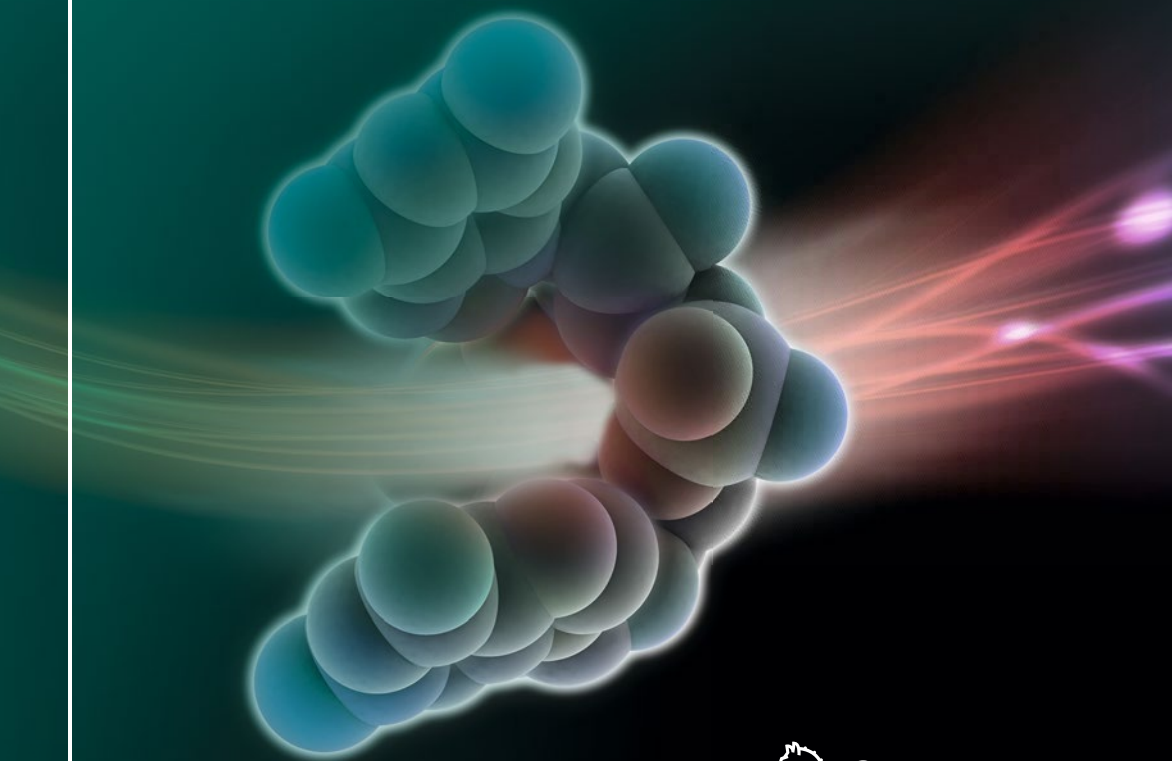
Shan-Ho Chou · Nicolas Guiliani
Vincent T. Lee · Ute Römling *Editors*

Microbial Cyclic Di-Nucleotide Signaling



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Cover Figure Caption: A space filling model of cyclic di-GMP, the first ever discovered cyclic di-nucleotide second messenger. The figure highlights the central and multifaceted role of this versatile second messenger as described in various chapters of the book. 2D render by Giorgio Giardina, Department of Biochemical Sciences, Sapienza University of Rome, Italy.

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Foreword

Role and Importance of Cyclic Di-Nucleotide Second Messenger Signaling

The discovery of nucleotide-based signaling molecules dates back to the work on the action of hormones carried out by Earl Sutherland in the 1950s of the last century. Sutherland had discovered that hormones like epinephrine, which act as global (first) messengers in the human body by conveying information between cells and organs, regulate cellular physiology via the production of a second, internal messenger. The observation that epinephrine never enters the cell but instead stimulates the formation of a distinct chemical substance in the cell membrane established a novel principle in cell biology. This was the birth of the second messenger concept. Sutherland painstakingly dissected this signaling cascade and identified cAMP as the new substance that serves as an intermediate during the function of the hormone (Sutherland, Nobel lecture 1971). Shortly after the discovery of cAMP, cGMP was isolated from the urine of rabbits and was later shown to play a role as second messenger, similar to cAMP. This far-reaching discovery was awarded with the Noble Prize in Physiology/Medicine in 1971.

The discovery of the larger, symmetric and possibly evolutionarily more ancient cyclic di-nucleotides (CDNs) shows some striking parallels to the pioneering work on cAMP. Moshe Benziman and his colleagues discovered cyclic di-GMP, the first representative of this family of signaling molecules, through meticulous biochemical experimentation carried out three decades after Sutherlands' findings. Driven by their goal to optimize the biotechnological production of cellulose, they identified an activator of cellulose synthase in the late 1980s that contained two GMP moieties linked by 3′–5′ bonds [1]. Activation of exopolysaccharide biosynthesis by cyclic di-GMP as discovered by Benziman turned out to be a fundamental principle in many bacteria and is one of the topics that is covered in this book in detail.

Moshe Benziman's pioneering contributions also prepared the ground for the identification and characterization of diguanylate cyclases and phosphodiesterases,

the catalysts responsible for the "makers and breakers" of cyclic di-GMP. Although it took more than a decade after the original discovery of cyclic di-GMP, genetic and biochemical studies eventually led to the identification of several large domain families, GGDEF, EAL, and HD-GYP, as the catalytic units of cyclic di-GMP metabolism [2–4]. This opened up rigorous structure/function analyses of these enzymes uncovering catalytic mechanisms, regulatory principles, and feedback control. Today, cyclic di-GMP is the front-runner of bacterial CDNs, with knowledge related to this compound being most advanced in this field. This is clearly due to its timely discovery, but also its broad distribution in the bacterial world, which includes several important model organisms of microbiology, cell biology, and infection biology.

But the discovery of cyclic di-GMP was only the first of a series of breakthroughs that gradually expanded the catalog of CDNs. Cyclic di-AMP was discovered in 2008 [5], cyclic AMP-GMP in 2012 [6], and mammalian cGAMP in 2013 [7]. The most recent discovery of a diverse range of novel di- and trinucleotides [8] argues that the chemical repertoire of these compounds is still incomplete and additional CDNs and related compounds might await their identification. The universal nature of CDNs together with their biomedical relevance has generated strong interest in this emerging field of research. In particular, the interaction of bacterial and mammalian CDNs with the human innate immune system and with inflammatory processes has attracted the interest of the pharmaceutical industry and drug makers in these compounds. Modulating the innate immune response with agonists or antagonists of CDNs is currently being looked at as a promising approach in the immunotherapy of cancer, viral infections, or autoimmune diseases [9]. At the same time, the important role of cyclic di-GMP and cyclic di-AMP for vital processes in bacteria, like virulence, biofilm formation, or stress response, also puts bacterial CNDs up for future scrutiny with the goal to develop novel antimicrobial treatment strategies. This is discussed in detail in one of the final chapters of this book.

The initial chapters of this book recapitulate the current knowledge of the "make and break" of bacterial CDNs, with a focus on cyclic di-GMP and cyclic di-AMP. By discussing the structural and catalytic properties of these enzymes and their sensory domains and signal input mechanisms, this part of the book retraces the important discoveries that have led to the current understanding of these potent and fascinating bacterial signaling molecules. The following chapters describe important cellular processes regulated by CDNs both in environmental bacteria like cyanobacteria, *Myxococcus xanthus*, or *Bacillus subtilis* and in important human pathogens like *Vibrio cholerae*, *Streptococcus pneumoniae*, or *Mycobacterium tuberculosis*. One of the central processes regulated by cyclic di-GMP is the transition between individual free-swimming bacteria in their planktonic state and surface-attached bacterial consortia engulfed in a self-produced matrix. The multifaceted behavioral changes that bacteria undergo when establishing biofilms or when resuming motility to escape from such communities demand coordinated multilevel control of various cellular processes.

CDNs are highly versatile signaling molecules with a wide range of physiological functions in bacteria. They can interfere with bacterial growth and behavior at

multiple levels ranging from gene expression to controlling the activity, interaction, stability, or cellular dynamics of proteins. Through this, CDNs can change bacterial physiology rapidly and globally and integrate numerous environmental and internal cues with other global regulatory networks. The observation that CDN networks respond to external signaling compounds like quorum-sensing molecules [10] makes them bona fide second messengers in the true conceptual sense originally established by Sutherland. In many bacteria, regulatory networks involving CDNs are highly complex, leaving countless interesting and relevant facets of these molecules to be discovered. This book provides an excellent compendium of the field's state of the art and thus represents the ideal launchpad for such endeavors.

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Urs Jenal

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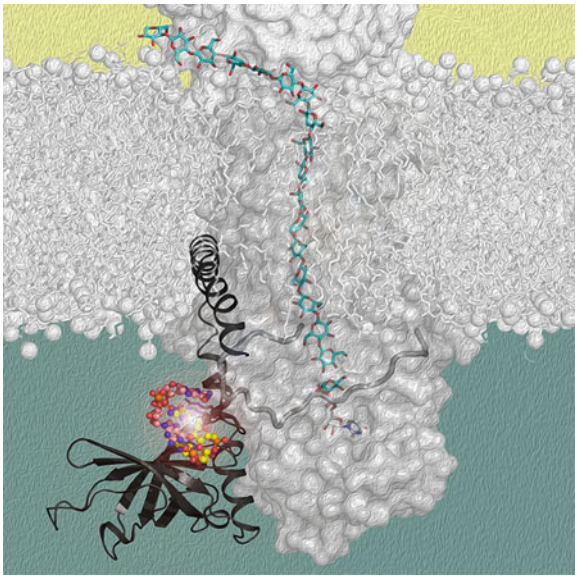
Preface

The discovery of the allosteric activator of a bacterial cellulose synthase and the enzymes involved in its synthesis and degradation was the result of rigorous scientific observations, persistence, recognizing the reality of the practicability of scientific approaches at that time, and, last but not least, hard work. A model organism, the fruit-degrading environmental bacterium *Acetobacter xylinum* (now reassigned as *Komagataeibacter xylinus*) producing high amounts of the polysaccharide cellulose, was needed as plants were too complex to be methodologically approached adequately at this time. The observation of the discrepancy between in vitro and in vivo cellulose production added the next puzzle piece indicating that a factor significantly enhancing the in vivo performance of the cellulose synthase enzyme was missing.



Many bacterial exopolysaccharides are synthesized by membrane-integrated processive glycosyltransferases. The enzymes catalyze polymer synthesis and membrane translocation. Cyclic di-GMP is an allosteric activator of exopolysaccharide biosynthesis and can bind directly to either the synthase or additional regulatory subunits associated with it. Shown is the cyclic di-GMP-activated state of the BcsA-B cellulose synthase complex. Cyclic di-GMP is shown in ball and sticks, cellulose as cyan and red sticks, and UDP-glucose at the enzyme's active site as sticks in gray and cyan for carbon atoms of the UDP and glucosyl moieties, respectively, by Jochen Zimmer, University of Virginia School of Medicine, Charlottesville, USA.

The wider impact of the outcome of this groundbreaking work by the Moshe Benziman group, the identification of cyclic di-GMP as the allosteric regulator of the cellulose synthase ("Regulation of cellulose synthesis in *Acetobacter xylinum* by cyclic diguanylic acid," Ross et al., Nature, 1987, 325, 279–281) and the identification of the enzymes that synthesize and degrade cyclic di-GMP ("Three *cdg* operons control cellular turnover of cyclic di-GMP in *Acetobacter xylinum*: genetic organization and occurrence of conserved domains in isoenzymes," Tal et al., Journal of Bacteriology, 1998, 180, 4416–4425), was scientific serendipity.

Gradually, the discovery of cyclic di-GMP and subsequent independently upcoming studies have changed our view on fundamental aspects of bacteriology. The volume of common bacterial cells is less than 10,000 the volume of a eukaryotic cell. In combination with the knowledge on second messengers, which at that time was more or less restricted to cAMP signaling in *Escherichia coli* with ppGpp called an alarmone, bacteria were simply thought not to require more complex diffusible second messenger systems. Second, bacteria were looked upon as being mostly unicellular organisms that occasionally and randomly form multicellular communities. Today we consider complex cyclic di-GMP signaling networks to modulate the transition between the association of self-replicating cells into multicellular communities and motility with all amalgamated morphological and physiological consequences.

The authoritative chapters in this book on "Microbial Cyclic Di-Nucleotide Signaling" provide an up-to-date comprehensive snapshot of our current knowledge on cyclic di-nucleotide-based second messenger signaling. Book chapters cover the three current cyclic di-nucleotide second messengers known to date in bacteria: well-investigated cyclic di-GMP (Chaps. 6, 16, and 23) and cyclic di-AMP (Chaps. 10, 11, and 17) and also recently discovered cyclic GAMP (Chaps. 34 and 35). The physiological roles of those ubiquitous second messengers in pathogenic and environmental Gram-negative and Gram-positive bacteria, including the first-discovered function of cyclic di-GMP in activation of biosynthesis of exopolysaccharides cellulose and alginate (Chaps. 13 and 14), are broadly presented in various chapters dedicated to individual genera or species. The global human pathogens *Mycobacterium tuberculosis* (Chaps. 1 and 26), *Vibrio cholerae* (Chap. 22), *Salmonella typhimurium* (Chap. 24), and *Streptococcus pneumoniae* (Chap. 27), the facultative pathogen *Pseudomonas aeruginosa* (Chap. 28), global plant pathogens as exemplified with *Xanthomonas campestris* (Chap. 25) and *Burkholderia* spp. (Chap. 30),

and the omnipresent *Bacillus* (Chap. 15), but also environmentally important photoautotrophic cyanobacteria (Chap. 19), multicellular *Myxococcus xanthus* (Chap. 18), and chemolithotrophic *Acidithiobacillus* (Chap. 21) are some of the representatives of the microbial kingdom that are described. The different aspects of bacterial physiology directed by cyclic di-nucleotide signaling systems such as biofilm formation and dispersal (Chap. 31), motility, virulence, fundamental metabolism (Chaps. 20 and 29), and osmohomeostasis are discussed in detail in the context of different microorganisms.

Cyclic di-nucleotide signaling systems are frequently horizontally transferred within the bacterial kingdom (Chap. 37) and, occasionally, even to eukaryotes (Chap. 32). Furthermore, book chapters dissectively describe the sophisticated catalytic activities of the multiple turnover enzymes and their regulation by external and intrinsic signals (Chaps. 2, 3, 4, 5 and 9). The (mostly) experimental discovery of the vast variety of effectors that cannot be recognized by bioinformatics, their metabolic and physiological consequences, and the contribution of the cyclic di-nucleotide second messenger networks to population heterogeneity are addressed by distinctly dedicated chapters (Chaps. 7, 8 and 12). Strategies for potential anti-biofilm therapies are also discussed (Chap. 33). Last but not least, novel honorary cyclic nucleotides such as 2′–3′ cyclic nucleotides, around for decades, with starting-to-be unraveled functions, for example in biofilm formation, are addressed (Chap. 36). Thus, the editors are confident that the collective contributions to this book will serve not only as a source of information, but also as inspiration to apply and expatiate on strategies to investigate currently known as well as upcoming cyclic di-nucleotide second messenger signaling systems.

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The editors would like to acknowledge the work of all individuals who contributed to the elucidation of the biological role of cyclic di-nucleotide signaling, irrespectively whether or not they contributed as authors to this book. The editors apologize that due to space constraints not all the excellent work done on cyclic di-nucleotide signaling could be considered for a contribution to this book. Appreciation also to those individuals who took the time and effort to review a book chapter.

Contents

Part I
Biochemistry/Structural Biology—Enzymes

Chapter 1
Cyclic Dinucleotide Signaling in Mycobacteria

Anushya Petchiappan, Avisek Mahapa, and Dipankar Chatterji

Abstract The success of a pathogen depends on its ability for long-term survival under hostile environmental conditions. In this regard, second nucleotide messengers like cyclic di-GMP and cyclic di-AMP play a major role. In mycobacteria, cyclic di-GMP has been shown to be involved in several fundamental phenotypes like cell division, biofilm formation, and antibiotic resistance. Compared to cyclic di-GMP, there is little information available regarding the physiological role of cyclic di-AMP in mycobacteria. However, both these second messengers are associated with the activation of immune response in the host. Most antibiotics target the key pathways of the central dogma, but bacteria evolve to become resistant to them. Therefore, auxiliary pathways, like the stress response pathways, can be putative targets for the development of novel therapeutics. *Mycobacterium smegmatis* encodes a single gene for cyclic di-GMP metabolism and a single gene each for cyclic di-AMP synthesis and hydrolysis. This makes it an ideal system to gain a deeper insight into the phenotypes affected by cyclic dinucleotides in mycobacteria. In this chapter, we have summarized the recent advances in the field of cyclic dinucleotide signaling in mycobacteria with a focus on their metabolism, regulation of activity, and the diversity of phenotypes governed by them. In the future, the research needs to address the important questions regarding the crosstalk between the second messengers as well as identification of new second messengers in mycobacteria.

Keywords Mycobacteria · Cyclic dinucleotides · Stress response · Crosstalk

Abbreviations

cAMP	Cyclic adenosine monophosphate
cyclic di-AMP	Cyclic di-adenosine monophosphate
cyclic di-GMP	Cyclic di-guanosine monophosphate

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CDN	Cyclic dinucleotide
cGAMP	Cyclic GMP-AMP
cGMP	Cyclic guanosine monophosphate
DAC	Diadenylate cyclase
DcpA	Diguanylate cyclase and phosphodiesterase A
DGC	Diguanylate cyclase
DisA	DNA integrity scanning protein A
DNC	Dinucleotide cyclase
HPLC	High-performance liquid chromatography
IFN	Interferon
LC MS/MS	Liquid chromatography with tandem mass spectrometry
(p)ppGpp	Guanosine pentaphosphate and tetraphosphate
PAMP	Pathogen-associated molecular pattern
PDE	Phosphodiesterase
pGpG	5′-linear dimeric GMP
RadA	Radiation-sensitive gene A
SPR	Surface plasmon resonance
STING	Stimulator of interferon genes

1.1 Introduction

Adaptability is the key to the survival of an organism in a dynamic environment. Signal transduction pathways enable organisms to sense changes in the environment and respond to them. Bacteria utilize dedicated nucleotide derivatives as "second messengers" to modulate the cellular response to environmental stimuli (first messengers) by relaying the signal from sensor molecules to the cellular targets. The repertoire of bacterial nucleotide second messengers known so far includes cyclic AMP (cAMP), cyclic GMP (cGMP), guanosine pentaphosphate and tetraphosphate ((p)ppGpp), cyclic di-GMP, cyclic di-AMP, and cyclic GMP-AMP (cGAMP) (Fig. 1.1) [1–7]. These ubiquitous molecules regulate cellular pathways related to replication, transcription, translation, cellular morphology, metabolism, and DNA repair among others. Additionally, they also play a major role in virulence, biofilm formation, persistence, quorum sensing, and antibiotic tolerance. Pathogens have to withstand several stress conditions inside the host including nutrient starvation, acidic pH, hypoxia, temperature fluctuation, genotoxic stress, reactive nitrogen intermediates, oxidative stress, and cell wall stress [8, 9]. Nucleotide second messengers are employed by bacteria under such hostile conditions to ensure cell survival, thus highlighting their importance in pathogenesis.

Stress response pathways have been linked to bacterial growth and persistence [10, 11]. Most antimicrobial agents function well only against rapidly growing cells and have decreased efficacy towards cells in stationary or dormant phase. The targets of most antibiotics are related to the central dogma and bacteria evolve to develop resistance against these antibiotics. Auxiliary pathways, like the second messenger signaling pathways, would make ideal targets for the development of new

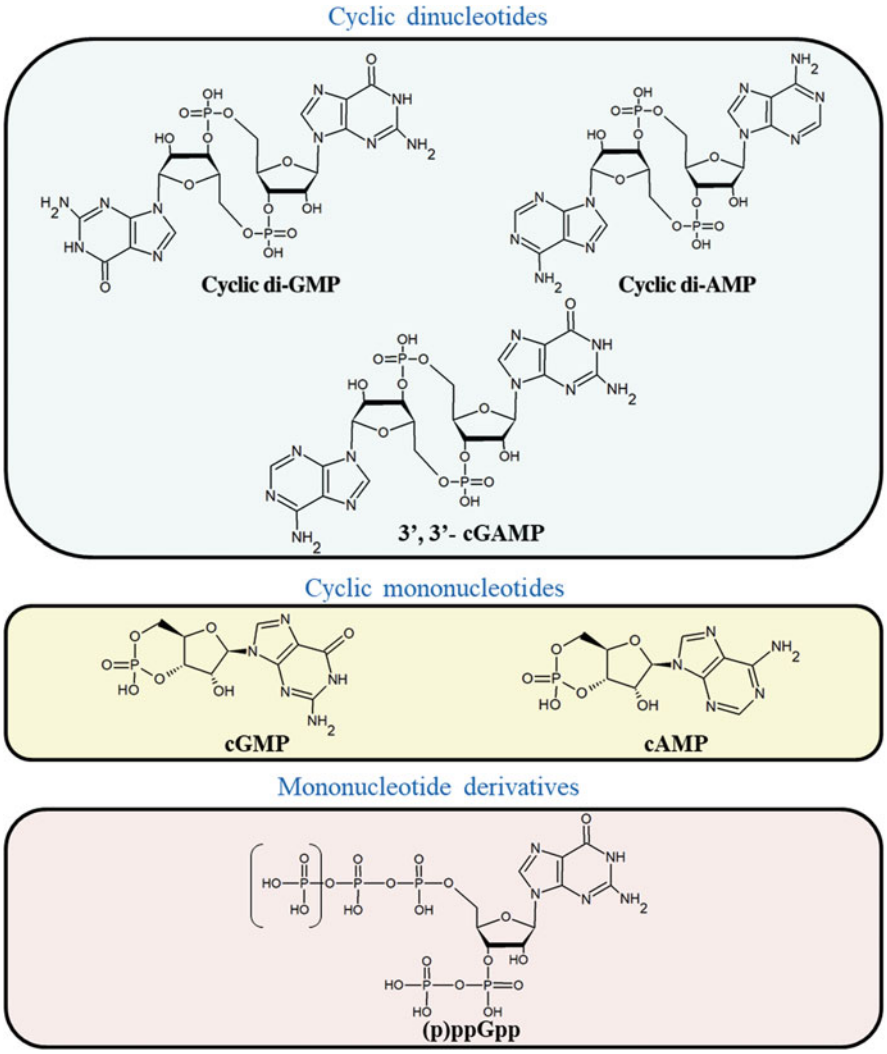

Fig. 1.1 Second messengers in bacteria. Cyclic di-GMP and cyclic di-AMP are cyclic di-nucleotide signaling molecules composed of two molecules of GMP and AMP, respectively. cGAMP is hybrid dinucleotide made of GMP and AMP. Cyclic AMP is a classical messenger found in most bacteria. The other cyclic nucleotide signaling molecule is cGMP. (p)ppGpp is synthesized from GTP or GDP and regulates stringent response in bacteria

antimicrobials as these would inhibit stress survival of bacteria and function against nondividing bacteria as well. With the ever-increasing threat of antibiotic-resistant infections, the study of second messenger signaling in bacteria is of paramount importance.

Though present in all kingdoms of life, there are species-specific differences in how second messenger signaling orchestrates cell behavior [1, 12]. Mycobacteria are Gram-positive and belong to the phylum Actinobacteria. Mycobacteria are of extreme medical relevance as they include highly relevant pathogens of mankind like *Mycobacterium tuberculosis* and *Mycobacterium leprae*. Other clinically relevant species include the non-tuberculous species like *Mycobacterium avium*, *Mycobacterium abscessus*, *Mycobacterium kansasii*, and *Mycobacterium fortuitum*. Most mycobacterial species are slow-growing, difficult to grow under laboratory conditions, and also challenging to manipulate genetically. A notable feature of mycobacterial species like *M. tuberculosis* is their complex cell envelope which is lipid-rich, is highly resistant to lysis, and has low permeability to several antibiotics [13]. More than a million people across the world fall prey to tuberculosis infections every year [14]. The success of *M. tuberculosis* as a pathogen is mainly due to its ability for long-term survival in a dormant stage inside human beings and its host immune evasion strategies. The global rise in the drug-resistant tuberculosis infections adds to the complexity of the problem. Second messengers aid mycobacterial survival under stress and contribute to their pathogenicity and antibiotic tolerance [15, 16]. Thus, second messenger signaling may prove to be the Achilles' heel for mycobacteria.

The role of classical second messengers cAMP and (p)ppGpp have been well documented in mycobacteria. In this chapter, we focus our attention on the cyclic dinucleotides (CDNs) of mycobacteria—cyclic di-GMP and cyclic di-AMP. Although cyclic di-GMP was first discovered in bacteria in 1987, its presence in mycobacteria was reported only in 2010 [5, 17, 18]. As a consequence, much less is known about cyclic di-GMP-related pathways in mycobacteria. Cyclic di-AMP was serendipitously discovered in bacteria more than two decades after cyclic di-GMP [6]. It was identified in mycobacteria in 2012 and its role in mycobacteria remains a relatively unexplored area [19]. Although it is reasonable to assume that the role of second messengers will be the same in all species of mycobacteria, conclusive evidence for the same is lacking.

Similar to other second messenger signaling pathways, CDN signaling is capable of integrating information from multiple extracellular cues and in turn transmitting them to a variety of cellular downstream targets [20]. A signaling pathway typically begins with the detection of the signal by a receptor followed by the synthesis of the CDN. The CDN subsequently binds and alters a downstream target, which could be either RNA or a protein, and results in a cellular response to the signal. We discuss each of these steps related to CDN signaling in mycobacteria and elaborate upon how the entire pathway is regulated within the cell. Much of the current literature available for CDN signaling in mycobacteria comes from studies carried out in *M. tuberculosis* or the model organism *Mycobacterium smegmatis*. The fortuitous presence of a single copy of a synthetase and hydrolase for each CDN in *M. smegmatis* genome makes it an ideal system for elucidating the CDN-associated cellular phenotypes. Furthermore, we attempt to identify the unresolved questions related to CDN signaling in mycobacteria which could be addressed by future research in the field.

1.2 Synthesis and Hydrolysis of CDNs

CDN signaling in bacteria can be initiated by any signal or stress like starvation, temperature or pH change, hypoxia, and DNA damage but the entire range of triggering signals for mycobacteria remains to be identified [21]. The intracellular levels of the signaling CDNs are modulated by distinct classes of enzymes—dinucleotide cyclases (DNCs) and phosphodiesterases (PDEs). Diguanylate cyclases (DGCs) and diadenylate cyclases (DACs) are responsible for the biosynthesis of cyclic di-GMP and cyclic di-AMP, respectively. On the other hand, PDEs are responsible for hydrolyzing these CDNs. The number of DNCs and PDEs varies across species but they all follow a similar catalytic mechanism. All DGCs bear a conserved GG(D/E)EF catalytic domain and synthesize cyclic di-GMP by the condensation of two GTP molecules [12, 22]. Many DGCs present in bacteria (including mycobacterial species like *M. tuberculosis*) also carry a regulatory GAF domain in addition to the GGDEF domain. The GAF domain can bind ligands like GDP to regulate the DGC activity [23]. Cyclic di-GMP-specific PDEs carry an EAL domain or a HD-HYP domain to hydrolyze cyclic di-GMP into the linear pGpG or two molecules of GMP, respectively.

DACs catalyze the synthesis of cyclic di-AMP from two molecules of AMP. There are five classes of DACs (DisA, CdaA, CdaS, CdaM, and CdaZ) which have been identified so far in bacteria [24]. Each one of them shares a common DAC (diadenylate cyclase) catalytic domain for cyclic di-AMP synthesis along with various regulatory domains. Four different types of cyclic di-AMP PDEs have been discovered till now—GdpP-type, DhhP-type, PgpH-type, and CdnP-type PDE. Each type of PDE has different domain architecture and catalyzes the conversion of cyclic di-AMP to either pApA or AMP. The types of DNCs and PDEs present in mycobacteria are described in detail in the subsequent section.

1.2.1 Cyclic di-GMP Metabolism

Two GGDEF domain containing proteins MSMEG_2916 (MSDGC-1, later renamed DcpA) and MSMEG_2774 (MSDGC-2) are encoded in the genome of *M. smegmatis* [18]. DcpA is a bifunctional multidomain protein containing a tandem arrangement of the catalytic GGDEF domain, EAL phosphodiesterase domain, and an N-terminal GAF domain. The GGDEF domain synthesizes cyclic di-GMP from GTP, whereas the EAL domain hydrolyzes it to pGpG. MSMEG_2774 is nonfunctional due to the presence of a mutation in the catalytic domain. No other cyclic di-GMP hydrolase is present in *M. smegmatis*. Thus, DcpA is the only enzyme responsible for cyclic di-GMP turnover in *M. smegmatis.* The ortholog of DcpA in *M. tuberculosis* is Rv1354c (MtbDGC) [17]. MtbDGC is also a bifunctional protein which can synthesize and hydrolyze cyclic di-GMP in vitro. Apart from MtbDGC, *M. tuberculosis* carries one more EAL domain containing PDE (Rv1357c or MtbPDE) to hydrolyze cyclic di-GMP to pGpG. *M. leprae* encodes three active

DGCs (ML1750c, ML1419c, and ML0397c) [25]. Among the DGCs, only ML1419c (DgcA) has been characterized and functionally analyzed. ML1750c is homologous to DcpA and Rv1354c. DgcA possesses three putative PAS-sensing domains, with two PAS domains containing conserved heme-binding sites hinting at a role related to oxygen tension or nitric oxide/carbon monoxide stress. *M. leprae* encodes an EAL domain containing PDE ML1752c (ortholog of Rv1357c), but its physiological functions are not known. In *Mycobacterium bovis* BCG, the gene BCG1416c encodes a cyclic di-GMP DGC, a homolog to Rv1354c in *M. tuberculosis* [26].

1.2.2 Cyclic di-AMP Metabolism

M. smegmatis possesses a single copy of DAC and PDE specific for the homeostasis of cyclic di-AMP. DisA (MSMEG_6080) is the sole cyclic di-AMP synthetase [27]. MsPDE (MSMEG_2630), the only PDE in *M. smegmatis*, consists of a DHH-DHHA1 domain and is able to convert cyclic di-AMP to pApA and AMP [28]. Similar to *M. smegmatis*, *M. tuberculosis* consists of one DAC, known as DisA (Rv3586) [19]. This is an ortholog of DisA from *Bacillus subtilis* which is a DNA integrity scanning protein [6]. DisA is capable of utilizing both ATP and ADP as a substrate for cyclic di-AMP synthesis in vitro. DAC from *M. tuberculosis* can also act as an ATPase or ADPase in vitro and this ATPase activity is suppressed by the DAC activity. The enzymatic activity occurs in a two-step mechanism and requires divalent metal ions such as Mg^{2+}, Mn^{2+}, or Co^{2+} [19, 29]. DisA has a conserved RHR motif, which interacts with ATP, and mutation in this motif severely affects the activity of this protein. Degradation of cyclic di-AMP in *M. tuberculosis* is carried out by Rv2837c (CnpB/CdnP), a DHH-DHHA1 domain containing phosphodiesterases [30, 31]. No GdpP-type PDE is present in *M. tuberculosis*. CnpB contains DxD motif, DHH motif, and GGGH motifs which are important for its activity. CnpB hydrolyzes cyclic di-AMP into AMP via a pApA intermediate in a metal-dependent reaction [29]. Structural and functional analysis suggested that CnpB is unable to distinguish between adenine and guanine and it can cleave cyclic di-NMPs other than cyclic di-AMP though at a lower rate [30]. Additionally, CnpB exhibits nanoRNase activity (3′–5′exonuclease) as well as a CysQ-like phosphatase (pAp hydrolysis) activity [32–34].

The domain architecture of some of the CDN synthetases and hydrolases of mycobacteria and their catalytic mechanism has been illustrated in Fig. 1.2. A list of DNCs and PDEs in mycobacteria has been presented in Table 1.1.

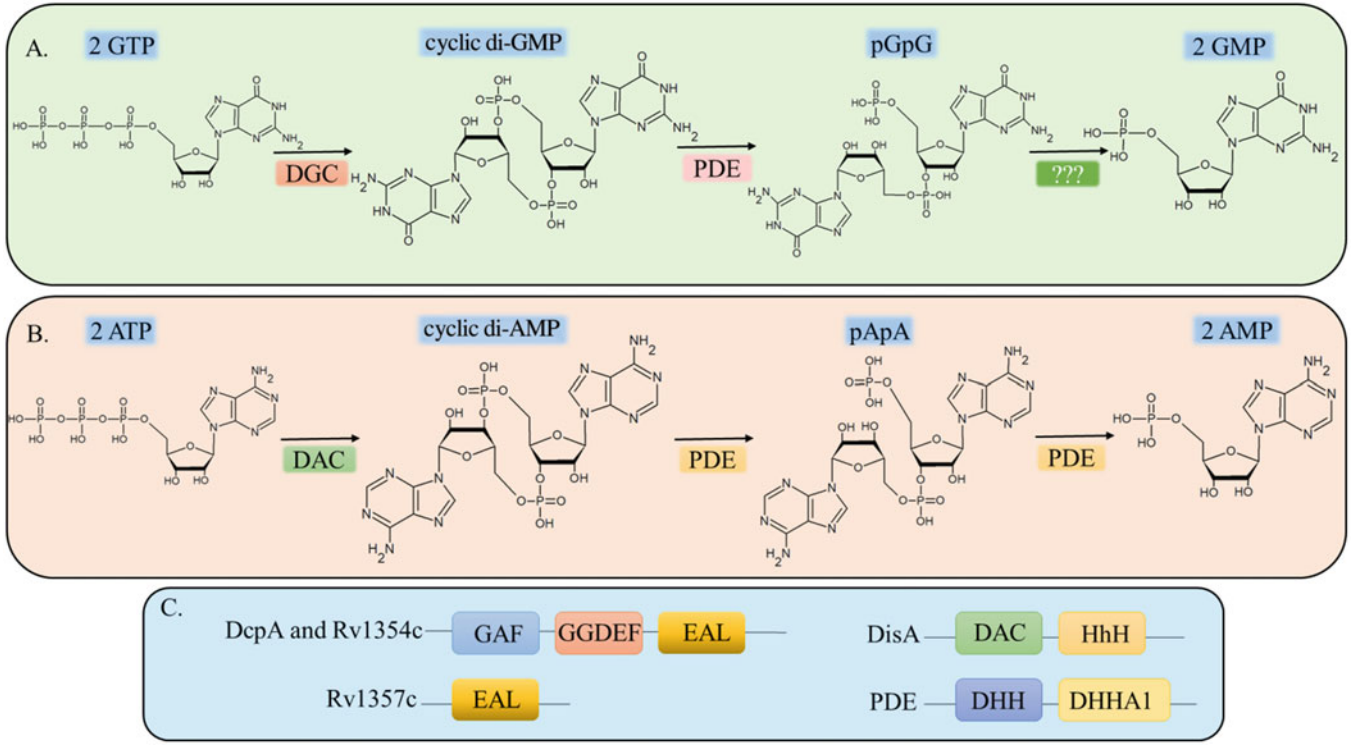

Fig. 1.2 CDN metabolism in mycobacteria. (**a**) Synthesis of cyclic di-GMP from two molecules of GTP by DGC and its subsequent hydrolysis to pGpG by a PDE is shown. The enzyme responsible for degradation of pGpG to GMP has not been identified so far. (**b**) Synthesis of cyclic di-AMP from two ATP molecules by DAC and its subsequent hydrolysis to pApA and two AMP molecules by a PDE is shown. (**c**) Domain architecture of some of the functionally characterized DNCs and PDEs is depicted. DcpA and Rv1354c are DGCs of *M. smegmatis* and *M. tuberculosis*, respectively. Both these proteins contain a GGDEF synthesis

1.3 CDN Effectors

Upon synthesis of the second messenger, effector binding generates a physiological response. As mentioned earlier, these effectors could be RNA or proteins. As these nucleotides modulate a variety of cellular phenotypes, a still growing wide range of proteins serves as receptors. This is facilitated by conformational adaptability of the CDNs, which can exist as monomers or dimers [20, 35]. Even the monomeric CDN can take a closed or open conformation. The binding of CDN could lead to a conformational change in the effector or even a change in oligomerization. Since the discovery of these CDNs, numerous targets have been pinpointed for them. In mycobacteria, however, the number of effectors identified so far remains limited. Cyclic di-GMP effectors mainly belong to the categories of PilZ domain containing proteins, mRNA riboswitches, transcription factors, proteins with degenerate GGDEF or EAL domains, and AAA+ ATPases [12, 20]. For example, dimeric cyclic di-GMP binds to the transcription factor VpsT in *Vibrio cholerae* leading to enhanced biofilm formation [36]. Cyclic di-GMP binds to inactive GGDEF-EAL domain containing LapD in *Pseudomonas aeruginosa* to modulate surface adhesion [37]. A cyclic di-GMP tetramer binds to a transcription factor BldD in *Streptomyces coelicolor* leading to its dimerization and altered activity [38]. A prominent example of cyclic di-AMP binding protein is KtrA, a potassium ion transporter, through which cyclic di-AMP modulates ion homeostasis in *Staphylococcus aureus* [39].

1.3.1 Cyclic di-GMP Binding Transcription Factor LtmA

The first cyclic di-GMP receptor characterized in mycobacteria is a transcription factor named LtmA [40]. To identify cyclic di-GMP targets in *M. smegmatis*, Li and He screened close to 500 putative regulatory genes for cyclic di-GMP binding. LtmA was identified as a putative cyclic di-GMP target and this binding was confirmed by cross-linking studies. SPR analysis of cyclic di-GMP-LtmA interaction revealed that the interaction is strong with K_d value close to 0.8 μM. However, LtmA does not contain any commonly known cyclic di-GMP motifs, such as those present in PilZ domain proteins. Therefore, it belongs to a novel class of cyclic di-GMP effectors and its interaction with cyclic di-GMP was validated by SPR. DNA footprinting and electrophoretic mobility shift assay (EMSA) binding studies revealed that cyclic di-GMP increases the DNA-binding ability of LtmA. A 12 bp

Fig. 1.2 (continued) domain, an EAL hydrolysis domain, and a regulatory GAF domain in tandem. Rv1357c is another EAL domain containing cyclic di-GMP PDE in *M. tuberculosis*. DisA, the DAC present in both *M. smegmatis* and *M. tuberculosis*, carries a DAC synthesis domain linked with a helix-hairpin-helix (HhH) domain. A PDE containing the DHH and DHHA1 domains is present in both mycobacterial species

Table 1.1 List of DNCs and PDEs in mycobacteria

Organism	Number of proteins with domain organization				
	GAF–GGDEF–EAL	GGDEF[a]	GGEEF[b]	EAL	Others[c]
M. tuberculosis H37Rv	1			1	
M. tuberculosis CDC1551	1				
M. bovis BCG Pasteur				1	
M. leprae TN	1	1		1	PAS-GGDEF-AraH
M. smegmatis mc^{2}155	1		1		
M. avium 104	2	4	1		

Organism	Number of proteins	
	DisA	PDE
M. tuberculosis H37Rv	1	1
M. tuberculosis CDC1551	1	
M. bovis BCG Pasteur	1	
M. smegmatis mc^{2}155	1	1

[a]GGDEF domain with one or more input sensory domain such as PAS, Rec, FlhA, or GAF
[b]GGDEF domain with conserved GGEEF or degenerate SDSEF motif
[c]Unusual domain organization arrangement, with EAL at the N terminus of GGDEF or an AraH (arabinose transport) output domain in the case of *M. leprae*

conserved palindromic motif GGACANNTGTCC is recognized by LtmA. LtmA binds to the promoter regions of several genes, including two genes which are in its vicinity, and positively regulates their expression. Among the various categories of genes whose expression is regulated by LtmA, there are 37 lipid transport and metabolism genes, 21 transcription regulators, and 13 cell wall/membrane biogenesis genes. Importantly, several genes were related to the metabolism of mycolic acids, which form a major component of the mycobacterial cell wall. LtmA overexpression, therefore, affects the colony morphology of the strain. In addition, LtmA affects the antibiotic resistance in *M. smegmatis*. As its ortholog is present in *M. tuberculosis*, cyclic di-GMP-mediated LtmA regulation has several implications.

1.3.2 Cyclic di-GMP Binding Transcription Factor HpoR

Subsequently, another transcription factor HpoR in *M. smegmatis* was identified as a target of cyclic di-GMP [41]. The *hpoR* operon contains mostly redox-related genes and is therefore important in the oxidative stress response of the bacteria. HpoR negatively regulates the expression of its own operon by binding to an upstream palindromic sequence (G/AGACANNTGTCC) upstream of it. Cyclic di-GMP can bind to HpoR ($K_d = 1.8$ μM). The DNA-binding ability of HpoR is enhanced by cyclic di-GMP, but at a cyclic di-GMP concentration higher than 270 μM

cyclic di-GMP inhibits the DNA binding by HpoR. Therefore, if cyclic di-GMP levels increase under oxidative stress, it will bind to HpoR and de-repress the negative regulation of its own operon thereby helping the cell survival. Interestingly, LtmA is a positive regulator of the *hpoR* operon and also physically interacts with HpoR [42]. This interaction is stimulated by cyclic di-GMP leading to enhanced DNA binding by LtmA and reduced DNA binding by HpoR. Therefore, cyclic di-GMP aids the oxidative stress response by increasing *hpoR* operon expression in three ways: first, by binding to LtmA and enhancing its binding at upstream region of *hpoR* operon; second, by binding to HpoR and relieving its binding at the upstream region of *hpoR* operon; and third, by stimulating the LtmA-HpoR interaction, thereby increasing LtmA binding at the *hpoR* operon.

1.3.3 Cyclic di-GMP Binding Transcription Factor EthR

EthR is another transcription factor found to bind cyclic di-GMP in *M. tuberculosis* [43]. Cyclic di-GMP binds EthR increasing its binding to the promoter of *ethA* gene. This represses the transcription of *ethA* gene. EthA is postulated to be involved in activating the prodrug ethionamide, an inhibitor of mycolic acid synthesis used as a second-line drug for tuberculosis. This finding suggests a role for cyclic di-GMP in the resistance towards ethionamide.

A global proteome microarray further identified more than 20 putative targets of cyclic di-GMP [44]. These include proteins like rhamnosyltransferase (Wbbl2), an ABC transporter (ProZ), and a polyphosphate kinase (PpnK). More studies need to be carried out in order to fully understand their cellular effects.

1.3.4 Cyclic di-AMP Binding Transcription Factor DarR

M. smegmatis DarR is the only transcription factor identified so far to bind cyclic di-AMP in mycobacteria [45]. SPR and cross-linking studies validated the binding of this CDN to DarR ($K_d = 2.8$ μM). DarR is a TetR family transcription factor which binds a 14 bp palindromic sequence motif (ATACTNNNNAGTAT). The binding of cyclic di-AMP leads to binding to its promoter resulting in repression of transcription. It downregulates the expression of three target genes—a major facilitator family transporter, a fatty acid-acyl coA ligase, and a cold shock family protein CspA. DarR, therefore, links cyclic di-AMP with fatty acid metabolism and cold shock response in *M. smegmatis*. This is substantiated by the fact that DarR overexpression is toxic to *M. smegmatis* and reduces levels of certain fatty acids.

1.3.5 Cyclic di-AMP Binding Protein RecA

Both the CDNs bind to *M. smegmatis* and *M. tuberculosis* RecA, though the binding of cyclic di-GMP is much weaker [46]. Cyclic di-AMP decreases DNA strand-exchange ability of *M. smegmatis* and *M. tuberculosis* RecA. The disassembly of *M. smegmatis* RecA filaments is enhanced by cyclic di-AMP but not those of *E. coli*, possibly due to the presence of a C-terminal tail in *M. smegmatis* RecA. Additionally, cyclic di-AMP also regulates transcription of RecA by translational repression of *recA* mRNA. Therefore, absence of intracellular cyclic di-AMP leads to lower levels of RecA leading to DNA damage in the presence of genotoxic agents. The binding site of cyclic di-AMP in RecA is not the same as that of ATP indicating the presence of allosteric regulation by the CDN. Thus, it appears that cyclic di-AMP is linked to the DNA repair pathway in mycobacteria.

Apart from bacterial targets, the CDNs also interact with the host cell receptors in humans. This will be described in a later section.

1.4 Physiological Roles of the CDNs

As mentioned earlier, CDNs have a global influence on the bacterial behavior. The binding of nucleotide second messengers to their effectors results in alteration of the cellular phenotypes in response to the initial stimuli. CDNs affect myriad processes in both Gram-negative and Gram-positive bacteria. This vast spectrum of physiological processes includes motility, biofilm formation, surface adhesion, virulence, toxin production, secretion system, cell morphology, and cell wall metabolism [12, 24]. In this section, we will describe the phenotypes affected by CDNs in mycobacteria.

1.4.1 Phenotypes Regulated by Cyclic di-GMP

In *M. smegmatis*, the deletion of cyclic di-GMP synthesizing gene DcpA affects long-term survival under nutrient starvation in addition to colony morphology and growth [18, 47]. The Δ*dcpA* strain of *M. smegmatis* is not capable of forming biofilm [16]. Reorientation of the bacterial phenotypes like sliding motility, colony morphology, and aggregation in liquid cultures is observed in the Δ*dcpA* strain. Chromatographic analysis of the lipid isolate from this knockout strain demonstrated that the amount of glycopeptidolipids and polar lipids (responsible for the maturation of the bacterial cell wall) are also depleted which leads to higher hydrophobicity of the cell wall. Further analysis revealed that cyclic di-GMP also regulates cell shape and division in *M. smegmatis* [47]. Microscopic analysis of the Δ*dcpA* strain displayed unique characteristics like elongated cell length and the presence of multiple nuclei

and septa thereby suggesting inhibition of cell division. Phenotypic microarray analysis of the Δ*dcpA* strain of *M. smegmatis* showed that the strain is resistant to several antibiotics [16]. All these phenotypes could be attributed to the role of cyclic di-GMP in influencing global gene expression pattern in bacteria. This is validated by the microarray analysis of the Δ*dcpA* strain which highlighted increased transcription of genes belonging to various functional categories like metabolism, virulence, and cell wall metabolism. It remains to be seen if the deficiency in cyclic di-GMP affects any other second messenger signaling or these cyclic di-GMP associated phenotypes could be compensated by other second messengers.

A *M. tuberculosis* strain devoid of cyclic di-GMP exhibits an increased dormancy phenotype [48]. Attenuation of virulence and pathogenicity in both human THP-1-derived macrophages and mouse model is observed in cyclic di-GMP PDE deletion strain of *M. tuberculosis.* The differences in phenotypes were observed under anaerobic conditions and this was validated by analyzing the changes in the transcriptome. This clearly links cyclic di-GMP to the pathogenicity and dormancy in *M. tuberculosis*.

Also, cyclic di-GMP deletion affects colony morphology and pellicle production in *M. bovis* [26]. Cyclic di-GMP affects oxidative stress response and antibiotic stress response in *M. smegmatis* and nitrosative stress response in *M. bovis*, further illustrating the importance of cyclic di-GMP in stress response in mycobacteria [26, 41, 43]. The identification of more CDN targets in the cell will aid to uncover the underlying mechanism behind the CDN-mediated phenotypes.

1.4.2 Phenotypes Regulated by Cyclic di-AMP

Cyclic di-AMP regulates fatty acid metabolism in *M. smegmatis*. Increased levels of the second messenger, resulting from either DisA overexpression or PDE deletion in *M. smegmatis*, increase the accumulation of C10–C20 fatty acids as well as lead to an abnormal colony morphology [28]. A higher level of cyclic di-AMP in *M. smegmatis* also leads to inhibition of motility and increased aggregation and expansion [27]. The regulation of DarR as well as other hitherto unknown effector proteins by cyclic di-AMP would explain these phenotypes. Deletion of DisA increases the sensitivity to genotoxic agents in *M. smegmatis* [45].

Removal of DisA inhibits cyclic di-AMP production, whereas deletion of the PDE *cnpB* (or *cdnP*) significantly increases cyclic di-AMP accumulation and secretion in *M. tuberculosis* [30]. As a phenotype, increased level of cyclic di-AMP also affects cell length in *M. tuberculosis*. Furthermore, elevated level of cyclic di-AMP (in *cnpB* knockout strain) induces production of higher levels of IFN-β during macrophage infection as compared to that of the wild-type strain. Mice infected with Δ*cnpB* strain have significantly reduced inflammation. Mycobacterial count is also lowered in the lungs and spleen cells compared with those infected with the parent strain. Furthermore, the deletion of PDE from the *M. tuberculosis* genome is associated with attenuation of the virulence in a mice model [49]. Mechanism of regulation of virulence by cyclic di-AMP in *M. tuberculosis* needs to be investigated.

Cyclic di-AMP has been shown to be essential in many bacteria under certain growth conditions [24]. It must be noted that though cyclic di-GMP and cyclic di-AMP play critical roles in mycobacterial physiology, neither of them are essential for mycobacteria. However, their continuous presence suggests beneficial roles in the life cycle of this bacterial genus.

1.5 CDN Homeostasis

Since the CDNs orchestrate a variety of phenotypes, it is of vital importance to tightly regulate their intracellular levels by fine-tuning their synthesis and hydrolysis. Bacteria regulate CDN synthesis at transcriptional level, by allosteric control, interaction with other proteins, feedback mechanism, oligomerization status of DNCs, as well as the cellular localization of DNCs [12, 20]. CDNs, being small molecules, can diffuse rapidly across the cell. However, a spatial organization of CDNs helps in orchestrating several modules of signaling simultaneously. There is a requirement of dimerization of DNCs due to the inherent twofold symmetry of the CDNs. The GGDEF domains of DGCs come together to form a functional dimer interface for catalyzing cyclic di-GMP synthesis in a metal-dependent manner [50, 51]. EAL domains also function mostly as higher oligomers though they may possess low activity as monomers. To restrict cyclic di-GMP synthesis, many DGCs contain an inhibitory I-site (RxxD motif) five amino acids upstream of the GGDEF motif which mediates allosteric regulation by cyclic di-GMP [12, 52]. Apart from these, many DNCs have regulatory domains in tandem or interact with other domains containing sensory domains for regulation of their activity.

1.5.1 Cyclic di-GMP Homeostasis

A signaling pathway is activated once an extracellular or intracellular signal is detected by a cellular receptor. Therefore, the spatial organization of DNCs becomes relevant in this regard. Little is known about the spatial organization of mycobacterial DNCs and PDEs, but since some of them (e.g., DcpA) have been shown to localize in membrane fractions, it is hypothesized that they bind to membrane receptors which trigger their activity under particular stress conditions, as is the case with other bacteria [53]. Their levels are further regulated at the transcription level [20].

DcpA is bifunctional and the presence of both the synthesis and hydrolysis domain in the same protein presents an enzymatic conundrum. This is analogous to the bifunctional (p)ppGpp synthetase Rel in mycobacteria [54]. How are each of the activities then regulated inside the cell? DcpA occurs in both dimeric and monomeric forms with the monomer preferentially conducting hydrolysis [53]. The GAF domain is involved in regulating the dimerization of GGDEF domain