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C-di-GMP signaling and implications for pathogenesis of *Mycobacterium tuberculosis*

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C-di-GMP is a ubiquitous bacterial second messenger that regulates a wide range of bacterial physiological processes including biofilm formation, virulence, motility and cell differentiation. Here, we have summarized our current knowledge on the upstream signaling factors and downstream effectors of c-di-GMP in addition to the interaction between c-di-GMP and eukaryotic organisms. New discoveries in these areas have enriched our understanding of the diversity of c-di-GMP signaling pathways and provide important clues for us to explore the roles of c-di-GMP signaling in human pathogens such as *Mycobacterium tuberculosis*.

c-di-GMP, second messenger, pathogenesis, *Mycobacterium tuberculosis*

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Bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) (Figure 1) is a ubiquitous bacterial second messenger that regulates a wide range of complex cellular processes [1,2] including biofilm formation [3], virulence [4], motility [5,6] and differentiation [7], and contributes to the pathogenesis of some types of bacteria. In most cases, a high level of intracellular c-di-GMP stimulates biofilm formation and cell cycle arrest while low c-di-GMP levels contribute to virulence, motility and cell division. Interestingly, although c-di-GMP is absent in eukaryotes, exogenous application of c-di-GMP can arrest cell cycle in eukaryotes [8,9] and stimulate the immune system of higher organisms [10,11].

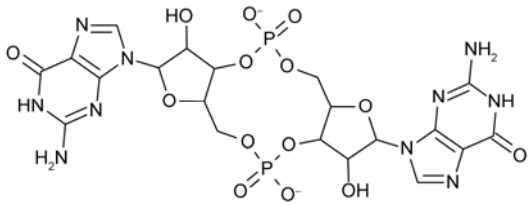

Figure 1 Structure of c-di-GMP.

1 Metabolism of c-di-GMP

The intracellular synthesis and degradation of c-di-GMP are mediated by diguanylate cyclase (DGC) and phosphodiesterase (PDE), respectively (Figure 2)[1,12]. The GGDEF domain of diguanylate cyclases produces a c-di-GMP molecule from two GTPs. The EAL and HD-GYP domains of phosphodiesterases on the other hand hydrolyze c-di-GMP into pGpG and two molecules of GMPs, respectively [13]. Although widespread in bacteria, the GGDEF, EAL and HD-GYP domains are absent in eukaryotes [13–15]. In addition, a newly identified protein called YybT in *Bacillus subtilis*, which contains a DHH/DHHA1 domain, can also hydrolyze c-di-GMP into pGpG [16]. Unlike the EAL domain, the DHH/DHHA1 domain is a multi-functional phosphodiesterase and can also hydrolyze c-di-AMP, a second messenger involved in DNA damage response [17], into pApA [16].

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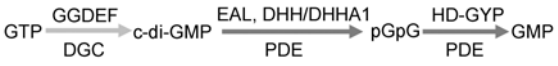

Figure 2 Synthesis and degradation of c-di-GMP.

C-di-GMP signaling has been studied heavily in recent years. Here, we summarize the latest discoveries on c-di-GMP signaling, including various upstream signaling factors and a variety of downstream effectors, in addition to the interaction between c-di-GMP and eukaryotic organisms. The potential roles of c-di-GMP signaling in *M. tuberculosis* are also discussed.

2 Signals regulating intracellular c-di-GMP level

Intracellular c-di-GMP levels are controlled by enzyme activities of diguanylate cyclases and phosphodiesterase. The GGDEF domain has feedback regulation. Besides an "A" site with catalytic activity, the GGDEF domain contains an "I" site which can inhibit diguanylate cyclases when bound to c-di-GMP [18–20].

In addition, c-di-GMP synthesis and degradation by diguanylate cyclases and phosphodiesterases can be regulated by various signals, such as phosphorylation [21,22], light [23] and nitric oxide [24]. The diversity in regulatory factors comes from the fact that GGDEF and EAL domains of diguanylate cyclases and phosphodiesterases, respectively, can interact with numerous signal molecules or regulatory domains [14,15,25]. Such domains include REC [22], GAF [26], PAS [27], BLUF [23] and others.

The response regulator PleD in *Caulobacter crescentus* contains an N-terminal phospho-receptor domain and a C-terminal GGDEF domain. Phosphorylation of the recevier domains causes the formation of dimers and activation of diguanylate cyclases [21]. BlrP1 of *Klebsiella pneumoniae* contains an N-terminal light-receptor BLUF domain and a C-terminal EAL domain. The BLUF domain senses blue light with FAD chromophore. BLUF can allosterically regulate the phosphodiesterase activity of EAL domains when stimulated by light [23].

Besides allosteric regulation, protein-protein interaction is another way by which the activity of c-di-GMP signaling proteins is regulated. In the *Shewanella woodyi* strain MS32, swDGC contains both diguanylate cyclase and phosphodiesterase domains. When bound by nitric oxide, H-NOX interacts with swDGC and increases its phosphodiesterase activity, thus influencing c-di-GMP metabolism [24].

3 C-di-GMP effectors

C-di-GMP has various downstream effectors including PilZ domain proteins [28], PelD of *Pseudomonas aeruginosa* [29], riboswitches [30], transcriptional factors [31,32], two-component histidine kinase [33], and polynucleotide phosphorylase [34]. C-di-GMP can also bind to the "I" site in the GGDEF domain of diguanylate cyclases [18] and degenerate GGDEF [35] and EAL [36] domains. These effectors include proteins, RNAs, enzymes and regulators.

Bioinformatic analysis indicated that PilZ domain proteins could be potential effectors of c-di-GMP [28]. Binding tests and mutation analyses of several PilZ proteins confirmed the prediction and identified RxxxR and D/NxSxxG as the sequence motifs required for c-di-GMP binding [37–40]. Additionally, recent studies have identified transcriptional factors [31,32] and riboswitches [30] as c-di-GMP effectors.

3.1 Transcriptional regulatory effectors

Currently identified c-di-GMP transcriptional regulatory effectors include the sigma-54 dependent transcriptional regulator FleQ of *P. aeruginosa* [31], the transcriptional factor Clp of *Xanthomonas axonopodis* [41,42], the LuxR family transcriptional regulator VpsT of *Vibrio cholerae* [32], the CRP/FNR family transcriptional regulator Bcam1349 of *Burkholderia cenocepacia* [43] and a special activator MrkH found in *Klebsiella pneumoniae* [44].

FleQ of *P. aeruginosa* was the first identified c-di-GMP responsive transcriptional factor [31]. FleQ contains three domains including an N-terminal FleQ domain, a AAAσ^{54} interaction domain and a C-terminal HTH domain. *In vitro* binding assays have shown that c-di-GMP can interact with the full length FleQ protein and its mutant fragnment that lack N-terminal FleQ domain. FleQ negatively regulates expression of genes involved in extracellular polysaccharide synthesis by binding to the *pel* operon. C-di-GMP binds to FleQ and inhibits the binding of FleQ to the *pel* operon, thus promoting expression of polysaccharide synthesis genes [31].

The global transcriptional regulator Clp controls the expression of a subset of virulence genes in *X. campestris*. This DNA-binding capability is abrogated by c-di-GMP, which binds to Clp with micromolar affinity [41,42].

VpsT of *V. cholerae* is a response regulator of typical two-component regulatory systems. However, two-component kinases that phosphorylate VpsT have not yet been identified. Early studies found that c-di-GMP affects regulation of VpsT but the mechanism was not clear. VpsT contains an N-terminal receiver domain and a C-terminal HTH domain. Recent studies have shown that two symmetrical c-di-GMP molecules form a dimer and interact with the receiver domain of VpsT. VpsT binds to c-di-GMP via a 4-residue motif W[F/L/M][T/S]R [32].

Bcam1349 of *B. cenocepacia* is a CRP/FNR family transcriptional regulator that contains an N-terminal cyclic nucleotide bound domain and a C-terminal HTH domain [43].

C-di-GMP binds to full length Bcam1349 through its cyclic nucleotide bound domain. C-di-GMP promotes the binding of Bcam1349 to the promoter of the cellulose synthase gene [43].

C-di-GMP stimulates the promoter-binding activity of the transcriptional activator MrkH in *K. pneumoniae* [44]. MrkH binds strongly to the promoter of *mrkA* only in the presence of c-di-GMP. Notably, MrkH does not contain the typical HTH domain and thus represents a new class of c-di-GMP response regulators [44].

3.2 Riboswitch

Riboswitch is a part of mRNA that regulates gene expression by binding to a small ligand molecule [45]. As PilZ domain proteins are absent in many species, it was initially assumed that riboswitches represent a new class of c-di-GMP receptors [2]. Subsequent studies confirmed this and identified two classes of riboswitches, Riboswitch-I [28] and Riboswitch-II [46], that bind c-di-GMP. Binding of c-di-GMP to Riboswitch-I could either activate or inhibit the expression of genes [28]. Structural analyses have indicated that c-di-GMP binds to riboswitch-I in a symmetrical dimer form [47,48]. Riboswitch-II is known to associate with self-splicing ribozymes. C-di-GMP binding to Riboswitch-II induces folding changes at atypical splice site junctions and modulation of RNA processing. Splice site variations can lead to changes in ribosomal binding sites and thus affect gene expression [46].

These studies clearly indicate that effectors of c-di-GMP are not limited to proteins but also include RNAs. Interestingly, a recently study also found that c-di-GMP interacts with polynucleotide phosphorylase to regulate RNA processing [34].

3.3 Two-component protein kinases

Besides allosteric regulation of enzymes and DNA binding of transcriptional regulators, c-di-GMP also controls protein localization by direct interaction. The histidine kinase SgmT of *Myxococcus xanthus* is one of the newly identified c-di-GMP effectors [33]. C-di-GMP interacts with the GGDEF domain of SgmT which does not affect its autophosphorylation and phosphate transfer activities. However, binding of c-di-GMP induces clustering of SgmT within the cell. SgmT mutation at the "I" site in the GGDEF domain leads to a more diffuse localization in the cell [33].

4 C-di-GMP and eukaryotes

The currently known GGDEF, EAL and HD-GYP domain proteins that regulate c-di-GMP metabolism are ubiquitous in bacteria but absent in eukaryotes [13–15]. However, early studies found several proteins, such as plant cellulose synthase [49] and human growth-promoting protein $p21^{ras}$ [8,50], that specifically bind to c-di-GMP with high affinity in both plants and animals. Recent studies indicate that c-di-GMP affects critical cellular processes including cellulose synthesis [49], DNA synthesis [50], cell cycle [8] and cell proliferation [9] in eukaryotes. Interestingly, growing evidence indicates that c-di-GMP also interacts with the immune system of eukaryotes [10,51]. Exogenous application of c-di-GMP induces expression of type I interferon [11] and can be directly sensed by the STING protein [52,53].

4.1 C-di-GMP affects cell cycle

C-di-GMP has been shown to bind to and activate cellulose synthase in plants using labeled c-di-GMP as probe [1,54,55]. Several studies have also suggested a link between c-di-GMP and cell cycle-related processes in animal cells [8,9,50]. C-di-GMP irreversibly enhances DNA synthesis but has no effect on cell replication in Molt4 cells [50]. In Jurkat cells, exogenous application of c-di-GMP induces blockage of cell cycle arrest at the S-phage and decrease in cell division [8]. C-di-GMP also inhibits basal and growth factor-stimulated human colon cancer cell proliferation [9]. C-di-GMP enters these cells and binds irreversibly to the growth-promoting protein $p21^{ras}$ [8,50]. It is possible that an irreversible binding of c-di-GMP to the $p21^{ras}$ protein results in a fixed active conformation of this protein, thereby affecting cell proliferation.

4.2 C-di-GMP stimulate the immune system

Several studies in rodents have shown that, owing to its interaction with the host immune system, c-di-GMP can produce antibacterial effects. C-di-GMP treatment reduces bacterial colonization by *Staphylococcus aureus* in a dose-dependent manner in a mouse model of mastitis infection. Interestingly, this effect is not mediated by inhibitory effects on *S. aureus* growth by c-di-GMP [56]. It has thus been proposed that the antibacterial effects come from the effect of c-di-GMP on the host immune system. By performing a systematical analysis of the response of the immune system to c-di-GMP, Karaolis confirmed that c-di-GMP is indeed an immunostimulatory molecule that induces a wide range of response pathways of the immune system [10]. Consistent with studies in *S. aureus* (a gram-positive pathogen), c-di-GMP also induces a significant protective immune response and improved bacteria clearance against a virulent strain of *K. pneumoniae* (a gram-negative pathogen) in a mouse model of bacterial penumonia [51].

Further studies exploring the mechanisms underlying the above observation indicate that c-di-GMP is sensed by a novel cytosolic immunosurveillance pathway. Responsiveness of c-di-GMP is independent of TLRs which monitor the signal stimulation. The transcriptional profile of host

cells provoked by c-di-GMP is similar to that which are triggered by extracellular DNA or RNA [11]. However, several known nucleic acid-sensing pathways are not required for responses to c-di-GMP, which suggests that there must be at least one additional nucleic acid sensor in the cytosol that responds well to c-di-GMP. Recent studies have found that STING is required for type I interferon to respond to c-di-GMP [52] and STING is a direct innate immune sensor of c-di-GMP [53]. These studies have provided valuable insights into the fundamental mechanisms by which the innate immune system responds to c-di-GMP.

5 C-di-GMP signaling in *M. tuberculosis*

5.1 Current progress

Two c-di-GMP signaling proteins Rv1354c and Rv1357c have been identified based on domain analysis in *M. tuberculosis*. Rv1354c contains three domains (GAF-GGEDF-EAL) while Rv1357c contains a single domain (EAL) (Figure 3). The "GGDEF" (residues 261–265 in Rv1354c) and "EAL" (residues 389–391 in Rv1354c, residues 89–91 in Rv1357c) amino acid residues are well-conserved in both proteins. In addition, the full length amino acid sequence and gene location are also well-conserved across the H37Rv, H37Ra and BCG strains except for a single mutation in the Rv1357c homolog in the BCG strain. Furthermore, the c-di-GMP metabolism activity of Rv1354c and Rv1357c has been validated *in vitro* [57].

Rv1354c has been found to be completely disrupted in clinical isolates of the *M. tuberculosis* Haarlem strain, which indicates that Rv1354c is not essential for survival and infectivity of *M. tuberculosis* [58]. However, disruption of Rv1357c by transposon insertion has been found to cause attenuation of BCG infection of macrophages, which reflects a link between c-di-GMP signaling and infectivity of *M. tuberculosis* [59]. Another gene expression analysis found that expression of both Rv1354c and Rv1354c was increased during infection of macrophages and reduced in H_2O_2-treated *M. tuberculosis* [60]. This suggests that c-di-GMP signaling plays as yet unknown roles in *M. tuberculosis*. Our current knowledge about c-di-GMP signaling in *M. tuberculosis* is still limited compared to that in other pathogens like *V. cholerae*, *P. aeruginosa* and *S. typhi*.

5.2 Potential roles of c-di-GMP in *M. tuberculosis*

Full length Rv1354c contains an N-terminal signal sensory GAF domain, and two domains—GGDEF and EAL—that have opposite enzymatic activities. The domain composition of Rv1354c shows a switch structure which implies that the GAF domain senses environmental signal and regulates the enzyme activities of GGDEF and EAL domains. A possible model of Rv1354c activation could be similar to that known for PleD [18]: when Rv1354c is stimulated by a signalling molecule or is phosphorylated, it may form dimers that exhibit diguanylate cyclase activity. However, when the environmental signal changes or when the protein is dephosphorylated, it may work as a monomer and exhibit phosphodiesterase activity.

Figure 3 Domain of c-di-GMP signaling proteins Rv1354c and Rv1354c in *M. tuberculosis*.

The sensory domain of a signaling protein can provide important clues for inferring its potential roles. The GAF domain is a ubiquitous signal sensory domain in both prokaryotes and eukaryotes [61]. It has been shown that the GAF domain can sense cGMP and cAMP [61]. A recent study has shown that c-di-GMP senses MetO as an indicator of oxidation and nutrition stress [62]. In addition, the GAF domain can sense NO [63]. It is worth noting that NO has been found to induce antimicrobial activity by affecting the eukaryotic host immune system as well as dormant *M. tuberculosis* [64,65]. Taken together, these studies show that the GAF domain is bound by a diverse set of ligands that includes signalling molecules that mediate both important host signaling and stress response in bacteria. Therefore, GAF domain-containing proteins such as Rv1354c may play important roles in stress response and pathogen-host interaction in bacteria. Interestingly, two other GAF domain-containing proteins, the two-component signal sensory histidine kinase DosT (Rv332c) and DosS (Rv2027c), have been shown to participate in regulating dormancy in *M. tuberculosis* [66,67]. Furthermore, MSMEG_2196, the Rv1354c homolog in *M. smegmatis*, has been shown to be critical for long-term survival of the bacteria under conditions of nutrition stress [68].

Analysis of the subcellular localization of Rv1354 in *M. tuberculosis* has revealed that it is an inner membrane protein [69]. Since a typical transmembrane region was not identified in Rv1354c using several bioinformatic tools, it is likely that Rv1354c physically associates with other membrane proteins. In our previous study, we predicted and validated interaction between Rv1354c and a group of ABC transporters [26]. Although this interaction may explain the membrane localization of Rv1354c, the cellular role of the interaction is still unclear. Considering that c-di-GMP regulates polysaccharides and colony morphology in several other bacteria, one possibility is that Rv1354c affects the extracellular matrix of *M. tuberculosis* by regulating the transport activity of the ABC transporters. In addition, since c-di-GMP has been shown to interact with the host immune system [10,11], the interaction of Rv1354c with ABC transporters may facilitate c-di-GMP to transport across the membrane if it is secreted by *M. tuberculosis*.

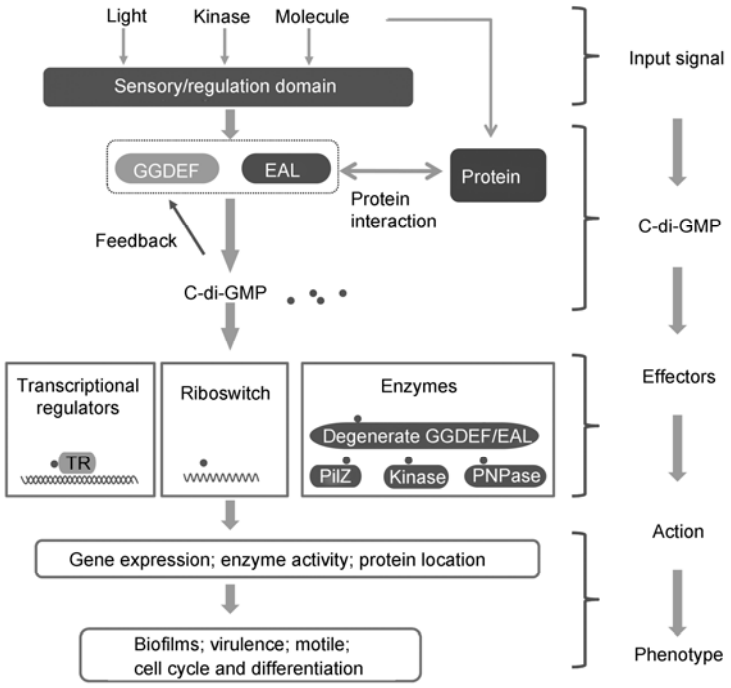

Figure 4 C-di-GMP signaling system.

6 Conclusions

C-di-GMP is a ubiquitous bacterial second messenger that regulates a wide range of cellular processes such as biofilm formation, virulence, motility and differentiation. Its functional diversity can be attributed to its diverse upstream ligands and downstream effectors. This review summarizes our recent understanding on c-di-GMP signaling (Figure 4).

After the initial discovery of PilZ domain proteins as their effectors, several transcriptional regulators and riboswitches were identified as c-di-GMP effectors. In addition, histidine kinase and polynucleotide phosphorylase have recently been identified as c-di-GMP effectors. These new findings indicate that c-di-GMP can affect cellular processes by several mechanisms including regulation of enzyme activity, gene expression and subcellular localization of other proteins (Figure 4). However, a general mechanism of effector recognition by c-di-GMP is still absent, which has limited identification of new effectors using bioinformatic methods. Although great strides in effector identification has been made, potential new types of effectors may exist considering that homologs of currently known effectors are absent in some species that employ c-di-GMP signaling.

Genomics, biochemistry and gene expression profiling approaches have revealed that c-di-GMP signaling is active in *M. tuberculosis*. However, our understanding of the downstream effectors of c-di-GMP and their physiological roles is still largely limited. Neither PilZ domain proteins nor other proteins that are homologous to current known effectors have been identified yet in *M. tuberculosis*. Although these areas need further research, bioinformatic analyses suggest that c-di-GMP may play important roles in regulation of dormancy and host interaction in *M. tuberculosis*.

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