



Tracking the homeostasis of second messenger cyclic-di-GMP in bacteria

Anushya Petchiappan¹ · Sujay Y Naik¹ · Dipankar Chatterji¹

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Abstract

Cyclic-di-GMP (c-di-GMP) is an important second messenger in bacteria which regulates the bacterial transition from motile to sessile phase and also plays a major role in processes such as cell division, exopolysaccharide synthesis, and biofilm formation. Due to its crucial role in dictating the bacterial phenotype, the synthesis and hydrolysis of c-di-GMP is tightly regulated via multiple mechanisms. Perturbing the c-di-GMP homeostasis affects bacterial growth and survival, so it is necessary to understand the underlying mechanisms related to c-di-GMP metabolism. Most techniques used for estimating the c-di-GMP concentration lack single-cell resolution and do not provide information about any heterogeneous distribution of c-di-GMP inside cells. In this review, we briefly discuss how the activity of c-di-GMP metabolising enzymes, particularly bifunctional proteins, is modulated to maintain c-di-GMP homeostasis. We further highlight how fluorescence-based methods aid in understanding the spatiotemporal regulation of c-di-GMP signalling. Finally, we discuss the blind spots in our understanding of second messenger signalling and outline how they can be addressed in the future.

Keywords C-di-GMP · Second messenger · Biofilm · Biosensor · FRET · Riboswitch

Introduction

Cyclic-di-GMP (c-di-GMP) is the first discovered cyclic dinucleotide second messenger in bacteria (Ross et al. 1987). It was identified more than 30 years ago by Benzimann and colleagues as an activator of cellulose synthesis in *Gluconacetobacter xylinum* and it has been found to be nearly ubiquitous across the bacterial kingdom (Jenal et al. 2017; Ross et al. 1987). Amongst other phenotypes, c-di-GMP regulates cell cycle control, cell morphology, exopolysaccharide production, antimicrobial tolerance, quorum sensing, chemotaxis, and virulence (Hecht and Newton 1995; Hengge 2016; Jenal et al. 2017; Lori et al. 2015; Matsuyama et al. 2016; Paul et al. 2010; Romling et al. 2013; Valentini and Filloux 2016). It also dictates the bacterial lifestyle by playing a key role in the transition from a planktonic form to a surface-attached biofilm. In this sense, c-di-GMP can be considered to be a ‘lifestyle messenger’. This versatile second messenger performs its role by binding to a diverse array of protein or RNA effectors which

includes PilZ domain-containing proteins, transcription factors, proteases, kinases, and riboswitches (Christen et al. 2007; Hengge 2009; Li and He 2012; Lori et al. 2015; Sudarsan et al. 2008). C-di-GMP assists the pathogens like *Mycobacterium tuberculosis*, *Vibrio cholerae*, *Salmonella Typhimurium*, and *Pseudomonas aeruginosa* withstand the challenging environmental conditions (Conner et al. 2017; Hong et al. 2013; Petersen et al. 2019; Valentini and Filloux 2016). Furthermore, c-di-GMP is identified by the human receptor STING leading to a host immune response (Burdette et al. 2011; Yin et al. 2012). Therefore, it is imperative to understand c-di-GMP signalling for developing strategies for combating drug-resistant bacterial infections.

C-di-GMP influences the decision-making in bacteria in response to the environmental signals they perceive. As its concentration determines the cellular fate of bacteria, it is necessary to understand how the c-di-GMP levels are fine-tuned under different conditions. Despite the substantial strides in understanding the multitude of functions regulated by c-di-GMP in the cellular context, many unresolved questions still remain to be addressed in this area. In this review, we briefly define how the bacterial proteins are regulated for balanced c-di-GMP turnover and discuss their spatiotemporal regulation. We further illustrate how fluorescence-based techniques can provide fruitful insights into this important field.

✉ Anushya Petchiappan
anushya@iisc.ac.in

¹ Molecular Biophysics Unit, Indian Institute of Science, Bangalore, India

The physical landscape of c-di-GMP metabolism

The intracellular concentration of c-di-GMP is maintained by balancing its synthesis by enzymes called diguanylate cyclases (DGCs) and its hydrolysis by phosphodiesterases (PDEs) (Hengge 2009; Jenal et al. 2017). DGCs contain a GGDEF amino acid motif and generate c-di-GMP from two molecules of GTP (Fig. 1a). PDEs are of two types- EAL-domain-containing PDEs and HD-GYP domain-containing PDEs. The former contain an EAL amino acid motif and degrade c-di-GMP into linear di-GMP (5'-pGpG) and the latter hydrolyzes c-di-GMP into two molecules of GMP.

Interestingly, GGDEF and EAL domains often reside in the same multi-domain protein (Fig. 1b) (Romling et al. 2013). Furthermore, the number of such proteins is in fact quite large

and a significant proportions of GGDEF and EAL proteins are actually examples of such 'hybrid' or 'fusion' proteins. Some of the organisms encoding such hybrid proteins include *Vibrio parahaemolyticus*, *Rhodobacter sphaeroides*, and *Mycobacterium smegmatis* (Bharati et al. 2012; Tarutina et al. 2006; Trimble and McCarter 2011).

Regulation of c-di-GMP homeostasis

As c-di-GMP plays such a prominent role in governing bacterial behaviour, even minor fluctuations in its concentration can have drastic consequences. Therefore, the cells must tightly regulate the rate of its synthesis and hydrolysis to maintain homeostasis. In addition to their expression being tightly regulated, the activity of c-di-GMP-metabolizing enzymes is

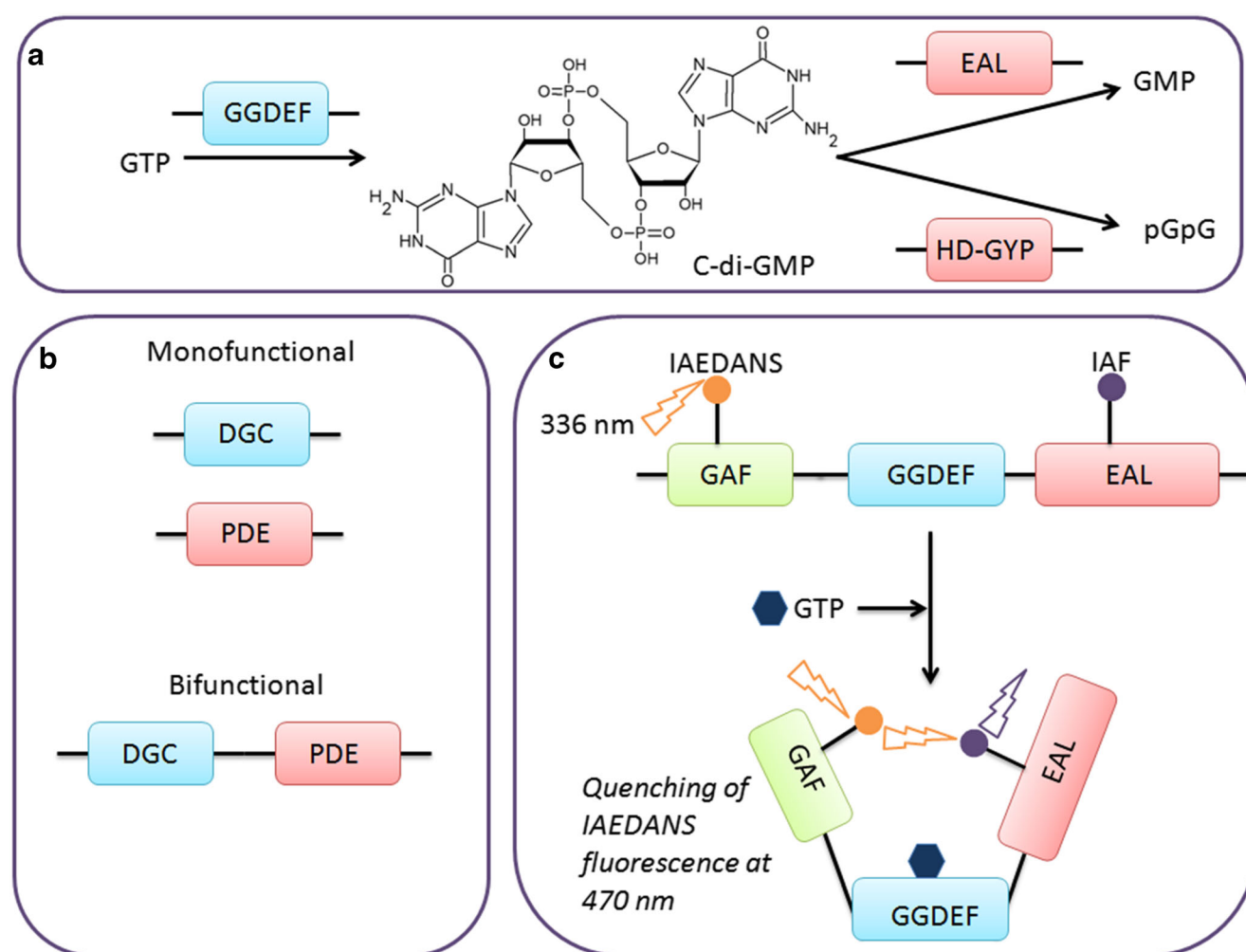


Fig. 1 C-di-GMP metabolism in bacteria. **a** Synthesis and hydrolysis of c-di-GMP. GGDEF-domain-containing enzymes synthesize c-di-GMP from two molecules of GTP. It is hydrolysed by EAL-domain proteins and HD-GYP domain proteins to GMP and pGpG respectively. **b** Domain architecture of DGCs and PDEs. Monofunctional enzymes have either synthesis (DGC) or hydrolysis (PDE) activity whereas bifunctional enzymes possess both activities. **c** Analysis of domain dependence in bifunctional proteins by FRET. *M. smegmatis* DcpA contains GGDEF

and EAL domains along with a regulatory EAL domain. The Cys124 in GAF domain and Cys579 in EAL domain are labelled with fluorophore IAEDANS (donor) and IAF (acceptor). Upon binding of its substrate GTP, there is a compaction of protein structure leading to the fluorophores coming proximal to one another. This leads to quenching of the IAEDANS fluorophore which can be observed as reduction in fluorophore intensity. The excitation and emission wavelength used are 336 nm and 470 nm

frequently modulated by various mechanisms (Jenal et al. 2017). Several DGC and PDE enzymes have a regulatory domain (like GAF, PAS) associated with them which modulate their activity and oligomerisation depending on the environmental stimuli (Chen et al. 2018). Allosteric regulation, feedback inhibition, protein-protein interactions, and post-translational modification are important regulatory mechanisms for these proteins (Chen et al. 2018; Jenal et al. 2017; Trimble and McCarter 2011). This is exemplified by the enzyme PleD from *Caulobacter crescentus* which needs to be phosphorylated to form its active dimeric form for c-di-GMP synthesis (Paul et al. 2007). The binding of the product c-di-GMP to PleD leads to an inactive conformation. Another prime example is the DgcZ protein from *E. coli* whose activity is regulated by zinc (Zähringer et al. 2013).

The curious case of the bifunctional protein

The presence of both DGC and PDE domains in the same protein presents an interesting scenario for studying c-di-GMP homeostasis. Several such hybrid proteins in fact have only one activity as one of the domains is degenerate and catalytically inactive (Romling et al. 2013). However, many composite proteins have been shown to have both DGC and PDE activity (Bharati et al. 2012). How do the cells solve the enzymatic conundrum presented by the presence of opposing activities in protein and avoid any futile cycles of c-di-GMP synthesis/hydrolysis?

Förster Resonance Energy Transfer (FRET)-based techniques can detect conformational changes in c-di-GMP metabolising enzymes upon ligand or protein binding (Christen et al. 2010; Greenwald et al. 2018). They can, therefore, provide critical insights into how the c-di-GMP synthesis and degradation by an enzyme is regulated in response to environmental signals and in the presence of inhibitors. This reveals clues as to how the intracellular c-di-GMP concentration is affected by that enzyme.

M. smegmatis contains a single bifunctional protein named DcpA for c-di-GMP turnover along with another inactive GGDEF-domain-containing protein (Bharati et al. 2012). DcpA has a GAF-GGDEF-EAL domain architecture and is transcribed more during starvation. It dynamically interconverts between monomeric and dimeric forms, with the dimer being preferred for DGC activity (Bharati et al. 2013; Sharma et al. 2014b). Binding of GDP to the GAF domain of DcpA enhances c-di-GMP hydrolysis and decreases synthesis (Chen et al. 2018). An important aspect of its activity regulation comes from the substrate-induced domain movement (Bharati et al. 2018). This was elegantly demonstrated by a Förster Resonance Energy Transfer (FRET)-based technique for studying relative domain movement (Fig. 1c). The protein was labelled at a Cys residue in its N-terminal GAF domain by the fluorophore IAEDANS (donor) and at a Cys residue in

its C-terminal EAL domain by the fluorophore IAF (acceptor). The change in Förster distance between the two fluorophores was estimated as a function of GTP binding. The reduction in the Förster distance showed that there is domain movement upon GTP binding which is important for its activity. A point mutation in the EAL domain not only abrogates the PDE activity but also reduces the DGC activity, further emphasizing the interdomain dependence. A similar regulatory mechanism may be present in other yet uncharacterised hybrid proteins. Additionally, these bifunctional proteins can also be regulated by the same regulatory mechanisms such as the monofunctional DGCs and PDEs. For example, the binding of a ligand might induce one activity over the other or the binding a protein might lead to a switch in activity.

Multiplicity of DGCs and PDEs

Monitoring c-di-GMP homeostasis is made complicated by the fact that many bacterial genomes encode multiple DGCs and PDEs. In some of the organisms, the number of DGCs and PDEs rises to 60 (Dahlstrom and O'Toole 2017). The *P. aeruginosa* genome encodes 18 GGDEF, 5 EAL, 16 GGDEF/EAL, and 3 HD-GYP-predicted proteins (Valentini and Filloux 2016). Similarly, *E. coli* possesses 29 proteins linked with c-di-GMP metabolism (Sarenko et al. 2017). This raises the following intriguing questions.

- Do all the DGCs and PDEs expressed within a cell play a role in c-di-GMP homeostasis?
- How do the bacteria tune their activities so as to maintain the necessary cellular c-di-GMP levels during balanced growth and during stress?
- How do bacteria modulate their c-di-GMP pools to ensure high-fidelity signalling? In other words, how do the bacteria integrate the various signals to give a common output? As c-di-GMP is a small molecule which can diffuse easily inside the cell, there must be a mechanism for activating only specific c-di-GMP-associated pathways at a given point of time while avoiding any undesirable crosstalk between the numerous c-di-GMP-binding partners.

In bacteria containing a multitude of DGCs and PDEs, many of them may be active in vivo and have an associated phenotype. However, not all of them will have a substantial impact on the global c-di-GMP concentration. The specificity in function of each enzyme indicates that each might be differentially regulated at the transcription or the protein level by one of the multiple mechanisms described earlier. One plausible explanation for the specificity in c-di-GMP signalling could be the differential affinity of the effectors to c-di-GMP (Dahlstrom and O'Toole 2017). Therefore, an effector protein with higher binding affinity to c-di-GMP will require lower c-

di-GMP for its function and a protein with lower binding affinity will need a higher threshold cellular concentration of this molecule. An alternate hypothesis for the high specificity signalling is the spatial and temporal sequestration of the c-di-GMP modules (Hengge 2009).

Spatial and temporal sequestration of c-di-GMP

Temporal sequestration means that not all DGC/PDE would be functionally active at the same time (Hengge 2009). This implies that their transcription and proteolysis would depend on the growth and environment. Consistent with this, it has been shown that many of the c-di-GMP-metabolising proteins are transcriptionally regulated according to the nutrients available in their environment (Dahlstrom et al. 2018). It has also been suggested that c-di-GMP acts as a toggle switch in which a spike in its concentration is enough to flip from one state to another and cells need not continuously synthesise the molecule throughout its life cycle (Rodesney et al. 2017).

The DGCs and PDEs could also be spatially or functionally sequestered by means of being localised at particular sites within the cell or be present in multi-protein complexes (Dahlstrom et al. 2015; Hengge 2009). Many DGCs and PDEs have transmembrane domains or are anchored to membrane proteins (Jenal et al. 2017; Valentini and Filloux 2016). In *V. parahaemolyticus*, the bifunctional ScrC is cytoplasmic membrane bound and acts as a DGC until the protein ScrB binds to it to induce its PDE activity (Trimble and McCarter 2011). In *B. subtilis* containing 3 DGCs, it has been shown that the cytoplasmic DGCs cannot complement the function of the membrane-bound DgcK unless overexpressed (Bedrunka and Graumann 2017). Spatial sequestration allows the operation of parallel c-di-GMP pathways with high specificity and less noise. This would also allow for the presence of 'local pools' of c-di-GMP associated with local signalling (Hengge 2009; Sarenko et al. 2017) (Fig. 2a). Also, different threshold concentrations of c-di-GMP are required for different bacterial behaviours which confirm the presence of local c-di-GMP pools.

Heterogeneity in c-di-GMP levels in single cells and in biofilms

Recent experiments in *C. crescentus* and *P. aeruginosa* have revealed an asymmetric distribution of c-di-GMP between the daughter cells after cell division (Fig. 2b) (Abel et al. 2013; Christen et al. 2010). This is achieved with the localisation of a specific PDE at the flagellated cell poles in *P. aeruginosa*.

During biofilm formation, c-di-GMP level increases, and it binds to the flagellar motility-associated proteins resulting in reduced motility and biofilm initiation (Nair et al. 2017). During the process of biofilm maturation, c-di-GMP positively regulates the polysaccharide synthesis. It has been observed that biofilm has physiological heterogeneity and c-di-GMP

level differs in distinct regions of the biofilm (Fig. 2c). This could be attributed mainly to the differential exposure of the cells at each biofilm layer to the nutrients (Klauck et al. 2018). The bacteria at the lower level of biofilm have access to a higher concentration of the nutrients but lower oxygen level than the bacteria in the upper layer of the biofilm. When propitious conditions are restored, it is reported that PDEs are activated and result in reduced c-di-GMP concentration in the dispersed cells (Fig. 2d) (Chua et al. 2014; Klauck et al. 2018).

A heterogeneous distribution of c-di-GMP has been observed in bacterial cultures (Fig. 2e). Such phenotypic heterogeneity in an isogenic population is considered as a bacterial bet-hedging strategy, and heterogeneity in (p) ppGpp levels in such cultures has been linked with increased persistence and antibiotic resistance (Valentini and Filloux 2016).

From test tube to living cell: estimating the concentration of c-di-GMP

In order to gain deeper insights into how c-di-GMP homeostasis is maintained and affects the bacterial phenotype, it is important to accurately monitor and estimate c-di-GMP levels. The c-di-GMP levels are usually in the nanomolar to micromolar range within the cell and require sensitive detection techniques (Hengge 2009; Kellenberger et al. 2013). In addition, the techniques need to be specific to c-di-GMP so that other second messengers do not interfere with the detection.

The in vitro activity of DGC and PDE can be estimated using radiolabeling, chromatographic, photometric, and fluorescence-based methods (Sharma et al. 2014a). However, this does not provide accurate information about their role in c-di-GMP homeostasis within the cell as multiple factors regulate its activity inside the cell which are not present in vitro. Therefore, to evaluate the role of any enzyme in modulating the c-di-GMP level, it is more useful to measure its impact on c-di-GMP level in vivo.

The initial method to detect and quantify the c-di-GMP relied on cellulose synthesis as an indirect measure of the c-di-GMP level inside the cell (Amikam et al. 2010). Other bacterial phenotypes like biofilm formation and swarming motility are correlated with c-di-GMP levels and are considered as proxies for c-di-GMP concentration within the cell (Jones and Wozniak 2017; O'Toole et al. 1999; Wolfe and Berg 1989). These methods are high-throughput but have low sensitivity. The 2D-TLC-based technique using incorporation of radioactive ³²P in media followed by extraction of radiolabeled c-di-GMP has also been previously adopted (Ross et al. 1986). Other techniques include circular dichroism (Stelitano et al. 2013). One of the most commonly used methods for c-di-GMP estimation is the HPLC-MS method (Bähre and Kaever 2017; Burhenne and Kaever 2013). The cellular quantification of c-di-GMP can be done by comparing with the known concentrations of c-di-GMP standard. C-di-

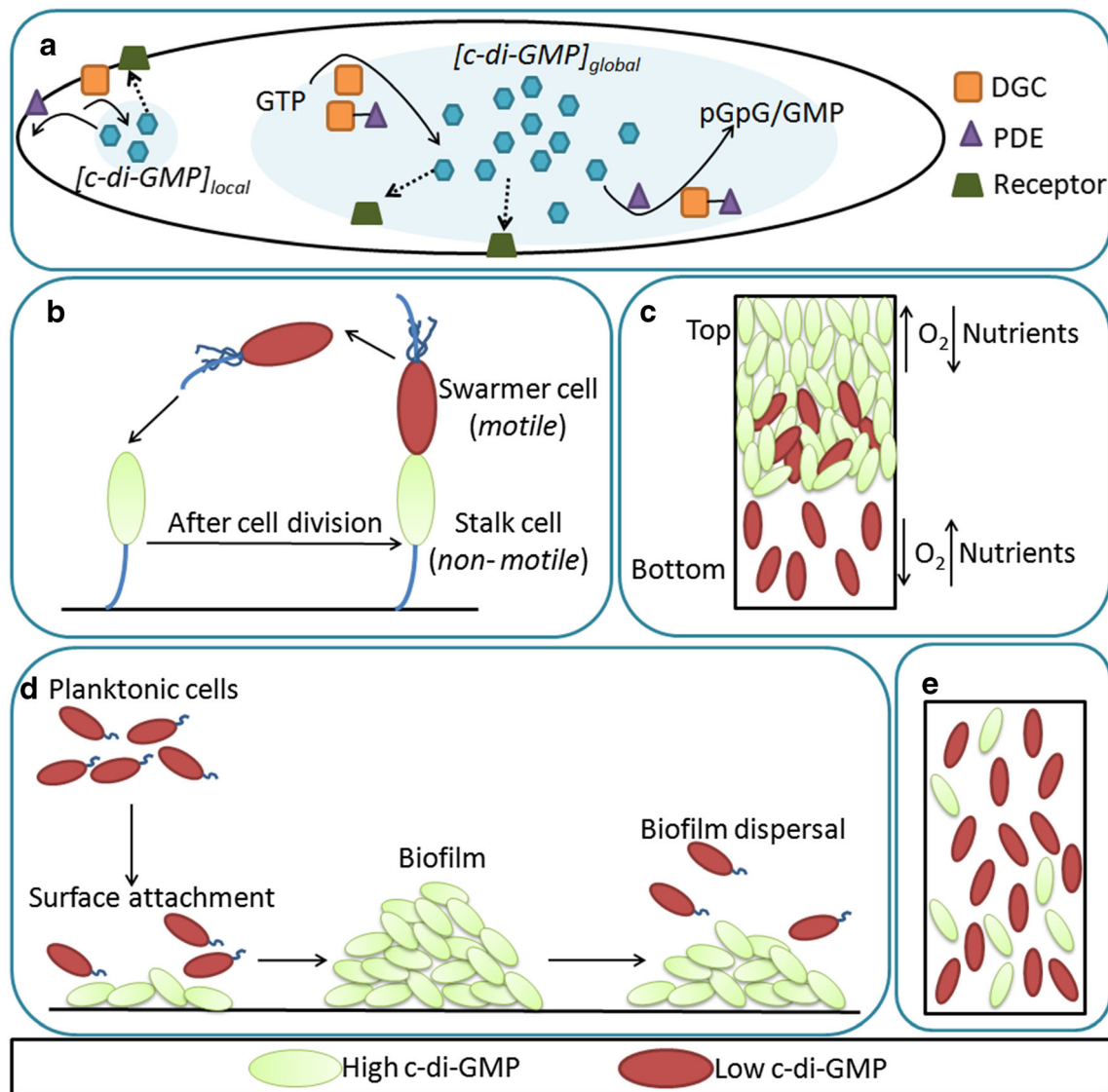


Fig. 2 Heterogeneity in c-di-GMP distribution in bacteria. **a** Spatial sequestration of c-di-GMP modules allows for global and local pools of c-di-GMP which affect bacterial phenotype by binding their receptors. **b** During the cell division in *C. crescentus*, the mother cell gives rise to daughter cells with an asymmetric distribution of c-di-GMP. The swarmer cell and the stalk cell contain low and high level of c-di-GMP immediately after division and this dictates their motility. **c** A cross-section of a biofilm is shown with a spatial distribution of c-di-GMP. Due to the

inherent differences in availability of oxygen and nutrients, the expression of PDE varies leading to differences in c-di-GMP concentration. **d** There is a temporal regulation of c-di-GMP during different stages of biofilm formation. The planktonic cells have low c-di-GMP concentration in contrast to biofilm cells. The c-di-GMP levels increase during surface adhesion and decrease during dispersal. **e** Isogenic cells in a culture display a bimodal distribution of c-di-GMP

GMP can be directly measured using LC-MS analysis by spiking a known concentration of c-di-GMP in mass spectrophotometer. The limit of detection could be as low as 1 fmol, making this technique a very sensitive approach for c-di-GMP quantification (De et al. 2008).

LC-MS and the other techniques can only reveal the cellular concentration of c-di-GMP from samples isolated at a given time from a bulk population. They also require tedious sample preparation and are not amenable to high-throughput screening (Yeo et al. 2017). The estimated concentration is an average of the total concentration of the cell and it will not take

into account the spatial variation in c-di-GMP pools. Moreover, it does not capture the dynamic changes in c-di-GMP turnover within the cells. These techniques do not provide for monitoring the heterogeneity of c-di-GMP at a single cell level or across the global architecture of a biofilm.

Fluorescence-based techniques to capture the c-di-GMP signalling

Fluorescence-based methods are being increasingly used for real-time mapping of c-di-GMP levels within cells. These

provide insights into how the c-di-GMP pools are modulated by bacteria and if there is heterogeneity in the c-di-GMP distribution. They have an added advantage that there is no requirement for sample homogenisation. Some of the fluorescence-based methods are listed below and have provided critical insights into how the c-di-GMP levels are modulated for highly specific and coordinated signalling.

- Reporter-based constructs

A c-di-GMP responsive promoter element fused to a fluorescent protein or luciferase can be used as a reporter for c-di-GMP levels in vivo. For a reporter cloned downstream of a positively regulated promoter, there is an enhanced expression of the reporter in the presence of c-di-GMP. This leads to an increase in fluorescence as a function of intracellular c-di-GMP concentration (Fig. 3a). The c-di-GMP concentration can be calculated by calibrating the fluorescence intensity with cellular c-di-GMP concentration estimated by LC-MS (Nair et al. 2017). The upstream promoter region of *cdrA* fused to the gene for GFP is a prime example of such a c-di-GMP reporter (Rybtke et al. 2012). A *P. aeruginosa* strain containing the pCdrA::gfp reporter has been previously used to analyse the c-di-GMP dynamics during surface attachment by time-lapse fluorescence confocal microscopy (Rodesney et al. 2017). This revealed that a high concentration of c-di-GMP is required for transition from planktonic to biofilm phase. A similar construct was used to visualise the spatial and temporal distribution of c-di-GMP in real time in a biofilm (Nair et al. 2017). Using CFP as a biomass indicator and GFP as c-di-GMP reporter, it was elucidated that there is a non-uniform distribution of c-di-GMP in the biofilm and that its concentration varies at different stages of biofilm development. Usage of a c-di-GMP pCsgD::gfp reporter in *E. coli* further emphasized the role of c-di-GMP and its heterogeneity in the three-dimensional landscape of a biofilm (Serra and Hengge 2019).

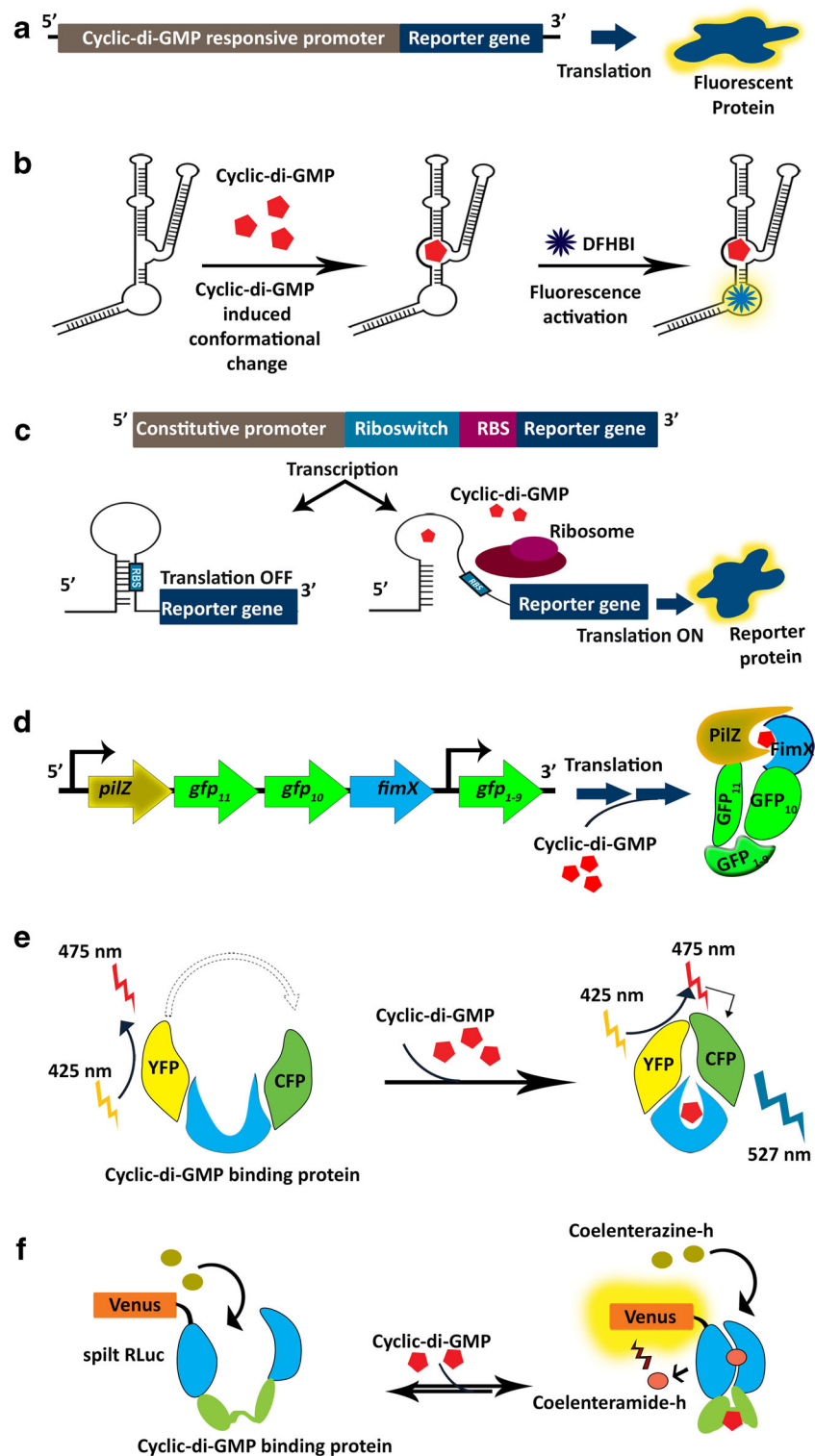
- Riboswitch-based biosensors

Riboswitches are regulatory elements present in the mRNA (often at the 5' untranslated region) which bind metabolites or signalling molecules to regulate their own expression (Villa et al. 2018). Multiple riboswitches have been identified in bacteria which specifically bind c-di-GMP at their aptamer domain leading to alteration in their secondary structure (Sudarsan et al. 2008). The aptamer domains of riboswitches can be easily synthesized and can be engineered to bind to their cognate ligand with high specificity (Gu et al. 2012). The identification of RNA aptamer constructs, which bind small weak fluorophores like 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI) to emit intense fluorescence, led to the development of riboswitch-based biosensors for c-di-

Fig. 3 Fluorescence-based methods for monitoring c-di-GMP homeostasis. **a** Reporter-based construct. The expression of a fluorescent reporter protein is dependent on the c-di-GMP-responsive promoter and hence the fluorescence intensity can be monitored to estimate the c-di-GMP concentration. **b** Riboswitch-based reporter. The expression of a fluorescent reporter protein is dependent on the c-di-GMP-binding riboswitch. The fluorescence is proportional to the c-di-GMP level. **c** A c-di-GMP-binding riboswitch is fused to a DFHBI-binding aptamer sequence. The binding of c-di-GMP leads to an altered conformation of the riboswitch which can subsequently bind DFHBI leading to fluorescence. **d**. Split-GFP reporter. FimX and PilZ are expressed as translational fusions with one of three non-functional portions of GFP, GFP₁₁, and GFP₁₀ respectively. GFP₁₋₉ is expressed separately. C-di-GMP increases the interaction between FimX and PilZ. GFP₁₁ and GFP₁₀ can subsequently reconstitute with GFP₁₋₉ to form a fluorescent GFP. The GFP fluorescence is an indicator of c-di-GMP concentration. **e** FRET-based biosensor. A c-di-GMP-binding protein is sandwiched between YFP and CFP. The binding of c-di-GMP causes structural alteration bringing YFP and CFP in close proximity. Excitation of YFP leads to FRET and the FRET signal can be measured to estimate c-di-GMP concentration. **f** CSL-BRET-based biosensor. A fluorescent protein (Venus) is expressed fused to a c-di-GMP-binding protein, which is fused between two non-functional parts of luciferase. In the presence of c-di-GMP, there is a structural alteration in the c-di-GMP-binding protein which causes reconstitution of the split luciferase. The luciferase converts its substrate coelenterazine-h to coelenteramide-h with concomitant light emission which is captured by Venus for fluorescence

GMP (Fig. 3b) (Nakayama et al. 2012). The binding of c-di-GMP to its aptamer leads to remodelling of the other aptamer domain fused to it. Consequently, DFHBI can bind to the fused aptamer domain leading to fluorescence which can be monitored to estimate c-di-GMP level. The initial biosensors consisted of a naturally occurring c-di-GMP-responsive riboswitch Vc2 aptamer domain linked in tandem with a DFHBI-binding Spinach aptamer (Nakayama et al. 2012). The next-generation biosensors have been engineered using variants of Vc2-Spinach constructs and are useful for live cell measurements (Kellenberger et al. 2013). The specificity of these biosensors depends on the c-di-GMP-responsive aptamer and can go to subnanomolar level. Furthermore, they have been shown to be useful in broader dynamic ranges as compared to fluorescent protein-based biosensors, especially under anaerobic conditions as they do not require much oxygen for fluorescence (Wang et al. 2016). These biosensors have been used for screening proteins for DGC activity in vivo and for live cell imaging. When used in conjunction with flow cytometry, they are capable of visualising temporal changes in c-di-GMP in single cells in any media with minimal perturbations to the cells (Yeo et al. 2017).

Another modification of a riboswitch-based reporter is the leader sequence of the lichenysin *lch* gene fused between a constitutively active promoter and the *yfp* gene (Fig. 3c) (Weiss et al. 2019). A *B. subtilis* culture transformed with this reporter was analysed by fluorescence microscopy and this revealed that there exists a bimodal distribution of c-di-GMP in the bulk population. A naturally occurring triple-



tandem riboswitch fused to a dual fluorescence reporter has been utilised for screening the activity of DGCs in vivo and provides a stringent and digital control as compared to a single riboswitch (Zhou et al. 2016). Allosteric ribozymes have been engineered as c-di-GMP reporters but so far have been successful only in cellular lysates (Gu et al. 2012).

• Split-protein reporters

A fluorescent split-protein reporter for c-di-GMP was designed using a tri-partite split GFP system (Fig. 3d). This reporter consists of the *X. campestris* proteins FimX and PilZ whose interaction is stimulated by the presence of c-di-

GMP (Chin et al. 2012). FimX and PilZ are expressed as translational fusions with one of the three non-functional portions of GFP, GFP₁₁, and GFP₁₀ respectively. The remaining non-functional portion of GFP (GFP₁₋₉) is expressed from a separate plasmid. In the presence of c-di-GMP, FimX and PilZ interact leading to a close proximity between GFP₁₁ and GFP₁₀ which reconstitute with GFP₁₋₉ to form a fluorescent GFP. The fluorescence intensity reflects the c-di-GMP concentration and heterogeneity in c-di-GMP levels in a population can be analysed by microscopy or flow cytometry (Mushnikov et al. 2019).

- FRET-based biosensors

This protein-based biosensor utilises the fact that many c-di-GMP effectors undergo a conformational change upon binding the nucleotide (Christen et al. 2010). Such c-di-GMP receptor proteins can be used as a sensor domain sandwiched between two fluorescent proteins to develop a FRET-based biosensor (Fig. 3e). The fluorescent proteins are selected such that when they are in proximity to one another, there is transfer of energy from one to the other leading to a FRET signal. Upon binding of c-di-GMP to the sensor domain, there is a conformational change which changes the distance between the two fluorescent proteins leading to a FRET signal. The change in intensity of FRET signal is an indicator of the c-di-GMP level. The FRET-based biosensors can be coupled to high-resolution microscopy to study the c-di-GMP concentration even at single-cell resolution (Christen et al. 2010).

A genetically encoded FRET-based sensor was used in a landmark study to demonstrate the cell-cycle dependent spatiotemporal fluctuations in c-di-GMP level in individual bacterial cells (Christen et al. 2010). The *S. Typhimurium* PilZ-domain-containing protein YcgR was used as the c-di-GMP receptor protein as it is known to have a significant conformational change upon binding c-di-GMP. YcgR was sandwiched between the cyan fluorescent protein CFP and the yellow fluorescent protein YFP. CFP and YFP have the ideal excitation and the emission wavelength such that excitation of CFP leads to a transfer of energy to YFP if they are in proximity. The emission wavelength of YFP is monitored for the FRET signal. In this case, the binding of c-di-GMP to the YcgR biosensor results in a low FRET signal due to the decreased proximity between the CFP and YFP subunits. A high FRET signal is an indicator of low c-di-GMP concentration. The FRET/CFP emission ratio was used as an indicator of the cellular c-di-GMP level and is correlated with standard binding curves to provide nanomolar sensitivity. This revealed that c-di-GMP is unevenly distributed between the daughter cells after cell division in *C. crescentus*, with the non-motile stalk cell having up to five times higher c-di-GMP concentration as compared to the swarmer cell. Additionally, time-lapse

microscopy using the same biosensor defined the dynamic changes in c-di-GMP levels in the cells during different stages of the cell cycle. The oscillation in the c-di-GMP concentration is important for regulating bacterial motility and can be attributed to the differential spatial and temporal distribution of the DGCs and PDEs in these bacteria. For example, the PDE PCH localises to the pole with the help of the chemotaxis machinery in *P. aeruginosa* during cell division thereby playing a role in generating the bimodal distribution of c-di-GMP in the daughter cells (Kulasekara et al. 2013).

The same biosensor was utilized in conjunction with flow cytometry in *S. Typhimurium* to decipher the changes in c-di-GMP concentration in response to different environmental nutrients in real-time high-throughput manner (Mills et al. 2015). FRET-based biosensors have also been engineered with other c-di-GMP effectors and with more stable fluorescent proteins. EAL-domain protein FimX and PilZ-domain proteins MrkH and VCA0042 have been used for developing biosensors with different ranges (Ho et al. 2013). A biosensor containing MrkH fused between mCerulean and mVenus was successfully used to understand the dynamics of c-di-GMP variations in *E. coli* upon treatment with biofilm-dispersal chemicals and within macrophages. The YcgR-based biosensor was recently optimised for c-di-GMP measurement in bacteria inside their eukaryotic host cells. CFP was replaced with teal fluorescent protein TFP which is brighter and more suitable in the eukaryotic cells having high blue-range background fluorescence. YFP was replaced by the kusabira orange variant KO2 as it is more pH stable. This TFP-KO2 biosensor demonstrated dynamic changes in c-di-GMP levels during the different stages of *S. Typhimurium* infection in macrophages and that there is a significant heterogeneity in the c-di-GMP level within the population (Petersen et al. 2019). C-di-GMP is important for the virulence of *S. Typhimurium* and its survival inside the host, and three of its PDEs are crucial for regulating the c-di-GMP homeostasis.

FRET-based biosensors have been extensively used to estimate the intracellular concentration of c-di-GMP with high specificity and sensitivity at a single cell as well as population level (Paul et al. 2010). Its advantages are manifold as demonstrated by its successful application in real-time measurement of c-di-GMP dynamics. These provide valuable information about the spatiotemporal changes in c-di-GMP distribution and can be performed in a high-throughput manner with no need for cell lysis and sample preparation.

- Bioluminescence Resonance Energy Transfer (BRET)-based biosensors

One of the drawbacks of the FRET-based biosensors is that they require external illumination due to which they cannot be used for long-term studies nor used within animal tissues (Dippel et al. 2018). To overcome this problem, BRET-

based sensors are being developed for c-di-GMP as they do not require any light excitation. Hammond and colleagues developed the first set of c-di-GMP chemiluminescent biosensors which combine BRET with the complementation of split luciferase (CLS) approach (Fig. 3f) (Dippel et al. 2018). This CLS-BRET-based biosensor consists of the c-di-GMP receptor YcgR fused between non-functional domains of the Renilla luciferase (RLuc). This in turn is fused to the fluorescent variant of the Venus protein such that RLuc sensor domain is sandwiched between the fluorescent protein (acceptor) and the luciferase (donor). Upon binding of c-di-GMP, there is an alteration in conformation of YcgR which leads to the reconstitution of the functional RLuc. RLuc oxidises its substrate coelenterazine with concomitant light emission which is captured by Venus when in close proximity leading to fluorescence. The presence of c-di-GMP, therefore, increases the proximity between reconstituted RLuc and Venus leading to a high BRET signal.

The CLS-BRET biosensor provides a reliable and high-throughput approach for monitoring c-di-GMP levels with greater sensitivity than FRET-based biosensors (Dippel et al. 2018). Additionally, they provide better signal to background ratio, fast activation in response to c-di-GMP and can be potentially used to study the cells in their natural environment. However, this technique was more suitable for measuring c-di-GMP concentration in cell lysates than live bacteria possibly due to limited diffusion of the substrate into the cells.

Hammond and colleagues have recently developed ratiometric, luminescent biosensors for c-di-GMP with possible application in live cell imaging (Dippel et al. 2019). This sensor consists of an engineered marine luciferase Nanoluc (NLuc) with increased signal intensity and stability as compared to RLuc. A variant of YcgR is sandwiched between a truncated Venus and NLuc variants. Binding of c-di-GMP leads to a large structural alteration in YcgR, which brings NLuc and Venus in close proximity resulting in a BRET signal. This sensor is amenable to high-throughput screening and detected even femtomolar concentration of c-di-GMP in *V. cholerae* cellular lysates which is comparable to LC-MS.

Lessons for tracking the homeostasis of other second messengers

In addition to c-di-GMP, bacteria contain a repertoire of nucleotide second messengers which assist in their growth and survival (Kalia et al. 2013). Some of them, like cAMP and (p) ppGpp, have been extensively studied by researchers across the world. Not much is known about the recently discovered second messengers like cGAMP, pGpp, and c-di-AMP (Corrigan and Gründling 2013; Li et al. 2019; Petchiappan et al. 2020). The regulatory mechanisms for many of the enzymes associated with second messenger turnover remain to be

elucidated. Though the mechanisms might vary, the general principles are very similar for all the signalling molecules. For example, many bacteria possess multiple synthetases and hydrolases for (p) ppGpp and c-di-AMP which raises a significant question about their function and regulation (Corrigan and Gründling 2013; Hauryliuk et al. 2015). Similarly, though bifunctional proteins present an enzymatic conundrum, bacteria employ many such proteins in signalling. Rel-SpoT family of proteins, which are responsible for (p) ppGpp turnover, are examples of such bifunctional proteins capable of making and breaking (p)ppGpp. Their activity is regulated such that they are either synthesis active or hydrolysis active. One form of regulation is through an interdomain communication between their N-terminal and their regulatory C-terminal domain (Jain et al. 2007).

The levels of second messengers in response to the environmental cues and many of the proteins involved have specific localisation patterns indicating that there could be spatial and/or temporal regulation of their activity. Additionally, it has been shown that there is uneven distribution of (p) ppGpp in isogenic bacterial cultures which plays an important role in persistence. The abovementioned fluorescence-based techniques used for monitoring c-di-GMP levels can be employed for answering the unresolved questions related to the new signalling molecules.

Conclusions and future perspectives

Bacteria utilise a complex network of DGCs and PDEs to fine tune their c-di-GMP level and this is achieved using a multi-modal approach. The activity of the c-di-GMP is tightly regulated, and there is compelling evidence for the presence of spatiotemporal regulation of c-di-GMP signalling modules. Any change in a c-di-GMP-associated phenotype could be due to variations in its global pool or an insulated signalling module. Therefore, it is of considerable importance that any method used to determine this variation in the c-di-GMP level should also account for the local c-di-GMP concentration.

Fluorescence-based techniques are ideal for mapping the spatial and temporal distribution of c-di-GMP with single-cell resolution and are useful for monitoring the real-time dynamics of c-di-GMP. Fluorescence-based reporters have helped unveil the heterogeneity in the c-di-GMP distribution in cultures and successfully captured the temporal oscillations of c-di-GMP levels during cell cycle and biofilm formation. However, many such sensors reflect only the free c-di-GMP concentration inside the cell and not the effector-bound c-di-GMP. Moreover, many of them are not optimised for monitoring long-term dynamics.

Many open questions remain about second messenger signalling in bacteria. One perplexing question is the role played by each member of the second messenger family and how often

they talk with each other. This necessitates the understanding of when and how each of these molecules are made and destroyed and what they bind. It is essential to develop techniques to identify how the bacteria integrates the multiple environmental input signals it receives to generate an appropriate output response. Programmable genetic circuits using c-di-GMP are being engineered for furthering our understanding of the complex signalling networks in bacteria. As c-di-GMP is of importance for quorum sensing, biofilm formation and pathogenesis, more tools need to be developed in the future to help visualise the c-di-GMP dynamics inside complex systems such as eukaryotic host tissues.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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