Critical Review

Quorum Sensing and Biofilm Formation in Mycobacteria: Role of c-di-GMP and Methods to Study This Second Messenger

Indra Mani Sharma
Anushya Petchiappan
Dipankar Chatterji*

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

Abstract

Bacteria have evolved to survive the ever-changing environment using intriguing mechanisms of quorum sensing (QS). Very often, QS facilitates formation of biofilm to help bacteria to persist longer and the formation of such biofilms is regulated by c-di-GMP. It is a well-known second messenger also found in mycobacteria. Several methods have been developed to study c-di-GMP signaling pathways in a variety of bacteria. In this review, we have attempted to highlight a connection between c-di-GMP and biofilm formation and QS in mycobacteria and several methods that have helped in better understanding of c-di-GMP signaling. © 2014 IUBMB Life, 66(12):823–834, 2014

Keywords: *microbial genetics; signal transduction; substrate and inhibitor recognition enzyme inhibitors as drugs; tuberculosis*

Introduction

Despite worldwide onslaughts since more than a millennium, the war on TB by mankind is still not over. The global burden of TB refuses to go down and every year more than 1.5 million people fall prey to the disease (1). Even with several antibiotics in hand, new forms of resistant bacteria are being discovered quite frequently. These include multidrug resistant, extensively drug resistant, and total drug resistant forms (2). Even more complex to understand is the persistence of the bacteria, which is mainly due to their ability to survive under stringent conditions, especially through the formation of "biofilms"(3). Biofilms are three-dimensional structures made up of extracellular matrix produced by bacteria. In these biofilms, the bacteria show a higher antibiotic tolerance leading to the emergence of persisters, which are a major cause of worry, as they are difficult to kill and survive for decades within the host (4,5). Importantly, the formation of biofilm is often regulated by a highly coordinated process called quorum sensing (QS; (6–9)).

QS, a cell to cell communication phenomenon, is involved in modulating the social behavior of bacteria (10). It is mediated by a variety of small molecules, cyclic-di-GMP being one of them (11,12). c-di-GMP, like cAMP, cGMP, (p)ppGpp, and c-di-AMP, is an important intracellular signaling molecule (13,14). It is a ubiquitous bacterial second messenger (15), recently discovered in a single cell eukaryote as well (16). Diguanylate cyclases (DGCs), with conserved GGDEF motif, synthesize one molecule of c-di-GMP using two molecules of GTP and phosphodiesterases (PDEs), with conserved EAL or HD-GYP motif, hydrolyze c-di-GMP into a linear pGpG or two GMP molecules (15). The level of c-di-GMP varies as a function of these two enzyme activities, which are regulated by extracellular or intracellular cues. The effective concentration of c-di-GMP facilitates desired phenotypes in the bacterial cells (17). These phenotypes vary from transition between motility and sessility, cell differentiation, virulence, long-term survival, and most importantly biofilm formation. A pictorial representation of c-di-GMP signaling in bacterial cells is shown in Fig. 1.

Because of the involvement of c-di-GMP in regulating biofilm formation (or persistence), unraveling the mechanism of c-di-GMP metabolism, and signaling can provide useful insights into the important physiological phenomena in mycobacteria.

Volume 66, Number 12, December 2014, Pages 823–834
Address correspondence to: Dipankar Chatterji, Molecular Biophysics Unit, Indian Institute of Science, Bangalore 560012, India. Tel: +22932836. Fax: +23600535. E-mail: dipankar@mbu.iisc.ernet.in
Conflict of Interest: Authors have no conflict of interest.
Received 19 October 2014; Accepted 4 December 2014
DOI 10.1002/iub.1339
Published online 26 December 2014 in Wiley Online Library (wileyonlinelibrary.com)

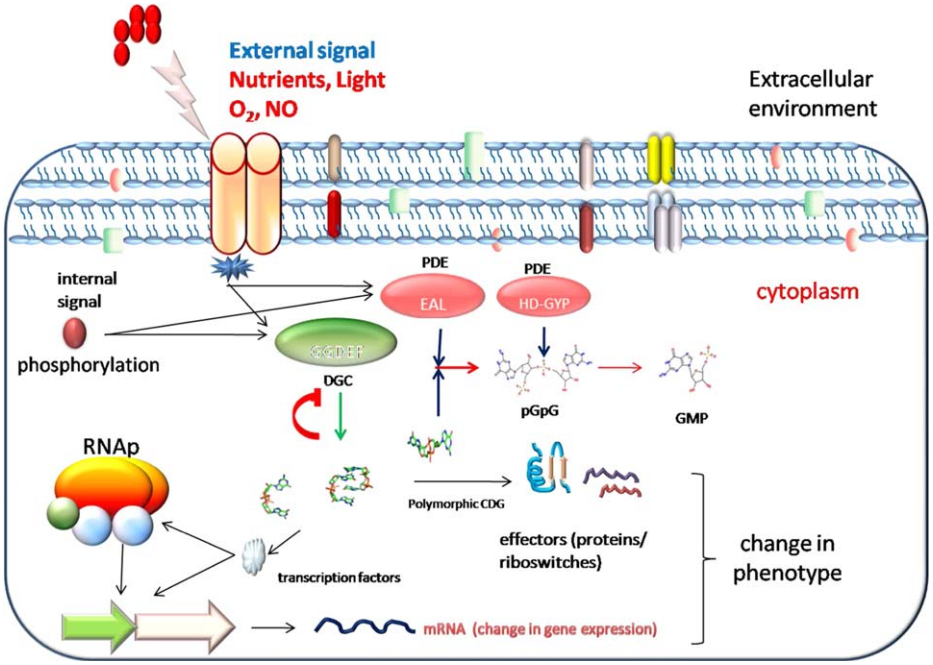

FIG 1 *Model depicting c-di-GMP based signaling network in the bacteria. Both DGC and cognate PDE can sense intra and extra cellular signals, which confer altered activity to them. c-di-GMP can inhibit its own synthesis by binding to product inhibition sites on many DGCs to set an upper level of cellular c-di-GMP concentrations. c-di-GMP shows structural polymorphism and these different polymorphic forms have been observed in bound form on different binding partners including proteins and RNAs to change the bacterial life style.*

A variety of methods have been developed to assay the activities of DGCs and PDEs as well as to quantify both intracellular and *in vitro* c-di-GMP levels. In this review, we have attempted to focus on QS and biofilm formation in mycobacteria with emphases on the possible involvement of c-di-GMP. We have further highlighted several important assays and methods for studying the various aspects of c-di-GMP signaling in different bacterial species with their possible implications in mycobacteria as and when required to yield a global picture of physiology and pathogenesis.

QS and Biofilm Formation in Mycobacteria

The phenomenon of QS has been well explored in Gram negative bacteria (18); relatively however, Gram positive bacteria remain poorly explored. Gram negative bacteria frequently use a LuxR-like system to sense and respond to fluctuating homoserine lactone levels as a function of changing environment or cell density (10,19). However, several Gram positive bacteria like *Bacillus subtilis* (20,21), *Streptococcus pneumoniae* (22), *Staphylococcus aureus* (23), and *Enterococcus faecalis* (24) use autoinducer peptides, which mediate the cellular cross-talk (25–27). However, the Gram positive mycobacteria remain a mystery with no clear evidence known about their canonical QS mechanism. Based on bioinformatics analysis, homologs of LuxR have been discovered in *Mycobacterium tuberculosis*, but the experimental supports are lacking (28). These homologs are Rv0195, Rv0386, Rv0491, Rv0890c, Rv0894, Rv2488c and Rv3133c. Some of these genes are ubiquitous across the mycobacterial species suggesting the possible existence of similar QS mechanisms. Additionally, because biofilm formation is a QS phenomenon, we argue that the "key players," which felicitate biofilm formation may be directly or indirectly involved in the QS process. Several genes involved in mycobacterial biofilm formation or persistence are WhiB3 (29), Rel_{mtb} (30), DcpA (31), DevR (32), and GroEL1 (33) among others.

The coordinated process of biofilm formation is a common mode of adaptation exhibited by a variety of bacteria and mycobacteria are no exception. Several species of mycobacteria have been shown to form biofilm under various conditions. Nontuberculous mycobacteria like *M. smegmatis* (33), *M. marinum* (34), *M. fortuitum* (35), *M. chelonae* (35), *M. ulcerans* (36), *M. abscessus* (37), *M. avium* (38), and *M. bovis* (39) are known to form biofilm under *in vitro* and sometimes *in vivo* conditions. Several factors like availability of nutrients (36), pH, oxygen, temperature (40), and iron (41), and sometimes mycobacterial pili (42) affect the biofilm formation and development. Mycobacterial biofilm formation appears to be a genetically programmed process as free mycolic acids are

FIG 2 *A typical biofilm formation by M. tuberculosis (5 weeks old). (Reproduced with permission from Kulka et al., J Vis Exp, 2012.) [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]*

released in the mycobacterial culture, which subsequently participate in maturation of the pellicle (43). Acetylated GPL derivatives and mycolyldiacylglycerol also participate in the biofilm formation (44,45). Mycobacterial biofilms not only provide recalcitrant environment but also provide opportunity for conjugal transfer of DNA to help bacteria to evolve according to their niche (46). Several genes related to DNA replication and repair, survival under carbon and oxidative stress, transport of solute across membrane and surface remodeling are up regulated during biofilm maturation of *M. smegmatis* (41). Similar to nontuberculous mycobacteria, Ojha and coworkers in their seminal work have shown that *M. tuberculosis* forms biofilm under *in vitro* conditions (47). The appearance of pellicle of *M. tuberculosis* is very similar to the aforementioned mycobacterial species (Fig. 2). Three genetic loci namely *pks16, helY and pks1* are known to be involved in *M. tuberculosis* biofilm formation (43,48). A summary list showing various genes/proteins involved in QS/biofilm formation/persistence of mycobacteria is given in Table 1. Islam et al. (4) have suggested that the infection of *M. tuberculosis* display characteristics of biofilm, and *in vivo M. tuberculosis* may form biofilm-like structures in liquefied lesions. They argued that the antibiotic tolerance and chronicity of *M. tuberculosis* share similar features displayed in the case of pathogenic biofilm infections. In addition, they have also suggested ways to investigate the *in vivo* occurrence and behavior of *M. tuberculosis* biofilm (4).

Cyclic-di-GMP Signaling in Mycobacteria

c-di-GMP was discovered in 1987 by Benziman and coworkers as an activator of cellulose synthesis in *Acetobacter xylinus* (49). Since then, it has been discovered in several other bacterial species including mycobacteria (17,31,50). The cellular level of c-di-GMP in *M. smegmatis* was found to be regulated by the activities of a protein called DcpA (earlier known as MSDGC-1), which is encoded by the gene with locus tag MSMEG_2196 (31). DcpA is a 615 amino acid long polypeptide harboring three domains *in tandem*, GAF, GGDEF and EAL. Intriguingly, both the GGDEF and the EAL domains are active, that is, they show synthesis and hydrolysis activities, respectively, in the presence of metal ions like Mg^{2+} (31).

Orthologs of DcpA are present in almost all mycobacterial species and they share a high degree of sequence similarity and domain architecture (31). In the case of *M. smegmatis* and *M. tuberculosis*, these proteins have been well characterized (31,50,51). DcpA and its ortholog from *M. tuberculosis*, MtbDGC (Rv1354c) share 60% sequence similarity with identical domain architecture and residues of catalytic importance. Both of these proteins are bifunctional and capable of c-di-GMP synthesis and hydrolysis. In the case of DcpA, the full length protein is required for dual activity under *in vitro* conditions. This was evident as the separated domains did not show any activity. Interestingly, the GGDEF-EAL domain of DcpA without GAF domain showed poor synthesis activity with complete loss of the hydrolysis activity, suggesting that GAF domain might be playing crucial role in attaining the optimal catalytic activities. Further, the regulation of bifunctional behavior of DcpA is intriguing (51). It exist both in monomeric and dimeric forms with the dimeric form showing both c-di-GMP synthesis and hydrolysis activities and the monomeric form showing only hydrolysis activity. Although there are four Cysteine residues distributed in the polypeptide of DcpA, the dimerization is due to noncovalent interactions and the dimeric and monomeric forms are interconvertible.

To function properly, DcpA localizes to the inner cytosolic membrane at cell poles but it does not have any predicted transmembrane helix. The altered level of DcpA affects cell length and colony morphology of *M. smegmatis* (51). These are surface related phenotypes affected by the product c-di-GMP. As a direct evidence, LtmA, a TetR-like transcription factor, which binds with c-di-GMP has been shown to affect the expression of genes involved in lipid metabolism of *M. smegmatis* (52). All these properties indicate that c-di-GMP may also be involved in biofilm formation of *M. smegmatis* under certain growth conditions. A pictorial representation of c-di-GMP signaling in *M. smegmatis* is shown in Fig. 3.

Since MSMEG_2196 is the only gene, which encodes for protein responsible for synthesis of c-di-GMP in *M. smegmatis*, its deletion results in the complete loss of cellular c-di-GMP. Such a deleted strain shows compromised long-term survival of *M. smegmatis* under conditions of carbon starvation (31). This suggests that c-di-GMP enhances mycobacterial capacity to resist carbon limiting conditions (51). The *M. tuberculosis* strain having the selective deletion of the GGDEF domain (dgc^{mut}) of Rv1354c and complete deletion of Rv1357c (Δ*pde*) showed increased and reduced dormancy respectively under anaerobic conditions (53). In addition, Δ*pde* showed decreased infectivity with human THP-1 cells and attenuated

TABLE 1 ***A summary list showing various genes/proteins vis-a-vis their function in mycobacteria***

Genes/proteins	*Domain architecture*	*Phenotype (persistence/biofilm formation/quorum sensing)*	*Factors affecting expression/activity*	*Connection with c-di-GMP*	*Experimental proof (E)/bioinformatics analysis (B)*	*Species*	*Reference*
Lux R homologues		Quorum sensing	Autoinducer peptides and small molecules	Yes	B	*M. tuberculosis*	[12,28]
Rv0195, Rv0386, Rv0491, Rv0890c, Rv0894, Rv2488c	GerE-HTH, GC-NB-ARC-GerE, RR, GerE, AAA ATPase, NB-ARC-GerE						
WhiB3	Whib	QS	Oxygen level	–	E	*M. tuberculosis*	[29]
Rel_{mtb}	HD-Rel/SpoT-TGS-ACT	Persistence	Nutrient stress	Yes	E	*M. tuberculosis*	[13,30,95,96]
Rv1354c	GAF-GGDEF-EAL	Persistence	Carbon stress/anaerobic conditions	Yes	E	*M. tuberculosis*	[50,53]
Rv1357c	EAL	Dormancy	Anaerobic conditions	Yes	E	*M. tuberculosis*	[53]
DcpA (earlier known as MSDGC-1)	GAF-GGDEF-EAL	Persistence	Carbon stress	Yes	E	*M. smegmatis*	[31]
DevR (Rv3133c-predicted LuxR homologue)	RR-GerE	Persistence	Hypoxia	–	E	*M. tuberculosis*	[32]
GroEL1	Cpn60-TCP1	Biofilm formation	Heat shock and infection	–	E	*M. smegmatis*	[33]
pks16, helY, pks1	–	Biofilm formation	Infection	–	E	*M. tuberculosis*	[43,48]

The metabolic pathway of the homologues of some of these genes/proteins like LuxR (12) and Rel are known to have a connection with c-di-GMP in the Gram negative bacteria (13,95,96). DcpA and Rv1354c are known to synthesize and hydrolyze c-di-GMP in M. smegmatis *and* M. tuberculosis *whereas Rv1357c can only hydrolyze c-di-GMP in* M. tuberculosis *(31,50).*
*AAA ATPase: ATPase Associated with diverse cellular activities; ACT: aspartokinase, chorismate mutase, TyrA domain; Cpn60-TCP1: TCP-1/cpn60 chaperonin family, HSP60 chaperone family and the TCP-1 (T-complex protein) family; GAF: cGMP-specific phosphodiesterases, adenylyl cyclases and FhlA; EAL: glutamate-alanine-leucine; GC: adenylate and guanylate cyclase catalytic domain; GerE-HTH: LuxR-type DNA-binding HTH domain (*B. subtilis *gerE is the transcriptional activator and repressor of spore formation; germination protein); GGDEF: glycine-glycine-aspartate-glutamate-phenylalanine; HD: metal dependent phosphohydrolases containing HD motif; NB-ARC: nucleotide-binding adaptor shared by APAF-1, certain R gene products and CED-4, a signaling motif present in both bacteria and eukaryotes; RR: response regulator receiver domain; TGS: ThrRS (threonyl tRNA-synthetase), GTPase, and SpoT domain; WhiB: transcription factor.*

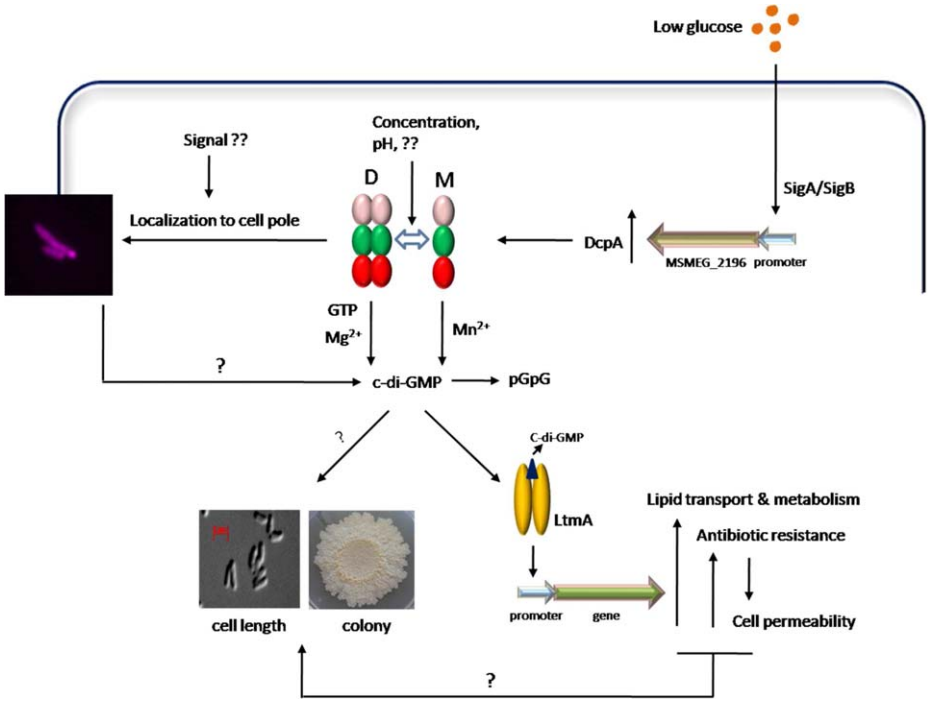

FIG 3

A schematic summary of c-di-GMP signaling in M. smegmatis. When the level of glucose reduces in the surrounding of a cell, it causes increased production of DcpA from its promoter by SigA/SigB (95). DcpA exists in both monomeric and dimeric forms, which are in equilibrium that is affected by proteins concentration, pH and by unknown factors. The dimeric DcpA synthesizes and hydrolyzes c-di-GMP in metal dependent manner whereas the monomeric form is able to hydrolyze c-di-GMP. By some unknown mechanism, dimeric, or monomeric form of DcpA is localized to the inner membrane at the cell poles to affect final c-di-GMP level. c-di-GMP then binds with LtmA, a transcription factor, which in turn activates lipid transport and metabolism genes that reduces cell wall permeability and increases antibiotic tolerance (52). Alterations in the level of DcpA affect cell length and colony morphology of M. smegmatis*. Some of the figures have been taken from Sharma et al. (51). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]*

pathogenicity in a mouse model. In other words, c-di-GMP is responsible for the negative regulation of bacterial virulence with infections of human macrophage-like cells and mice (53). In a different study, Rv1357c (the stand-alone PDE in *M. tuberculosis*) was introduced in BCG Pasteur strain (54). Such a modified strain served as a vaccine against TB in murine model. In comparison to the control strain, the Rv1357c:BCG Pasteur strain was more phagocytosed and showed halted replication, which withstood TB challenges.

Because c-di-GMP plays an important role in the mycobacterial survival (31,53) and is present in both pathogenic and nonpathogenic species and possibly involved in the biofilm formation, the selective control of c-di-GMP signaling may help in clearing mycobacterial infection. In fact, in a structure based modeling and drug discovery work, Cui et al. (55) shortlisted few molecules that can inhibit the diguanylate cyclase (Rv1354c) of *M. tuberculosis*. Hence, in this review, we have highlighted various methods, which can be used to study the steps in the c-di-GMP signaling pathways in various bacterial species including mycobacteria. This may help in advancing our understanding of c-di-GMP signaling system in mycobacteria and possibly lead to discovery of novel inhibitors.

Methods of c-di-GMP Synthesis

To efficiently study c-di-GMP signaling, one would first require a large quantity of pure c-di-GMP. There are two major approaches to synthesize c-di-GMP in bulk.

Enzymatic Method

Although c-di-GMP can be synthesized using any active diguanylate cyclase, factors like feed back inhibition, enzyme stability and requirement of accessory factors might limit its overproduction. To circumvent these problems, Liang and coworkers engineered a diguanylate cyclase named etDGC having a GGDEF domain from *Thermotoga maritima*, TM1788 (56). This protein showed almost 100% conversion of GTP into c-di-GMP with a high turnover number. Under optimum conditions, 10 mg of this enzyme yielded 200 mg of c-di-GMP. Similarly, Schirmer and coworkers used a GGDEF domain containing protein YdeH from *E. coli* to synthesize c-di-GMP on a large scale (57).

Chemical Method

The protocols for chemical synthesis of c-di-GMP are continuously evolving. Jones and coworker reported an amenable solution-phase method to produce milligrams of c-di-GMP (58).

The reagents used in each step of this method do not interfere with the reagents used in subsequent steps, making the synthesis possible in a single flask.

Diguanylate Cyclase and Phosphodiesterase Assays and Estimation of c-di-GMP Levels

The first protein with a GGDEF domain to be assayed for DGC activity was PleD from *Caulobacter crescentus* (59). Biochemical assays using radioactive GTP showed that PleD can convert $GT^{32}P$ to c-di-$GM^{32}P$ *in vitro*. Since then several direct and indirect methods have been employed to monitor the DGC and PDE activities and to detect changes in c-di-GMP concentrations. These methods have been discussed further in the text below.

Thin Layer Chromatography (TLC)

It is a highly sensitive batch method wherein the determination of enzymatic activities of DGCs and PDEs has been performed using radioactive substrates, $GT^{32}P$ and c-di-$GM^{32}P$, respectively (31,50,51,59–61). The enzyme to be studied is incubated with the radioactive substrate in a suitable buffer and the reaction products are separated on a thin layer chromatographic plate (PEI-cellulose) using one-dimensional chromatography in a mobile phase and then imaged using phosphorimagers. Nevertheless, detection of intracellular c-di-GMP using this method is extremely difficult because of the low intracellular concentration and the presence of several other nucleotides. However, Tischler and Camilli (61) have been able to detect c-di-GMP in bacterial cell using two-dimensional TLC.

Reverse Phase HPLC and Mass Spectrometry

Reverse phase HPLC and mass spectrometry based methods have been used most extensively to determine the levels of c-di-GMP and in turn analyze the activities of several DGCs and PDEs (62–66). Simm et al. (66) were able to detect up to femptomolar levels of c-di-GMP in bacterial extracts indicating the high sensitivity of this method.

Colorimetric Detection of Pyrophosphate and Inorganic Phosphate

The steady state kinetics of DGCs can be monitored using commercially available pyrophosphate detection colorimetric kits. This method can be performed in a high-throughput fashion using multi-well microplates, however, the upper limit of detection of pyrophosphate (PPi) is 5 μM. It is also sensitive to Pi contamination. The proteins namely XCC4471, WspR, and PleD and have been analyzed using this method (67–69). Similarly, a malachite green coupled colorimetric assay has been developed by Chan et al. (70) to monitor release of Pi in batches. An enzymatic inactivation step is however required before the analysis.

C-di-GMP-Proflavine-Hemin Complex: Nucleotidezyme

C-di-GMP can form G-quadruplex in the presence of proflavine. Such a complex interacts with hemin and acts as a nucleotidezyme (71). This nucleotidezyme can in turn oxidize the colorless compound ABTS to the colored radical cation ABTS. This provides a simple method for colorimetric detection of c-di-GMP at a very low concentration.

Thiazole and c-di-GMP Complex

In the presence of planer intercalators like thiazole orange, c-di-GMP exhibits structural polymorphism. The resultant thiazole orange, c-di-GMP complex shows enhanced fluorescence (72). This property has been elegantly used to determine the cellular levels of c-di-GMP. To do this, c-di-GMP was over-produced in *E. coli*, the cells were lysed and nucleotide extracts were mixed with thiazole orange. This resulted in fluorescence emission, which was characteristic of c-di-GMP and thiazole orange complex. This method is possible due to the extraordinary fact that thiazole orange is able to induce structural polymorphism specifically to c-di-GMP in a mixture containing other nucleotides.

Circular Dichroism (CD) Based Activity Assay

This method is based on the simple fact that manganese ions can selectively induce the formation of intercalated dimer of c-di-GMP in solution. Such a dimer consists of four mutually intercalated guanines stabilized by stacking interactions among themselves. Importantly, such a structure exhibits an intense sigmoidal CD spectrum in the near-UV region. This property is not shown by either GTP or pGpG. Therefore, Cutruzzola and coworkers used the unique property of c-di-GMP to study activity kinetics of DGCs and PDEs in real time (73). As a function of DGC or PDE activity, the concentration of c-di-GMP and hence the concentration of intercalated dimeric c-di-GMP varies in solution and can be monitored using CD experiments at 282 nm. The activities of PleD and RocR have been analyzed using this method. These analyses yielded similar results to that obtained in HPLC based activity assay.

Allosteric Ribozymes

Several ribozymes are known to bind with c-di-GMP in various bacterial species. Using *in vitro* selection methods, a series of allosteric ribozymes were generated that can sense c-di-GMP. Two types of ribozymes were then selected whose self-cleavage was induced or repressed in the presence of c-di-GMP. These ribozymes were sensitive to micromolar levels of c-di-GMP from *E. coli* cell lysate (74).

Fluorescent c-di-GMP Analogs

Recently we have synthesized and characterized a fluorescent analog of c-di-GMP called MANT-(c-di-GMP) (75). Its use is harmless but highly sensitive. Using MANT-(c-di-GMP), we successfully monitored the hydrolysis activity of DcpA in real time. We observed that the bound product MANT-pGpG displays lower emission than the bound substrate MANT-(c-di-GMP). Hence, a decrease in the fluorescence emission could be monitored as a function of phosphodiesterase activity of DcpA.

Use of 3′,3′-cG(d2AP)MP

Sintim and coworkers have recently synthesized a fluorescent 2-aminopurine (2AP)-containing cyclic dinucleotide analogue

called 3′,3′-cG(d2AP)MP (76). Two such molecules can self associate in the presence of Mn^{2+}. But more importantly, it can also associate with c-di-GMP to form heterodimer in the presence of Mn^{2+}. Such a heterodimerization results in the quenching of the fluorescence of 3′,3′-cG(d2AP)MP. This provides a convenient method to monitor both DGC and PDE activities.

Fluorescent Biosensors

Fluorescent biosensors have been designed to monitor concentrations of c-di-GMP both *in vitro* and *in vivo*. The EAL_{FimX} protein has been shown to bind with c-di-GMP with very high affinity, which leads to the local structural changes in the protein. When this protein was labeled with environment sensitive fluorescent dyes namely MDCC and 6-IAF, the binding of c-di-GMP to it resulted in change in the fluorescence emission. Thus, it could be used as a biosensor to quantitate c-di-GMP level *in vitro* (77).

YcgR, a PilZ domain containing protein that binds with c-di-GMP, was fused with YFP and CFP and expressed in *Caulobacter crescentus*. The biosensor thus produced helps in determining the spatiotemporal distribution of c-di-GMP as a function of cell division (78). Similarly, when bonafide c-di-GMP receptors MrkH and VCA0042 were tagged with mCerulean and mVenus at their N- and C-terminus, respectively, they acted as biosensors for changes in intracellular c-di-GMP concentration in *E. coli* (79). In another study, a c-di-GMP responsive promoter was fused with the GFP gene (green fluorescent protein) in *Pseudomonas aeruginosa* (80). A change in the c-di-GMP level could then be monitored as a function of the change in the fluorescence.

Congo Red Dye Binding Assay

Congo red dye has the ability to bind with polysaccharides with the resultant complex appearing red even after several washes. Therefore, the Congo red dye has been widely used to quantitate extra-polysaccharide produced by bacteria. It has been shown by several groups that c-di-GMP affects polysaccharide production in bacteria (49). Therefore, the Congo red binding assay can be used to indirectly approximate the intracellular levels of c-di-GMP. Since the intracellular level of c-di-GMP is maintained by a balance between activities of DGCs and PDEs, one can also correlate their activity to the extent Congo red binds with the extra-polysaccharides (81). Recently, we have shown that this assay can be used to assign c-di-GMP synthesis activity of dimeric form of DcpA in the cell (51).

Discovery of c-di-GMP Receptors and Determination of Binding Parameters

Several existing methods have been adapted to discover c-di-GMP binding partners (proteins or RNA) and to investigate the interaction between these two. We have described here some elegant methods in the text below.

Cellular Fractionation and UV-Cross Linking

To discover a novel c-di-GMP binding partner from a cell, to verify binding of c-di-GMP with a protein or to probe the interacting amino acids, cellular fractionation coupled with UV-cross linking method has been widely used (82).

Pull Down Assays (Affinity Chromatography)

Recently, a functionalized analog of c-di-GMP called 2′AHC-c-di-GMP has been chemically synthesized (83). This analog is immobilized on NHS-activated sepharose beads and pull down experiments were performed. The bacterial cell lysate was allowed to bind with affinity matrix for 2 hours at 4 °C with gentle rotation. After extensive washing, the resin was incubated with excess c-di-GMP. The eluted proteins were then precipitated with chloroform/methanol and subsequently separated by SDS-PAGE and analyzed by high resolution mass spectrometry. Further, to validate and quantify the interaction with a test protein, 2′-AHC-c-di-GMP was also employed in SPR study.

Fluorescence Spectroscopy

Fluorescent analogs of c-di-GMP called Di-MANT-c-di-GMP and MANT-c-di-GMP (MANT-CDG) have been developed by Biolog Co. Germany and by us (75), respectively. MANT-CDG can be excited by irradiation of light at 355 nm that results in a fluorescence emission at ~440 nm. The fluorescence of MANT-CDG is sensitive to changes in the microenvironment, which helped us to study its interaction with representative proteins from three major classes of c-di-GMP binding receptors. These proteins were PleD (a bonafide diguanylate cyclase from *Caulobacter crescentus*), DcpA (a bifunctional protein from *Mycobacterium smegmatis*), and YcgR (a PilZ domain containing receptor). Two approaches were adapted to investigate the interaction; (a) measurement of direct fluorescence and (b) measurement of fluorescence resonance energy transfer. The method (a) is suited for proteins with or without tryptophan residue and (b) is suited only for the proteins having tryptophan residue. Method (a) was used to investigate interaction of PleD, DcpA, and YcgR whereas method (b) was implied in the case of DcpA and YcgR. The specificity of interaction was confirmed by monitoring the competition of native c-di-GMP with MANT-CDG for a binding site on these proteins. This competition was followed by a decrease in the fluorescence emission of MANT-CDG bound with protein.

Differential Radial Capillary Action of Ligand Assay (DRaCALA)

This is a rapid method (completed in 5 Sec), which can be performed in a single set or in a high throughput manner to quantitatively measure the extent of interaction of c-di-GMP and its receptors in purified form or from cell lysate (84). The selection of nitrocellulose membrane and its ability to discriminate between bound and free ligand is the key to the success of this method. In this method, the ligand should be preferably radioactive; though any ligand that has intrinsic signal like fluorescence can also be used. When a free ligand is spotted on a

nitrocellulose membrane, it is mobilized by bulk movement of the solvent through capillary action. This results in the appearance of relatively large round spot with weak signal. However, when a ligand bound to protein is spotted, it is sequestered at the site of application forming a relatively small round but intense spot. If the ligand does not interact with the protein, it forms a spot similar to in the absence of protein. Additionally, if the interaction is specific, one would observe disappearance of the intense spot in the presence of excess of nonradioactive ligand. A variety of ligands [small molecules like cAMP, cGMP, (p)ppGpp, c-di-GMP (84), c-di-AMP (85), and small DNA or RNA fragments (86)] can be screened using this method.

Optogenetic Applications of Light Regulated c-di-GMP Synthesis Module

Recently, an engineered chimeric photo-activated c-di-GMP synthesis module (a synthetic operon consisting of three genes namely bphS, bphO, and yhjH) has been reported by Ryu and Gomelsky (87). The gene bphS produces a PAS-GAF-PHY-GGDEF domain containing protein, whereas bphO and yhjH produce a heme oxygenase and a c-di-GMP phosphodiesterase, respectively. The protein heme oxygenase produces biliverdin, a cofactor that binds with PAS-GAF-PHY-GGDEF domain protein (or BphS) to make it photoactive. Upon irradiation of light at 756 nm, BphS is activated by ~11 fold, which results in ~50 fold increment in the cellular c-di-GMP pool. A fine tuned production of the phosphodiesterase YhjH helps in maintaining the level of c-di-GMP in the cell to avoid any spill over. This method of maintaining c-di-GMP level by light provides an opportunity for orthogonal regulations. Use of photoactivation module of bacteriophytochrome is additionally advantageous because it requires a light irradiation in near infrared window (NIRW), which span from 680 to 800 nm. This light has the ability to penetrate deep eukaryotic tissues with least side effects. Application of such a light regulated module to influence DGC and PDE activity in *M. tuberculosis* inside host would help in understanding the effect of c-di-GMP in the bacterial survival and pathogenesis.

Discovery of Inhibitors

With the ever increasing number of antibiotic-resistant infections of *M. tuberculosis*, there is a pressing need to discover new alternatives to combat them. Targeting the c-di-GMP signaling pathways is therefore a promising strategy because it is found in the bacteria but absent in humans, which makes its associated pathways important targets to explore. A glycosylated triterpenoid saponin (GTS) from *Pisum sativum* was the first reported inhibitor to DGCs (88). However, due to its inability to cross bacterial membranes, it could not be used as a drug. Subsequently, two approaches have been popularly taken to identify the possible drugs; one by designing new c-di-GMP structural analogs to be allosteric inhibitors of DGCs and another by screening the available chemical compound libraries for any DGC or biofilm inhibitors.

Although there are few known structural analogs of c-di-GMP, like endo-S-c-di-GMP (89) known to inhibit PDE activity *in vitro*, they have limited bioactivity and do not exhibit properties required in a drug. Consequently, several high throughput screening (HTS) methods have been used to identify novel compounds. One such strategy involved using a Congo red dye-based primary assay, followed by a biofilm formation assay using crystal violet and a reporter gene assay for DGC activity (90). The antimetabolite drug sulfathiazole was identified as a biofilm inhibitor in this manner. This provided a simple, rapid and a whole-cell based HTS assay for screening compounds without the need to perform time-consuming procedures like mass spectrometry and HPLC.

Another HTS strategy employed the clever use of a gene expression system with two plasmids in a *Vibrio cholerae* strain (91). The first plasmid encoded a DGC under the control of an IPTG inducible promoter whereas the second plasmid contained a c-di-GMP responsive promoter fused to the *lux* reporter gene. Thus, any change in c-di-GMP levels due to the presence of an inhibitor was reflected in the luciferase expression, which could be monitored easily. Biofilm formation was quantified under static conditions using a crystal violet assay on microtiter plates similar to the previous HTS procedure and under flow conditions with disposable flow cells. *In vitro* inhibition of DGC activity was studied by the EnzChek assay (Invitrogen). Several thousands of molecules were effectively screened by this approach leading to the identification of seven compounds, which inhibit DGC activity and reduce biofilm formation. These molecules inhibit the biofilm formation by either directly inhibiting the DGCs involved in biofilm formation or else reducing the transcription of c-di-GMP responsive genes (Fig. 4).

Furthermore, DRaCALA was recently developed as a novel HTS method to screen drug candidates (92). Using pin transfer for the simultaneous transfer of hundreds compounds followed by the direct measurement of radioactivity, it provides for rapid and sensitive detection of specific inhibitors. Ebselen, a compound with known pharmacological and toxicity properties, was identified through DRaCALA as an inhibitor of DGCs through covalent modification. Fluorescence anisotropy using the c-di-GMP analogs and scintillation proximity assays are the other possible methods for identification of novel antimicrobial agents. In the future, adaptations of the available c-di-GMP analysis methods on HTS platforms would lead to discovery of other possible strategies for discovering new interventions.

Conclusion and Future Perspectives

QS facilitates biofilm formation in bacteria and appears to be operational in mycobacteria as well. Biofilm

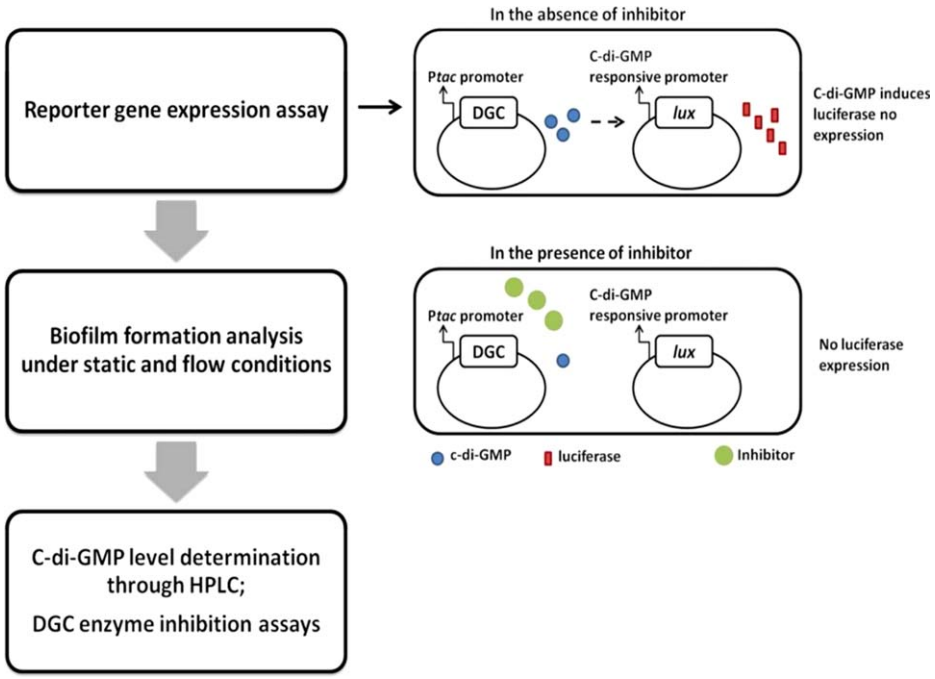

FIG 4 *An example of screening strategy for DGC inhibitors (adapted from Sambanthamoorthy et al., Antimicrob Agents Chemother, 2012, 56, 5202–5211). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]*

formation is a unique survival strategy, which is regulated by c-di-GMP in several bacteria and possibly in mycobacteria as well. These suggest a connection between c-di-GMP, biofilm formation, and QS. Therefore, investigating the c-di-GMP signaling in mycobacteria may provide unique opportunity to understand the complex QS related phenomenon. Since c-di-GMP appears to play a very important role in mycobacterial physiology, we have summarized several elegant methods to dissect the stages in the c-di-GMP signaling pathways (Table 2). Some of the methods like DRaCALA are indeed helpful for investigating new c-di-GMP binding proteins, for determination of affinity for ligand protein interaction and discovery of new inhibitors. Additionally, one should also consider understanding the process of biofilm dispersion while studying biofilm formation as the dispersed cells are soft targets of antibiotics. Finally, a clear picture of QS and biofilm formation in mycobacteria will help in the treatment of chronic mycobacterial infections (93,94).

TABLE 2 ***Various methods to study c-di-GMP signaling pathways***

Techniques	*Activities*	*Concentration of c-di-GMP*	*References*
HPLC/LC-MS	DGC and PDE, intracellular levels of c-di-GMP	Upto femptomolar	(62–66)
DraCALA	Protein-ligand interactions	Upto nanomolar	(84)
TLC	DGC and PDE, intracellular levels of c-di-GMP	Submicromolar	(31,50,51,59–61)
Fluorescent methods	DGC and PDE	Submicromolar	(75–80)
Ribozyme	*In vitro* and intracellular levels of c-di-GMP	Submicromolar	(74)
CD	DGC and PDE	Micromolar	(73)
Colorimetric detection	DGC and PDE	Micromolar	(67–71)

Acknowledgements

This work was supported by a Center of Excellence Grant, Department of Biotechnology, Government of India, India to DC. The authors apologize to several authors whose contributions could not be included due to lack of space.

References

[1] Dye, C., Lonnroth, K., Jaramillo, E., Williams, B. G., and Raviglione, M. (2009) Trends in tuberculosis incidence and their determinants in 134 countries. Bull. World Health Organ. 87, 683–691.

[2] Phillips, L. (2013) Infectious disease: TB's revenge. Nature 493, 14–16.

[3] Costerton, J. W., Stewart, P. S., and Greenberg, E. P. (1999) Bacterial biofilms: a common cause of persistent infections. Science 284, 1318–1322.

[4] Islam, M. S., Richards, J. P., and Ojha, A. K. (2012) Targeting drug tolerance in mycobacteria: a perspective from mycobacterial biofilms. Expert Rev. Anti Infect. Ther. 10, 1055–1066.

[5] Smith, I. (2003) Mycobacterium tuberculosis pathogenesis and molecular determinants of virulence. Clin. Microbiol. Rev. 16, 463–496.

[6] Davies, D. G., Parsek, M. R., Pearson, J. P., Iglewski, B. H., Costerton, J. W., et al. (1998) The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science 280, 295–298.

[7] Cvitkovitch, D. G., Li, Y. H., and Ellen, R. P. (2003) Quorum sensing and biofilm formation in Streptococcal infections. J. Clin. Investig. 112, 1626–1632.

[8] Garcia-Aljaro, C., Melado-Rovira, S., Milton, D. L., and Blanch, A. R. (2012) Quorum-sensing regulates biofilm formation in Vibrio scophthalmi. BMC Microbiol. 12, 287.

[9] Waters, C. M., Lu, W., Rabinowitz, J. D., and Bassler, B. L. (2008) Quorum sensing controls biofilm formation in Vibrio cholerae through modulation of cyclic di-GMP levels and repression of vpsT. J. Bacteriol. 190, 2527–2536.

[10] Miller, M. B. and Bassler, B. L. (2001) Quorum sensing in bacteria. Annu. Rev. Microbiol. 55, 165–199.

[11] Ueda, A. and Wood, T. K. (2009) Connecting quorum sensing, c-di-GMP, pel polysaccharide, and biofilm formation in Pseudomonas aeruginosa through tyrosine phosphatase TpbA (PA3885). PLoS Pathog. 5, e1000483.

[12] Srivastava, D., Harris, R. C., and Waters, C. M. (2011) Integration of cyclic di-GMP and quorum sensing in the control of vpsT and aphA in Vibrio cholerae. J. Bacteriol. 193, 6331–6341.

[13] Kalia, D., Merey, G., Nakayama, S., Zheng, Y., Zhou, J., et al. (2013) Nucleotide, c-di-GMP, c-di-AMP, cGMP, cAMP, (p)ppGpp signaling in bacteria and implications in pathogenesis. Chem. Soc. Rev. 42, 305–341.

[14] Bharati, B. K. and Chatterji, D. (2013) Quorum sensing and pathogenesis: role of small signaling molecules in bacterial pathogenesis. Curr. Sci. 105, 643–656.

[15] Hengge, R. (2009) Principles of c-di-GMP signalling in bacteria. Nat. Rev. Microbiol. 7, 263–273.

[16] Chen, Z-H. and Schaap, P. (2012) The prokaryote messenger c-di-GMP triggers stalk differentiation in Dictyostelium. Nature 488, 680–683.

[17] Romling, U., Galperin, M. Y., and Gomelsky, M. (2013) Cyclic di-GMP: the first 25 years of a universal bacterial second messenger. Microbiol. Mol. Biol. Rev. 77, 1–52.

[18] Whitehead, N. A., Barnard, A. M., Slater, H., Simpson, N. J., and Salmond, G. P. (2001) Quorum-sensing in Gram-negative bacteria. FEMS Microbiol. Rev. 25, 365–404.

[19] Parsek, M. R. and Greenberg, E. P. (2000) Acyl-homoserine lactone quorum sensing in gram-negative bacteria: a signaling mechanism involved in associations with higher organisms. Proc. Natl. Acad. Sci. USA 97, 8789–8793.

[20] Magnuson, R., Solomon, J., and Grossman, A. D. (1994) Biochemical and genetic characterization of a competence pheromone from B. subtilis. Cell 77, 207–216.

[21] Solomon, J. M., Magnuson, R., Srivastava, A., and Grossman, A. D. (1995) Convergent sensing pathways mediate response to two extracellular competence factors in Bacillus subtilis. Genes Dev. 9, 547–558.

[22] Haverstein, L. S. and Morrison, D. A. (1999) "Quorum sensing and peptide pheromones in Streptococcal competence for genetic transformation." In *Cell–Cell Signaling in Bacteria*. (Dunney, G. M. and Winans, S. C., eds.), pp. 9–26, ASM Press, Washington DC.

[23] Novick, R. P. (1999) "Regulation of pathogenecity in Staphylococus aureus by a peptide-based density-sensing system." In *Cell–Cell Signaling in Bacteria*. (Dunny, G. M. and Winans, S. C., eds.), pp. 129–146, ASM Press, Washington DC.

[24] Qin, X., Singh, K. V., Weinstock, G. M., and Murray, B. E. (2000) Effects of Enterococcus faecalis fsr genes on production of gelatinase and a serine protease and virulence. Infect. Immun. 68, 2579–2586.

[25] Kleerebezem, M., Quadri, L. E., Kuipers, O. P., and de Vos, W. M. (1997) Quorum sensing by peptide pheromones and two-component signal-transduction systems in Gram-positive bacteria. Mol. Microbiol. 24, 895–904.

[26] Lazazzera, B. A. and Grossman, A. D. (1998) The ins and outs of peptide signaling. Trends Microbiol. 6, 288–294.

[27] Sturme, M. H., Kleerebezem, M., Nakayama, J., Akkermans, A. D., Vaugha, E. E., et al. (2002) Cell to cell communication by autoinducing peptides in gram-positive bacteria. Antonie van Leeuwenhoek 81, 233–243.

[28] Chen, J. and Xie, J. (2011) Role and regulation of bacterial LuxR-like regulators. J. Cell Biochem. 112, 2694–2702.

[29] Banaiee, N., Jacobs, W. R., Jr., and Ernst, J. D. (2006) Regulation of Mycobacterium tuberculosis whiB3 in the mouse lung and macrophages. Infect. Immun. 74, 6449–6457.

[30] Primm, T. P., Andersen, S. J., Mizrahi, V., Avarbock, D., Rubin, H., and Barry, C. E., III. (2000) The stringent response of Mycobacterium tuberculosis is required for long-term survival. J. Bacteriol. 182, 4889–4898.

[31] Bharati, B. K., Sharma, I. M., Kasetty, S., Kumar, M., Mukherjee, R., et al. (2012) A full-length bifunctional protein involved in c-di-GMP turnover is required for long-term survival under nutrient starvation in Mycobacterium smegmatis. Microbiology 158, 1415–1427.

[32] Gupta, R. K., Thakur, T. S., Desiraju, G. R., and Tyagi, J. S. (2009) Structure-based design of DevR inhibitor active against nonreplicating Mycobacterium tuberculosis. J. Med. Chem. 52, 6324–6334.

[33] Ojha, A., Anand, M., Bhatt, A., Kremer, L., Jacobs, W. R., Jr., et al. (2005) GroEL1: a dedicated chaperone involved in mycolic acid biosynthesis during biofilm formation in mycobacteria. Cell 123, 861–873.

[34] Bardouniotis, E., Ceri, H., and Olson, M. E. (2003) Biofilm formation and biocide susceptibility testing of Mycobacterium fortuitum and Mycobacterium marinum. Curr. Microbiol. 46, 28–32.

[35] Hall-Stoodley, L., Keevil, C.W., and Lappin-Scott, H.M. (1999) Mycobacterium fortuitum and Mycobacterium chelonae biofilm formation under high and low nutrient conditions. J. Appl. Microbiol. 85, 60–69.

[36] Marsollier, L., Brodin, P., Jackson, M., Kordulakova, J., Tafelmeyer, P., et al. (2007) Impact of Mycobacterium ulcerans biofilm on transmissibility to ecological niches and Buruli ulcer pathogenesis. PLoS Pathog. 3, e62.

[37] Rhoades, E. R., Archambault, A. S., Greendyke, R., Hsu, F. F., Streeter, C., et al. (2009) Mycobacterium abscessus Glycopeptidolipids mask underlying cell wall phosphatidyl-myo-inositol mannosides blocking induction of human macrophage TNF-alpha by preventing interaction with TLR2. J. Immunol. 183, 1997–2007.

[38] Johansen, T. B., Agdestein, A., Olsen, I., Nilsen, S. F., Holstad, G., et al. (2009) Biofilm formation by Mycobacterium avium isolates originating from humans, swine and birds. BMC Microbiol. 9, 159.

[39] Adetunji, V. O., Kehinde, A. O., Bolatito, O. K., and Chen, J. (2014) Biofilm formation by Mycobacterium bovis: influence of surface kind and temperatures of sanitizer treatments on biofilm control. Biomed Res. Int. 2014, 210165.

[40] Falkinham J. O. III. (2002) Nontuberculous mycobacteria in the environment. Clin. Chest Med. 23, 529–551.

[41] Ojha, A. and Hatfull, G. F. (2007) The role of iron in Mycobacterium smegmatis biofilm formation: the exochelin siderophore is essential in limiting iron conditions for biofilm formation but not for planktonic growth. Mol. Microbiol. 66, 468–483.

[42] Ramsugit, S., Guma, S., Pillay, B., Jain, P., Larsen, M. H., et al. (2013) Pili contribute to biofilm formation in vitro in Mycobacterium tuberculosis. Antonie van Leeuwenhoek 104, 725–735.

[43] Ojha, A. K., Baughn, A. D., Sambandan, D., Hsu, T., Trivelli, X., et al. (2008) Growth of Mycobacterium tuberculosis biofilms containing free mycolic acids and harbouring drug-tolerant bacteria. Mol. Microbiol. 69, 164–174.
[44] Recht, J. and Kolter, R. (2001) Glycopeptidolipid acetylation affects sliding motility and biofilm formation in Mycobacterium smegmatis. J. Bacteriol. 183, 5718–5724.
[45] Ojha, A. K., Trivelli, X., Guerardel, Y., Kremer, L., and Hatfull, G. F. (2010) Enzymatic hydrolysis of trehalose dimycolate releases free mycolic acids during mycobacterial growth in biofilms. J. Biol. Chem. 285, 17380–17389.
[46] Nguyen, K. T., Piastro, K., Gray, T. A., and Derbyshire, K. M. (2010) Mycobacterial biofilms facilitate horizontal DNA transfer between strains of Mycobacterium smegmatis. J. Bacteriol. 192, 5134–5142.
[47] Kulka, K., Hatfull, G., and Ojha, A. K. (2012) Growth of Mycobacterium tuberculosis biofilms. J. Vis. Exp. 60, e3820, doi:10.3791/3820.
[48] Pang, J. M., Layre, E., Sweet, L., Sherrid, A., Moody, D. B., et al. (2012) The polyketide Pks1 contributes to biofilm formation in Mycobacterium tuberculosis. J. Bacteriol. 194, 715–721.
[49] Ross, P., Weinhouse, H., Aloni, Y., Michaeli, D., Weinberger-Ohana, P., et al. (1987) Regulation of cellulose synthesis in Acetobacter xylinum by cyclic diguanylic acid. Nature 325, 279–281.
[50] Gupta, K., Kumar, P., and Chatterji, D. (2010) Identification, activity and disulfide connectivity of C-di-GMP regulating proteins in Mycobacterium tuberculosis. PLoS One 5, e15072.
[51] Sharma, I. M., Prakash, S., Dhanaraman, T., and Chatterji, D. (2014) Characterization of a dual active enzyme, DcpA, involved in cyclic-di-guanosine monophosphate turnover in Mycobacterium smegmatis. Microbiology 160, 2304–2314.
[52] Li, W. and He, Z. G. (2012) LtmA, a novel cyclic di-GMP-responsive activator, broadly regulates the expression of lipid transport and metabolism genes in Mycobacterium smegmatis. Nucleic Acids Res. 40, 11292–11307.
[53] Hong, Y., Zhou, X., Fang, H., Yu, D., Li, C., et al. (2013) Cyclic di-GMP mediates Mycobacterium tuberculosis dormancy and pathogenecity. Tuberculosis (Edinb) 93, 625–634.
[54] Flores-Valdez, M. A., Aceves-Sanchez, M. J., Montero-Perez, S. A., Sanchez-Lopez, A. D., Gutierrez-Pabello, J. A., et al. (2012) Vaccination of mice with recombinant bacille Calmette-Guerin harboring Rv1357c protects similarly to native BCG. Int. J. Tuberc. Lung Dis. 16, 774–776.
[55] Cui, T., Zhang, L., Wang, X., and He, Z. G. (2009) Uncovering new signaling proteins and potential drug targets through the interactome analysis of Mycobacterium tuberculosis. BMC Genomics 10, 118.
[56] Rao, F., Pasunooti, S., Ng, Y., Zhuo, W., Lim, L., et al. (2009) Enzymatic synthesis of c-di-GMP using a thermophilic diguanylate cyclase. Anal. Biochem. 389, 138–142.
[57] Zahringer, F., Massa, C., and Schirmer, T. (2011) Efficient enzymatic production of the bacterial second messenger c-di-GMP by the diguanylate cyclase YdeH from E. coli. Appl. Biochem. Biotechnol. 163, 71–79.
[58] Gaffney, B. L. and Jones, R. A. (2012) One-flask synthesis of cyclic diguanosine monophosphate (c-di-GMP). Curr. Protoc. Nucleic Acid Chem. 14, 11–17.
[59] Paul, R., Weiser, S., Amiot, N. C., Chan, C., Schirmer, T., et al. (2004) Cell cycle-dependent dynamic localization of a bacterial response regulator with a novel di-guanylate cyclase output domain. Genes Dev. 18, 715–727.
[60] Paul, R., Abel, S., Wassmann, P., Beck, A., Heerklotz, H., et al. (2007) Activation of the diguanylate cyclase PleD by phosphorylation-mediated dimerization. J. Biol. Chem. 282, 29170–29177.
[61] Tischler, A. D. and Camilli, A. (2004) Cyclic diguanylate (c-di-GMP) regulates Vibrio cholerae biofilm formation. Mol. Microbiol. 53, 857–869.
[62] Ryan, R. P., Fouhy, Y., Lucey, J. F., Crossman, L. C., Spiro, S., et al. (2006) Cell-cell signaling in Xanthomonas campestris involves an HD-GYP domain protein that functions in cyclic di-GMP turnover. Proc. Natl. Acad. Sci. USA 103, 6712–6717.
[63] Rao, F., Qi, Y., Chong, H. S., Kotaka, M., Li, B., et al. (2009) The functional role of a conserved loop in EAL domain-based cyclic di-GMP-specific phosphodiesterase. J. Bacteriol. 191, 4722–4731.
[64] Sawai, H., Yoshioka, S., Uchida, T., Hyodo, M., Hayakawa, Y., et al. (2010) Molecular oxygen regulates the enzymatic activity of a heme-containing diguanylate cyclase (HemDGC) for the synthesis of cyclic di-GMP. Biochim. Biophys. Acta 1804, 166–172.
[65] He, Y. W., Boon, C., Zhou, L., and Zhang, L. H. (2009) Co-regulation of Xanthomonas campestris virulence by quorum sensing and a novel two-component regulatory system RavS/RavR. Mol. Microbiol. 71, 1464–1476.
[66] Simm, R., Morr, M., Remminghorst, U., Andersson, M., and Romling, U. (2009) Quantitative determination of cyclic diguanosine monophosphate concentrations in nucleotide extracts of bacteria by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry. Anal. Biochem. 386, 53–58.
[67] Yang, F., Tian, F., Sun, L., Chen, H., Wu, M., et al. (2012) A novel two-component system PdeK/PdeR regulates c-di-GMP turnover and virulence of Xanthomonas oryzae pv. oryzae. Mol. Plant Microbe Interact. 25, 1361–1369.
[68] De, N., Pirruccello, M., Krasteva, P. V., Bae, N., Raghavan, R. V., et al. (2008) Phosphorylation-independent regulation of the diguanylate cyclase WspR. PLoS Biol. 6, e67.
[69] Wassmann, P., Chan, C., Paul, R., Beck, A., Heerklotz, H., et al. (2007) Structure of BeF3- -modified response regulator PleD: implications for diguanylate cyclase activation, catalysis, and feedback inhibition. Structure 15, 915–927.
[70] Chan, C., Paul, R., Samoray, D., Amiot, N. C., Giese, B., et al. (2004) Structural basis of activity and allosteric control of diguanylate cyclase. Proc. Natl. Acad. Sci. USA 101, 17084–17089.
[71] Nakayama, S., Roelofs, K., Lee, V. T., and Sintim, H. O. (2012) A C-di-GMP-proflavine-hemin supramolecular complex has peroxidase activity-implication for a simple colorimetric detection. Mol. Biosyst. 8, 726–729.
[72] Nakayama, S., Kelsey, I., Wang, J., Roelofs, K., Stefane, B., et al. (2011) Thiazole orange-induced c-di-GMP quadruplex formation facilitates a simple fluorescent detection of this ubiquitous biofilm regulating molecule. J. Am. Chem. Soc. 133, 4856–4864.
[73] Stelitano, V., Brandt, A., Fernicola, S., Franceschini, S., Giardina, G., et al. (2013) Probing the activity of diguanylate cyclases and c-di-GMP phosphodiesterases in real-time by CD spectroscopy. Nucleic Acids Res. 41, e79.
[74] Gu, H., Furukawa, K., and Breaker, R. R. (2012) Engineered allosteric ribozymes that sense the bacterial second messenger cyclic diguanosyl 5′-monophosphate. Anal. Chem. 84, 4935–4941.
[75] Sharma, I. M., Dhanaraman, T., Mathew, R., and Chatterji, D. (2012) Synthesis and characterization of a fluorescent analogue of cyclic di-GMP. Biochemistry 51, 5443–5453.
[76] Roembke, B. T., Zhou, J., Zheng, Y., Sayre, D., Lizardo, A., et al. (2014) A cyclic dinucleotide containing 2-aminopurine is a general fluorescent sensor for c-di-GMP and 3′,3′-cGAMP. Mol. Biosyst. 10, 1568–1575.
[77] Ho, C. L., Koh, S. L., Chuah, M. L., Luo, Z., Tan, W. J., et al. (2011) Rational design of fluorescent biosensor for cyclic di-GMP. Chembiochem 12, 2753–2758.
[78] Christen, M., Kulasekara, H. D., Christen, B., Kulasekara, B. R., Hoffman, L. R., et al. (2010) Asymmetrical distribution of the second messenger c-di-GMP upon bacterial cell division. Science 328, 1295–1297.
[79] Ho, C. L., Chong, K. S., Oppong, J. A., Chuah, M. L., Tan, S. M., et al. (2013) Visualizing the perturbation of cellular cyclic di-GMP levels in bacterial cells. J. Am. Chem. Soc. 135, 566–569.
[80] Rybtke, M. T., Borlee, B. R., Murakami, K., Irie, Y., Hentzer, M., et al. (2012) Fluorescence-Based Reporter for Gauging Cyclic Di-GMP Levels in Pseudomonas aeruginosa. Appl. Environ. Microbiol. 78, 5060–5069.
[81] De, N., Navarro, M. V., Raghavan, R. V., and Sondermann, H. (2009) Determinants for the activation and autoinhibition of the diguanylate cyclase response regulator WspR. J. Mol. Biol. 393, 619–633.
[82] Christen, M., Christen, B., Allan, M. G., Folcher, M., Jeno, P., et al. (2007) DgrA is a member of a new family of cyclic diguanosine monophosphate receptors and controls flagellar motor function in Caulobacter crescentus. Proc. Natl. Acad. Sci. USA 104, 4112–4117.

[83] Duvel, J., Bertinetti, D., Moller, S., Schwede, F., Morr, M., et al. (2012) A chemical proteomics approach to identify c-di-GMP binding proteins in Pseudomonas aeruginosa. J. Microbiol. Methods 88, 229–236.

[84] Roelofs, K. G., Wang, J., Sintim, H. O., and Lee, V. T. (2011) Differential radial capillary action of ligand assay for high-throughput detection of protein-metabolite interactions. Proc. Natl. Acad. Sci. USA 108, 15528–15533.

[85] Rebecca, M. C., Campeotto, I., Jeganathan, T., Roelofs, K. G., Lee, V. T., et al. (2013) Systematic identification of conserved bacterial c-di-AMP receptor proteins. Proc. Natl. Acad. Sci. USA 110, 9084–9089.

[86] Donaldson, G. P., Roelofs, K. G., Luo, Y., Sintim, H. O., and Lee, V. T. (2012) A rapid assay for affinity and kinetics of molecular interactions with nucleic acids. Nucleic Acids Res. 40, e48.

[87] Ryu, M. H. and Gomelsky, M. (2014) Near-infrared light responsive synthetic c-di-GMP module for optogenetic applications. ACS Synth. Biol. 3, 802–810.

[88] Ohana, P., Delmer, D. P., Carlson, R. W., Glushka, J., Azadi, P., et al. (1998) Identification of a novel triterpenoid saponin from Pisum sativum as a specific inhibitor of the diguanylate cyclase of Acetobacter xylinum. Plant Cell Physiol. 39, 144–152.

[89] Wang, J. X., Zhou, J., Donaldson, G. P., Nakayama, S., Yan, L., et al. (2011) Conservative change to the phosphate moiety of cyclic diguanylic monophosphate remarkably affects its polymorphism and ability to bind DGC, PDE, and PilZ proteins. J. Am. Chem. Soc. 133, 9320–9330.

[90] Antoniani, D., Bocci, P., Maciag, A., Raffaelli, N., and Landini, P. (2010) Monitoring of diguanylate cyclase activity and of cyclic-di-GMP biosynthesis by whole-cell assays suitable for high-throughput screening of biofilm inhibitors. Appl. Microbiol. Biotechnol. 85, 1095–1104.

[91] Sambanthamoorthy, K., Sloup, R. E., Parashar, V., Smith, J. M., Kim, E. E., et al. (2012) Identification of small molecules that antagonize diguanylate cyclase enzymes to inhibit biofilm formation. Antimicrob. Agents Chemother. 56, 5202–5211.

[92] Lieberman, O. J., Orr, M. W., Wang, Y., and Lee, V. T. (2014) High-throughput screening using the differential radial capillary action of ligand assay identifies ebselen as an inhibitor of diguanylate cyclases. ACS Chem. Biol. 9, 183–192.

[93] Hentzer, M., Riedel, K., Rasmussen, T. B., Heydorn, A., Andersen, J. B., et al. (2002) Inhibition of quorum sensing in Pseudomonas aeruginosa biofilm bacteria by a halogenated furanone compound. Microbiology 148, 87–102.

[94] Kalia, V. C. (2013) Quorum sensing inhibitors: an overview. Biotechnol. Adv. 31, 224–245.

[95] Bharati, B. K., Swetha, R. K., and Chatterji, D. (2012) Identification and characterization of starvation induced msdgc-1 promoter involved in the c-di-GMP turnover. Gene 528, 99–108.

[96] Dalebroux, Z. D. and Swanson, M. S. (2012) ppGpp: magic beyond RNA polymerase. Nat. Rev. Microbiol. 10, 203–212.