from its default kinase to the S-phase-specific phosphatase state.
CsrB and CsrC, which in turn antagonize the translation inhibitor CsrA. (d) Biofilm escape mechanism in *P. fluorescens* Pf0-1. The LapA surface protein mediates *P. fluorescens* surface adhesion and contributes to the stabilization of biofilms. Under phosphate starvation conditions LapA is degraded by the periplasmic protease LapG resulting in biofilm escape. If enough phosphate is available, LapG is sequestered by its partner LapD in its c-di-GMP bound conformation. When phosphate becomes limited the RapA PDE is expressed through the phosphate control system Pst/PhoRB, leading to a drop of c-di-GMP, a conformational change of apo-LapD and the release of the protease.

**Figure 4: Role of c-di-GMP in virulence of *Clostridium difficile*.** *C. difficile* virulence is regulated by c-di-GMP-specific riboswitches. Type-I riboswitches and type-II riboswitches control the expression of factors involved in motility, surface attachment and virulence. Type-I riboswitches (OFF-switches) inhibit translation upon c-di-GMP binding, while type-II-riboswitches (ON-switches) promote translation of target genes when bound to c-di-GMP. Increasing levels of c-di-GMP stimulate the expression of adhesion factors such as type-4-pili (T4P) and collagen-binding proteins (CBP) and inhibit the expression of flagellar genes and the CBP protease. The gene encoding the sigma factor SigD is co-regulated with flagellar genes. Thus, when the c-di-GMP concentration is low, cells not only express motility and anti-adhesion genes, but also express the SigD-dependent Cdiff toxins TcdA and TcdB.

**Figure 5: General concepts of c-di-GMP signaling modules.** Effectors (E) (c-di-GMP binding proteins), diguanylate cyclases (DGC) and phosphodiesterases (PDE) are labeled. c-di-GMP molecules are indicated as black circles or as spatial gradient in (c). (a) Evolutionary diagram of recruiting cellular processes into an existing CDN network. Minor modifications of the surface of a specific protein can mediate specific binding of c-di-GMP, which in turn can modulate the protein’s activity, stability or interaction with a partner. (b) and (c) Network architecture involved in pathway-specific signaling. C-di-GMP-dependent processes can be specifically regulated by temporal (b) or spatial (c) separation. Temporal regulation relies on effector proteins with different ligand affinities and on DGCs and/or PDEs with specific inhibition and activation constants, respectively. This allows establishing precise cellular thresholds of c-di-GMP thereby activating specific downstream effectors and pathways. Spatially separated signaling relies on some form of compartmentalization, for example with a specific
DGC/PDEs module interacting with its specific effector. To avoid unwanted crosstalk with other effectors and cellular pathways, spatially confined modules need to be effectively insulated. This can occur via the action of the module-specific PDE or by a general cellular PDE that restrains leakage of c-di-GMP. (d) C-di-GMP can interfere with the same biological process at different levels of control. E.g. c-di-GMP can control gene expression (transcription/translation) or control the activity of one of the resulting proteins as indicated. Expression and allosteric control can be mediated by the same module comprising a DGC and PDE (arrows) or can be modulate independently by different DGC/PDEs modules.

Acknowledgements:

This work was supported by grants of the Swiss National Science Foundation grant (310030B_147090) to U.J. and by an ERC Advanced Research Grant (322809) to U.J.

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motility, adherence, biofilm, virulence, development, cell-cycle progression

Figure 1

Rec1 Rec2
GGDEF

PleD

DgcZ
CZB
GGDEF

[c-di-GMP]_2

BcsA
PilZ
**Figure 2**

(a) Flagellar assembly

- TipF•cdG
- Pfl/FliG
- cdG
- chromosome
- new cell pole

(b) TipF degradation  CtrA activation

- PdeA
- CckA kinase
- PDE?
- ClpXP
- TipF
- ChpT
- pGpG
- Cori
- CckA phosphatase

(c) CtrA/PdeA degradation

- CtrA
- DGC?
- Cori
- PdeA
- ClpXP
- cdG
- DivJ
- PleD
- PloA•cdG

(d) CtrA inactivation

- CtrA
- DGC?
- Cori
- ChpT
- CckA phosphatase
- PdeA
- ClpXP
- DivJ
- PleD

[cdG] <50 nM
[cdG] 300-500 nM
type-II riboswitch
ON

type-I riboswitch
OFF

fla genes
pilA
tcd genes

sigD

cbp-protease

TcdA
TcdB
type-4 pili (T4P)

C. difficile

Host cell

CDP

collagen

Figure 4
Figure 5

[Diagram showing biochemical processes and interactions involving DGC and PDE modules, with equations and kinetic parameters.]