Cyclic Dinucleotides in Bacterial Behavior

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Contributor: Cristiane Guzzo

Cyclic dinucleotides (CDNs) constitute intracellular signaling second messenger systems, they determine the regulation of multiple bacterial phenotypes. Most notably, c-di-GMP, along with proteins related to its synthesis, sensing, and degradation, was identified as playing a central role in the switching from biofilm to planktonic modes of growth.

Keywords: GGDEF ; SMODS ; DAC ; c-di-GMP ; cGAMP ; c-di-AMP

1. Introduction

In the mid-2000s, the idea emerged that c-di-GMP molecules, cyclic-bis(3′→5′)-dimeric GMP, could be second messengers ubiquitous in bacteria, in which proteins containing GGDEF and EAL or HD-GYP domains were at the center of this regulation, being involved in the synthesis and degradation of c-di-GMP, respectively [1]. In the following years, different papers were published showing the central role of c-di-GMP orchestrating different signaling networks such as regulation of the flagellar rotor, bacterial motility such as twitching, exopolysaccharide synthesis, and regulation of bacterial biofilm formation. Nevertheless, c-di-GMP was first identified in 1987 as an allosteric activator of cellulose synthase in the cellulose-producing bacterium *Komagataeibacter* (*Gluconacetobacter) xylinus* [2]. It was the first c-di-GMP receptor described, and nowadays a huge range of different receptors have been identified, including RNA structures known as riboswitches. Therefore, a cyclic dinucleotide neglected in the microbiology area for 20 years emerged as a regulator of the bacterial cell lifestyle.

Recently, this research area has been under expansion, with the discoveries of new intracellular signaling cyclic dinucleotides (CDNs) in bacteria. In 2008, it was demonstrated that bacteria can produce not only c-di-GMP, but also c-di-AMP, cyclic-bis(3′→5′)-dimeric AMP, by an enzyme known as DisA that possess a DAC domain [3]. In 2012, a novel cyclic dinucleotide has been found to be a second bacterial messenger, cGAMP, cyclic guanosine (3′→5′) monophosphate-adenosine (3′→5′) monophosphate, synthesized by proteins containing SMODS domain such as the DncV protein [4][5]. At the moment, c-di-GMP, c-di-AMP and c-GAMP have been described as the main bacterial second messengers. Nevertheless, different classes of cyclic oligonucleotides, such as c-UAMP, c-di-UMP, c-UGM, c-CUMP, and c-AAGMP, have also been found in bacteria [2][3][5][6]. These molecules include not only di-purines but also hybrids of purine and pyrimidines and cyclic trinucleotides [6].

The cyclisation between two nucleotides of the most common bacterial CDNs involves the formation of a phosphodiester bond that links the C3’ of one pentose ring with the C5’ of another, resulting in a 3’-5’ cyclic dinucleotide (3′→5′). Despite their chemical similarities, there are specific enzymes involved in the synthesis and degradation of different CDNs. Furthermore, bacteria have different classes of CDN receptors that are specific to only one type of CDN. However, how the receptors differentiate one CDN from another is still unclear. Given the specificity of the receptor, since this is the molecule responsible for directly or indirectly regulating different bacterial phenotypes, changes in a single base of the CDN can lead to quite divergent biological responses, as described below.

Molecules of c-di-GMP generally coordinate the transition of a bacterium’s lifestyle, from a mobile single cell undergoing planktonic growth to a multicellular community in biofilm structures, a form of sessile growth. Regulation of these transitions are mediated by controlling the bacterial motility through the regulation of the flagellar rotor [7] and the twitching motility machinery [8]. Alternatively, in Streptomycetes, c-di-GMP regulates the transition from vegetative mycelial growth to the formation of reproductive aerial mycelium [9]. This dinucleotide is also involved in the regulation of bacterial adhesion, cell cycle progression and division, biofilm formation, quorum sensing [10], regulation of the type II (T2SS) [11], type III (T3SS) [12], and type VI (T6SS) [13] secretion system machineries, as well as the synthesis and secretion of virulence factors and pathogenesis [14][15][16][17][18]. Similarities in the roles of eukaryotic cyclins and bacterial c-di-GMP molecules have also been suggested. In eukaryotes, cyclins drive the cell cycle by regulating the activity of cyclin-dependent kinases and promoting the asymmetric replication of future cells [19].

2. GGDEF, SMODS, and DAC Domains Do Not Share Structural Similarities and Probably Perform the Nucleotide Cyclization Catalysis by Different Mechanisms

At the moment, three different classes of prokaryotic proteins are known to synthesize CDN molecules: (i) proteins containing GGDEF domains (Pfam family: PF00990); (ii) CD-NTases enzymes that have the catalytic domain known as SMODS (PF18144) [4]; and (iii) DAC proteins that have a catalytic domain called DAC domain (DisA_N domain, PF2457).

Proteins containing GGDEF domains synthesize mainly 3'-5' c-di-GMP (c-di-GMP) molecules, while proteins containing SMODS domain synthesize preferentially 3'-5' cGAMP (cGAMP) molecules and proteins containing DAC domain synthesize mainly 3'-5' c-di-AMP (c-di-AMP) molecules. Even though CDN molecules are mainly synthesized by prokaryotic cells, eukaryotic cells also synthesize CDNs such as 2'-3' cGAMP by cGAS enzymes. These three classes of CDN synthetases do not share structural similarities, have different residues involved in substrate binding, and possess different catalytic mechanisms. Therefore, they are not homologs and probably evolved independently to catalyze analogous chemical reactions.

Members of families within the CD-NTases superfamily, such as SMODS and cGAS, often do not share detectable primary sequence similarity but adopt a Pol-β-like nucleotidyl transferase fold, suggesting a common origin followed by divergent evolution [5][6][20][21]. cGAS and enzymes containing-SMODS domain use a single active site to sequentially form two separate phosphodiester bonds and release one cyclic nucleotide product. On the other hand, proteins containing DAC or GGDEF domains require homodimerization to perform catalysis. DACs adopt a unique, particular fold, while GGDEF domains are homologous to adenylyl/guanylyl cyclase catalytic domains and to the palm domain of DNA polymerases; see below [1][22].

Proteins containing GGDEF domains require an accessory domain that sense different signals to regulate the GGDEF homodimerization and consequently its enzymatic activity [23]. Each GGDEF domain binds one molecule of GTP and its dimerization positions the two GTP molecules in an antiparallel manner to enable their condensation into c-di-GMP with the release of two pyrophosphate molecules [24]. Therefore, proteins containing GGDEF domains are Bi Ter (two substrates, three products) enzymes and cannot be described by a Michaelis–Menten model [25][26]. A similar enzymatic mechanism seems to happen for proteins containing DAC domains.

3. Cyclic Dinucleotide Receptors

Different classes of CDN receptors have been described and are involved in the regulation of a broad range of bacterial behaviors, while, in eukaryotic cells, they are associated with the activation of innate immune response through interactions with STING proteins. In order to analyze the residues involved in CDN ligand and to compare the ligand structure inside of the protein pocket, we focused on the CDN receptors with three-dimensional structures solved and deposited in the Protein Data Bank (PDB) (**Table 1**).

C-di-GMP receptors are the most studied CDN receptors, probably because it was the first CDN identified as a second bacterial messenger. Therefore, most of the receptors with the structure solved in complex with the ligand analyzed in this review are c-di-GMP receptors. Examples of these are STING proteins and proteins containing PilZ domains, degenerate EAL domains, allosteric site of DGC enzymes containing GGDEF domains, and RNA structures such as c-di-GMP I and c-di-GMP II riboswitches. Other examples of c-di-GMP receptors are the C-terminal domain of the master regulator of *Streptomyces* cell development BldD, REC domain of the transcriptional regulator VspT protein, N-terminal domain of the ATPase of the Type II secretion system MshE protein (T2SSE_N domain), MerR domain of BrlR protein, AAA+ ATPase domain (Sigma54_activat, PF00158) of the transcriptional regulator FleQ protein, and CA domain (HATPase_c, PF02518) of the cell cycle kinase CckA protein (**Table 1**).

Different classes of c-di-AMP receptors have also been described, such as proteins containing STING domains, Aldo-keto reductase domain of oxidoreductase RECON proteins, cyclic-di-AMP receptor domain of PII-like signal transduction protein (PtsA), pyruvate carboxylase domain (HMGL-like domain, PF00682) of *L. monocytogenes* pyruvate carboxylase (LmPC) or *Lactococcus lactis* pyruvate carboxylase (LIPC), TrkA_C domain of potassium transporter A (KtrA), CBS domain of OpuC carnitine transporter, and RNA structures such as ydaO-yuaA riboswitches (**Table 1**). For cGAMP, the receptors analyzed in this review are only STING proteins and c-di-GMP I riboswitches.

The function of each CDN receptor, as well as the residues involved in ligand binding, are described in more detail in **Table 1**. It is notable that most of CDN receptors are specific to their ligands, with the exception of receptors involved in mammalian cell innate immunity, such as STING that interact with different CDNs such as c-di-GMP, c-di-AMP and 3'-5'

and 2’-3’ cGAMP molecules (**Figure 1**B). Interestingly, even though the CDNs are chemically different, the STING binding pockets for each kind of CDN are very similar and the residues involved in ligand binding for each CDN are almost the same (**Figure 1**A,C). This suggests that STING adjusts the ligand binding site for each CDN by placing or removing water or magnesium molecules.

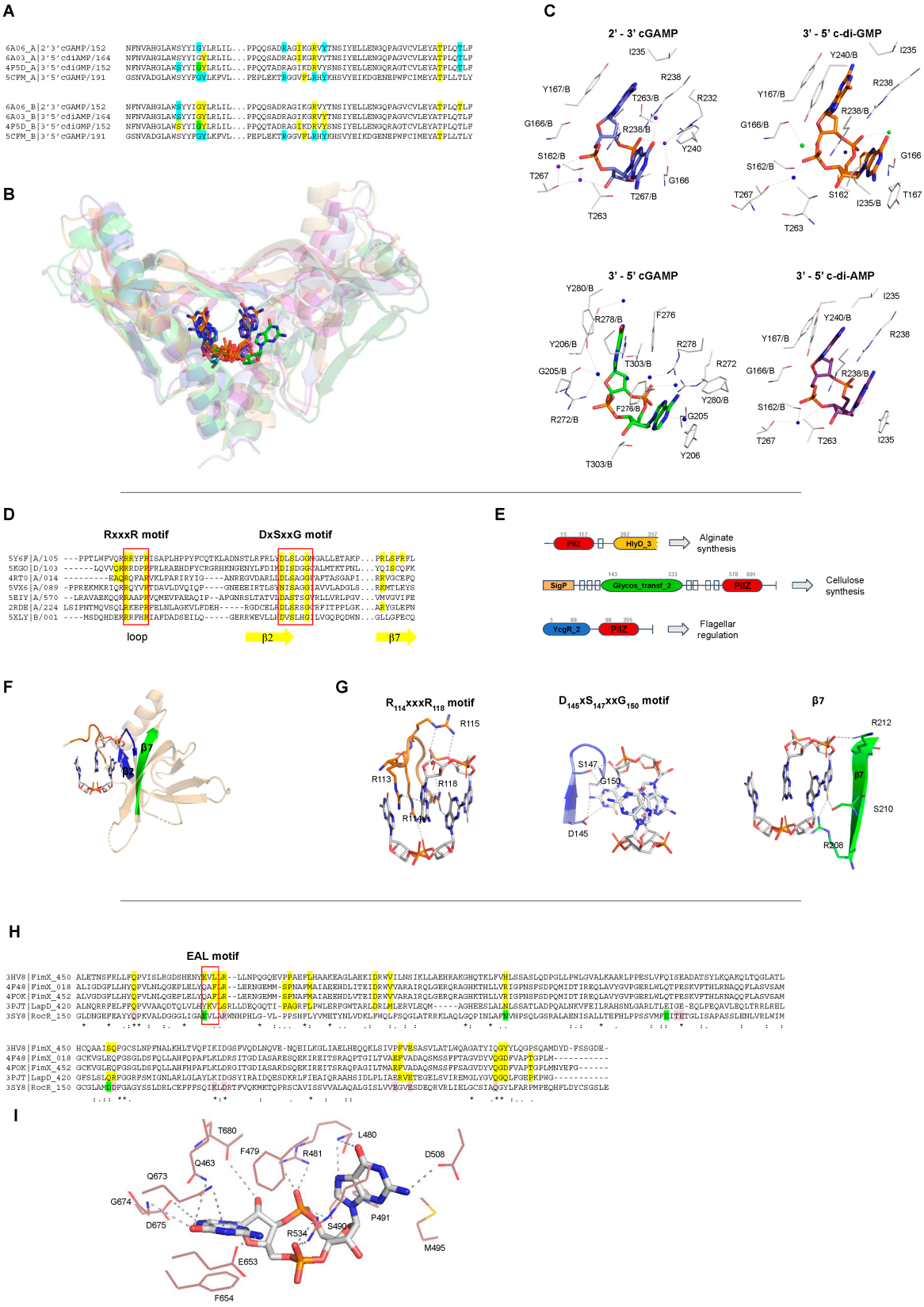

Figure 1. Some CDN receptors: STING, PILZ, and degenerate EAL domains. (**A**) multiple sequence alignment of STING proteins in complex with different CDNs: monomeric 3’-5’ c-di-GMP (PDBID: 4F5D), 2’-3’ cGAMP (PDBID: 6A06), 3’-5’ c-di-AMP (PDBID: 6A03), and 3’-5’ cGAMP (PDBID: 5CFM). The residues highlighted in yellow are involved with direct interaction with the ligand, those in green are involved with interaction with the ligand via magnesium ions, and those in cyan interact via water molecules; (**B**) structural superposition of STING proteins (domain TMEM173) in complex with different CDNs bound at the same protein interface. The ligands are colored by element: nitrogen atoms are in dark blue and oxygens are in red. Carbons are colored according to the ligand: 3’-5’ c-di-GMP in orange; 2’-3’ cGAMP in blue, 3’-5’ c-di-AMP in purple and; 3’-5’ cGAMP in green; (**C**) residues involved in interactions with the different ligands are the same

one highlighted in panel (**A**). The residues are colored by element, with carbon in white, nitrogen in dark blue, and oxygen in red, while the ligands are colored as described in panel (**B**). Water molecules are shown as blue spheres, while magnesium cations are shown as green spheres. (**D**) multiple sequence alignment of the PilZ domains that had their structures solved in complex with c-di-GMP molecules. Residues highlighted in yellow are involved in interactions with the ligand. The secondary structure elements shown belong to the PilZ domain of YcgR from *E. coli* (PDBID: 5Y6F). The motifs conserved within PilZ members are placed in red boxes; (**E**) some domain organizations found in proteins containing PilZ domains (Alg44, BcsA, and YcgR) and their related functions; (**F**) structural representation of a PilZ domain as a cartoon (PilZ domain of YcgR, PDBID: 5Y6F). Residues belonging to the “RxxxR” motif are colored in orange, the “DxSxxG” motif is colored in blue, and the β strand 7 is colored in green. The dimeric c-di-GMP is shown as sticks. The interaction network presented in this figure is shown in more details in panel (**G**); (**H**) multiple sequence alignment of proteins containing degenerate EAL domains (PDBID: 4F48, 4FOK, and 3PJT) and a catalytic EAL domain (PDBID: 3SY8) that had their structure solved in complex with monomeric c-di-GMP. The residues highlighted in yellow are involved with direct interactions with the ligand, those in green are involved with interactions with the ligand via magnesium ions. In the case of RocR, which has a catalytic EAL domain, the residues highlighted in salmon were experimentally demonstrated to be important for catalysis [27].The consensus “EAL” motif is placed in a red box; (**I**) interaction network of the binding site of a FimX degenerate EAL domain (PDBID: 4FOK). Gray dotted lines represent hydrogen bonds. The residues and the c-di-GMP molecule are colored by element. The multiple sequence alignments were performed using the CLUSTAL W server [28].

STING proteins are localized on the endoplasmic reticulum membrane of eukaryotic cells and are CDN sensors that, when bound, regulate the induction of type I interferons (IFN-α and IFN-β), thus eliciting the intracellular signals of the invasion by bacteria and/or viruses, and activating the innate immune response to attack the pathogen. STING proteins can directly sense the pathogen invasion by interaction with bacterial CDNs (3’-5’ c-di-GMP, 3’-5’ c-di-AMP or 3’-5’ cGAMP) or indirectly by binding to eukaryotic 2’-3’ cGAMP through its C-terminal domain (TMEM173, PF15009). It is controversial whether STING binds 2′-5′ cGAMP preferentially in relation to other CDNs, or binds all of them with the same affinity [29].

Other important c-di-GMP receptors are proteins containing PilZ domains (PF07238). PilZ domains regulate twitching and swarming motility via the flagellar regulator YcgR protein [7], but proteins containing different domain architectures are related with other functions, such as the regulation of the synthesis of cellulose by BcsA in *Rhodobacter sphaeroides*, or chemotaxis by MapZ protein and alginate secretion by Alg44 to promote biofilm formation in *Pseudomonas aeruginosa* (**Figure 1**E and **Table 1**). PilZ domain is found associated with different domains that could be sensor domains, such as GAF, Cache, and PAS domains, and catalytic domains such as GGDEF, EAL, and Peptidase_S8. Therefore, proteins containing PilZ domain could be classified based on their domain architecture and function in different paralogous families [30][31]. The Pfam database describes 221 different domain architectures containing PilZ domains [32], showing the diversity of signaling networks in which c-di-GMP can be involved and have not yet been explored.

It is interesting that two proteins containing PilZ domains that are c-di-GMP receptors have been found to be involved in the production of different exopolysaccharides to produce bacterial biofilms: cellulose and alginate. Moreover, another c-di-GMP receptor is also involved in exopolysaccharide production, the PelD of *Pseudomonas aeruginosa* that regulates the synthesis of the Pel exopolysaccharide (**Table 1**).

PilZ proteins interact with c-di-GMP by two conserved sequence motifs: RxxxR and DxSxxG motifs (**Figure 1**D). In the RxxxR motif located in a loop at the N-terminal part of the PilZ domain, each arginine is interacting with the phosphate group and the base of the ligand. In the case of DxSxxG motif, the aspartic acid, serine and glycine residues bind the base and the pentose ring of the c-di-GMP molecule (**Figure 1**G). Other residues not conserved within members of the PilZ family are also involved in ligand binding and some of them are located at the β-strand 7 of the PilZ protein (**Figure 1**D,F,G). Some PilZ proteins lost their canonical residues to bind c-di-GMP and are not c-di-GMP receptors anymore but may work as protein–protein adaptors, as happens with the complex FimX-PilZ-PilB that regulates the twitching motility in *Xanthomonas citri* [8]. This ternary complex is an example of a full set of "degenerate" GGDEF, EAL, and PilZ domains, in which GGDEF does not synthesize c-di-GMP, PilZ does not bind c-di-GMP, and the EAL domain does not cleave c-di-GMP but kept the ability to bind it [8].

Degenerate EALs proteins lost their ability to cleave c-di-GMP to pGpG, and some of them still bind c-di-GMP molecules but do not cleave them changing its function from enzyme to a CDN receptor. The residues involved in c-di-GMP interaction are described in **Table 1** and **Figure 1**H,I. The loss of the EAL domain catalytic function seems to be related with a change in the residues important for the coordination of a magnesium cation (**Figure 1**H,I).

In *Xanthomonas citri*, *Xanthomonas campestris*, and *Pseudomonas aeruginosa*, FimX proteins regulate twitching motility by sensing c-di-GMP levels via interaction with degenerate EAL domain and regulates type IV pilus machinery [8]. LapD from *Pseudomonas fluorescens* is a transmembrane protein that binds c-di-GMP through its C-terminal degenerate EAL domain to prevent cleavage of the surface adhesin LapA and therefore activates biofilm formation [33].

Different classes of RNA riboswitches sense different kinds of CDNs (**Table 1**). Riboswitches are structured RNAs located in the 5'-untranslated regions of mRNAs and some can sense CDNs molecules to change its structure to regulate expression of downstream genes that could be involved with virulence, motility, biofilm formation, cell wall metabolism, synthesis and transport of osmoprotectants, sporulation, and other important biological processes [34][35].

There are three distinct classes of riboswitches that bind specific CDNs and have had their structures solved in complex with their ligand and deposited in the Protein Data Bank: c-di-GMP I riboswitch (RF01051), c-di-GMP II riboswitch (RF01786), and c-di-AMP riboswitch (ydaO-yuaA riboswitch, RF00379). C-di-GMP I riboswitch and c-di-GMP II riboswitch bind c-di-GMP molecules while c-di-AMP riboswitch binds c-di-AMP molecules [36][37][38]. The c-di-GMP I riboswitch was originally annotated as a conserved RNA-like structure of Genes Related to the Environment, Membranes and Motility (GEMM motif) and later another c-di-GMP riboswitch class was identified, the c-di-GMP II riboswitch. They have the same function but do not share any sequence motif or structural similarities. The c-di-AMP riboswitch is one of the most common riboswitches in various bacterial species and is found in the vicinity of genes related to cell wall metabolism, sporulation in Gram-positive bacteria, and other important biological processes [34][35]. These structures reveal that the RNAs use different ways to bind CDNs.

The TetR-like transcriptional factor, DarR, from *Mycobacterium smegmatis* was the first c-di-AMP receptor discovered [39], where c-di-AMP stimulate the DNA binding activity of this protein. DarR is a repressor that negatively regulates the expression of its target genes [39]. Another protein that interacts with c-di-AMP by a poorly understood mechanism is KdpD/KdpE that controls the potassium uptake in situations where the potassium concentrations are extremely low and other uptake systems wouldn't be enough to give the cell all potassium it requires. In *Escherichia coli*, there are three systems responsible for potassium uptake, namely, Trk, Kdp, and Kup. In the case of Trk system, four genes are constitutively expressed and TrkA is the predominant potassium transporter at neutral pH. The Kdp-ATPase system is induced at low potassium concentrations and under conditions of osmotic stress. The Kup, formerly TrkD, is activated when TrkA and Kdp activities are not sufficient [40][41][42]. In *Bacillus subtilis*, a novel high-affinity transporter KimA (formerly YdaO) has recently been characterized and the expression of KimA and KtrAB is negatively regulated by c-di-AMP riboswitches [36]. When the concentration of potassium is high in the cell, the concentration of c-di-AMP increases inhibiting potassium uptake by two ways, by binding to c-di-AMP riboswitches that will avoid the expression of proteins involved in transport, and by direct interactions with regulatory subunits of KtrAB and KtrCD causing the inhibition of potassium transport [43]. A similar process seems to happen in *Staphylococcus aureus*, where c-di-AMP binds to the KtrA protein and to the universal stress protein (USP) domain of the KdpD sensor kinase inhibiting the expression of Kdp potassium transporter components. In this manner, c-di-AMP appears to be a negative regulator of potassium uptake in different Gram-positive bacteria [44][43].

One of the most well understood receptors for c-di-AMP is KtrA, which binds c-di-AMP through its C-terminal domain (RCK_C or TrkA_C) to cause inactivation of the KtrA function (**Table 1**). c-di-AMP binds to the interface of the KtrA homodimer, and the residues involved in the ligand interaction are described in **Table 1**. Another c-di-AMP receptor is the c-di-AMP receptor domain (PF06153) of the PII-like signal transduction protein, PstA. PstA is a homotrimer and, in each protein interface, one c-di-AMP molecule is bound. The residues involved in ligand binding in PstA are also described in **Table 1**.

c-di-AMP is also related with negative control of aspartate and pyruvate pools in *Lactococcus lactis* by a pyruvate carboxylase, LlPC protein, and *Listeria monocytogens* pyruvate carboxylase, LmPC protein, respectively. In both cases, c-di-AMP binds to the pyruvate carboxylase domain (HMGL-like domain in the Pfam) (**Table 1**). LIPC forms a tetramer and each c-di-AMP molecule binds the protein dimer interface at the carboxyltransferase (CT) domain in a binding site pocket containing residues that are poorly conserved among pyruvate carboxylases [45].

The huge repertoire of CDN receptors demonstrates the complexity of CDN signaling networks in bacteria. Additionally, CDNs may regulate different bacterial behaviors at different speeds through regulation of gene transcription by transcriptional factors, protein translation by riboswitches, and directly by regulating the function of different classes of protein.

Table 1. List of the bacterial c-di-GMP, c-di-AMP, cGAMP, and eukaryotic cGAMP receptors that had their structure solved in complex with their ligand and deposited in the Protein Data Bank (PDB). The Pfam/Rfam and, in some cases, the InterPro domain is described. The residues involved in ligand binding are also described for a representative of each receptor.

Receptor Class (Pfam/Rfam)	Organism (PDBID)	Receptor Function	Ligand Binding Site	Ref.
3′-5′ c-di-GMP				
STING (TMEM173, PF15009)	*Homo sapiens* (4EF4, 4EMT, 6RM0, 6S86, 4F9G, 4F5D, 4F5Y)	Members of Transmembrane Protein 173 (TMEM173) family, also known as Stimulator of Interferon Genes (STING), are an important component of the immune system. STING proteins are responsible for regulating the induction of type I interferon via activation of INF-β gene transcription.	STING proteins interact with c-di-GMP at the protein dimer interface in a perfectly symmetrical manner increasing the homodimer stability. This binding involves a hydrophilic core, that in the human STING (PDB 4F5D) corresponds to, S162, G166, Y167, R238, Y240, S241, N242, E260, T267, and the presence of two Mg^{2+} ions and two water molecules (**Figure 1**A–C). STING proteins bind monomers of c-di-GMP that are stabilized in the protein pocket at *intermediate* or *closed* conformations, **Figure 2**.	[47] [48] [49] [50] [51] [52]
	Sus scrofa (6A04)	Human STING (carrying the more common R232 allele) binds eukaryotic 2′-3′ cGAMP with high affinity compared with bacterial CDNs such as c-di-GMP, c-di-AMP, and 3′-5′ cGAMP [46]. Nevertheless, it is controversial whether STING binds 2′-5′ cGAMP preferentially since others STINGs binds CDNs with the same affinity [29].		[53]
	N. vectensis (5CFL, 5CFP)			[54]

Receptor Class (Pfam/Rfam)	Organism (PDBID)	Receptor Function	Ligand Binding Site	Ref.
3′-5′ c-di-GMP				
c-di-GMP I Riboswitch (RF01051)	*V. cholerae* (3MXH, 3MUT, 3MUR, 3MUM, 3IRW)	c-di-GMP Riboswitches, also known as GEMM (Genes for the Environment, Membranes and Motility), are structured RNAs located in the 5′-untranslated regions of mRNAs that sense c-di-GMP molecules to regulate expression of downstream genes that could be involved with virulence, motility and biofilm formation. Despite having the same function, the c-di-GMP I Riboswitch and c-di-GMP II Riboswitch do not share any sequence motifs or structural features.	GEMM Riboswitches interacts with c-di-GMP by an uncharacterized motif with high affinity, at the picomolar range, compared to c-di-GMP protein receptors, with nanomolar to micromolar affinities. In the case of c-di-GMP I Riboswitch (PDB 3IRW) the nucleotides involved in ligand binding are: G14, C15, A16, C17, A18, G19, G21, C46, A47, A48, A49, G50. c-di-GMP II riboswitch (PDBID 3Q3Z) binds to c-di-GMP through the nucleotides: A13, A14, U37, G39, U60, A61,C68, A69, A70, C71, C72, G73, and A74. Riboswitches can recognize the guanine base of the ligand in different ways. The ligand was found as *closed monomers*, **Figure 2**.	[55] [56]
	Geobacter (4YB0)			[57]
	E. coli (3IWN)			[58]
c-di-GMP II Riboswitch (RF01786)	*C. acetobutylicum* (3Q3Z)			[56]

Receptor Class (Pfam/Rfam)	Organism (PDBID)	Receptor Function	Ligand Binding Site	Ref.
3′-5′ c-di-GMP				
PilZ domain (PF07238)	*V. cholerae* (2RDE)	VCA0042 is an important protein for the efficient infection of mice by *V. cholerae*. This PilZ-containing protein senses the bacterial second messenger c-di-GMP and controls virulence factors.	This PilZ domain interacts with monomeric c-di-GMP via two main sequence motifs: RxxxR and DxSxxG motifs (PDBID: 2RDE), **Figure 1**D, E. The ligand was found as *intermediate monomers*, **Figure 2**.	[30]

Receptor Class (Pfam/Rfam)	Organism (PDBID)	Receptor Function	Ligand Binding Site	Ref.
3′-5′ c-di-GMP				
	R. sphaeroides (5EIY, 5EJ1, 5EJZ, 4P00, 4P02)	BcsA, Bacterial cellulose synthase A, is a component of a protein complex that synthesizes and translocates cellulose across the inner membrane. The binding of c-di-GMP to a complex BscA and BcsB releases the enzyme from an autoinhibited state, generating a constitutively active cellulose synthase.	Most PilZ domains interact with dimeric c-di-GMP, in which one molecule interacts with two main sequence motifs on the β-barrel surface, DxSxxG and RxxxR motifs (PDBI: 5EIY, 5EJ1, 5EJZ, 4P00, 4P02, 5Y6F, 5Y6G, 5VX6, 5KGO, 5EJL, 5XLY, 2L74, 5Y4R, 4RT0, 4RT1). In the PilZ domain of YcgR (PDBID: 5Y6F) the “DxSxxG” motif corresponds to D145, S147 and G150, and the “RxxxR” motif corresponds to R114 and R118, **Figure 1**D,E. The ligand was found as *closed dimers*, **Figure 2**. One PilZ was found to interact with a trimeric c-di-GMP (PDBID: 4XRN), **Figure 2**B.	[59] [60]
	E. coli (5Y6F, 5Y6G)	YcgR like proteins such as the motility inhibitor (MotI) protein is a diguanylate receptor that binds c-di-GMP, acting as a molecular clutch on the flagellar stator MotA to inhibit swarming motility.		[61]
	B. subtilis (5VX6)	The PilZ domain of MrkH, also a YcgR like protein, is transcriptional regulator protein, and binds c-di-GMP as well as DNA sequences to regulate type 3 fimbriae expression and biofilm formation.		[62]
	K. pneumoniae. (5KGO, 5EJL)	YcgR proteins regulate motility and biofilm formation by sensing c-di-GMP.		[63] [64]

Receptor Class (Pfam/Rfam)	Organism (PDBID)	Receptor Function	Ligand Binding Site	Ref.
3′-5′ c-di-GMP				
	P. aeruginosa. (5XLY, 2L74, 5Y4R)	MapZ in complex with c-di-GMP interacts directly with a chemotaxis methyltransferase, CheR1, and inhibits its activity. In this manner, it regulates chemotaxis in *Pseudomonas aeruginosa*.		[65] [66] [67]
	P. aeruginosa (4RT0, 4RT1)	The alginate biosynthesis protein Alg44 regulates alginate secretion to promote biofilm formation by sensing dimeric c-di-GMP molecules.		[68]
	P. aeruginosa (4XRN)	Unknown function	The ligand is in an unusual trimeric oligomerization state, in which the six guanine bases are oriented almost parallel to each other, **Figure 2**B.	[69]

Receptor Class (Pfam/Rfam)	Organism (PDBID)	Receptor Function	Ligand Binding Site	Ref.
3′-5′ c-di-GMP				
I-site of GGDEF domains (PF00990)	*P. fluorescens* (5EUH for GcbC)	Proteins containing GGDEF domains are DGCs and some of them are regulated by feedback regulation by interaction of c-di-GMP to their allosteric site (I-site).	Proteins with GGDEF domain act as receptor proteins when c-di-GMP binds their allosteric site via the RxxD motif. In the WspR GGDEF (PDB 3BRE) this motif corresponds to Arg242, Ser243, Ser244 and Asp245. The ligand was found as *closed dimers*, very similar to the PilZ proteins, **Figure 2**.	[70]
	P. aeruginosa (3BRE and 3I5C for WspR; 4EUV, 4ETZ, 4EU0 for PelD)			[71] [72] [73]
	P. syringae (3I5A for WspR)			[71]
	M. hydrocarbonoclasticus (3IGN for MqR89a)			[74]
	T. maritima (4URG, 4URS for TM1788)			[75]
	C. vibrioides (1W25, 2WB4, 2V0N for PelD)			[76] [77] [78]
	E. coli (3TVK, 4H54 for DgcZ)			[79]
	P. aeruginosa (4DN0)	PelD is a membrane protein in which the cytoplasmatic GGDEF domain binds c-di-GMP to regulate the synthesis of the PEL exopolysaccharide.		[80]

Receptor Class (Pfam/Rfam)	Organism (PDBID)	Receptor Function	Ligand Binding Site	Ref.
3′-5′ c-di-GMP				
Degenerate EAL domains (PF00563)	*X. citri* (4FOK, 4FOJ, 4FOU)	The FimX protein regulates twitching motility by sensing c-di-GMP molecules through its EAL domain and regulates the type IV pilus machinery.	Proteins with EAL domain, such as FimX (PDB 4FOK), interact with the c-di-GMP by Q463, F479, L480, R481, S490, P491, M495, D508, R534, E653, F654, Q673, G674, D675 and T680. The $A_{478}F_{479}L_{480}$ residues belong to a degenerate EAL motif, **Figure 1**H and I. The ligand was found always as *open or intermediate monomers*. Different EAL containing proteins bind the most diverse c-di-GMP conformation states analyzed in this review, **Figure 2**.	[81]
	P. aeruginosa (3HV8)			[82]
	X. campestris (4F3H, 4F48)			[83]
	V. cholerae (6PWK, 6IH1)	The transmembrane receptor LapD is a multidomain protein, in which the C-terminal EAL domain binds c-di-GMP to prevent cleavage of the surface adhesin LapA, inhibiting biofilm dispersal.		[84] [85]
	P. fluorescens (3PJT, 3PJU)			[33]
C-terminal domain of BldD (PF not defined)	*S. venezuelae* (5TZD, RsiG protein: 6PFJ and, RsiG-σ^{WhiG} complex: 6PFV)	BldD is a master regulator of cell development. BldD represses the transcription of close to 170 sporulation genes during vegetative growth controlling morphological differentiation and also directly control expression of antibiotics. BldD has an N-terminus helix-turn-helix motif (HTH), while the C-terminal domain binds four c-di-GMP molecules to regulate cell differentiation.	The C-terminal domain of BldD (PDB 5TZD) interacts with a tetramer of c-di-GMP, forming a $BldD_2$-$(c\text{-}di\text{-}GMP)_4$ complex, by two motifs: $R_{114}G_{115}D_{116}$ and $R_{125}Q_{126}D_{127}D_{128}$. The ligand was found as *closed tetramers*, **Figure 2**. A dimer of RsiG or RsiG in complex σ^{WhiG} binds $(c\text{-}di\text{-}GMP)_2$ at the dimer interface and the ExxxSxxRxxxQxxxD motif of each helix of a coiled coil are involved in the ligand binding. The two repeats are: $E_{64}xxxS_{68}xxR_{71}xxxQ_{75}xxxD_{79}$ and $E_{162}xxxS_{166}xxR_{169}xxxQ_{173}xxxD_{177}$. The residues D106, S108, H110, S112 and R115 of RsiG also bind $(c\text{-}di\text{-}GMP)_2$ as well as the K57, G61 and R62 of σ^{WhiG}. The ligand was found as *intermediate dimer*.	[86] [87]
	S. coelicolor (4OAZ)			[9]

Receptor Class (Pfam/Rfam)	Organism (PDBID)	Receptor Function	Ligand Binding Site	Ref.
3′-5′ c-di-GMP				
REC domain (Response_reg, PF00072)	*V. cholerae* (3KLO)	VpsT is transcriptional regulator that binds c-di-GMP at its REC domain to control biofilm formation and motility. VpsT is described as a master regulator for biofilm formation and consists of an N-terminal REC domain and a C-terminal HTH domain.	A c-di-GMP_2 binds into the VspD interface between two REC domains; the REC dimerization is required for ligand binding. Proteins with the REC domain of VpsT (PDB 3KLO) interact with two molecules of c-di-GMP by a K and a W[F/L/M][T/S]R motif that correspond to: K120, W131, L132, T133 and R134. The ligand was found as *closed dimers*, **Figure 2**.	[88]
Pseudo-receiver Domain	*C. vibrioides* (6QRL)	ShkA has a pseudoreceiver domain (Rec1) that binds c-di-GMP to allow the autophosphorylation and subsequent phosphotransfer and dephosphorylation of the protein. The c-di-GMP binds to the protein to release the C-terminal domain to step through the catalytic cycle.	C-di-GMP binds to the Rec1-Rec2 linker that contain the DDR motif. The residues involved in the ligand binding are: R324, Y338, I340, P342, R344, S347, Q351. The D369, D370 and R371 from the DDR motif located in a loop are inside of the c-di-GMP binding site in the apo form of the protein suggesting that c-di-GMP compete with this protein loop.	[89]
T2SSE_N domain (PF05157)	*V. cholerae* (5HTL)	MshE is an ATPases associated with the bacterial type II secretion system, homologous to the type IV pilus machinery. Its N-terminal domain binds c-di-GMP and cGAMP with different affinities, while the C-terminal catalytic domain binds ATP. The MshE N-terminal domain (T2SSE_N) binds c-di-GMP (*Kd* of 0.5 μM) with higher affinity than cGAMP (*Kd* of 330 μM).	The N-terminal domain of MshE (locus tag VC0405, PDB 5HTL) interacts with c-di-GMP by mainly two similar motifs spaced by five residues. These motifs have a similar sequence, RLGxx(L)(V/I)xxG(I/F)(L/V)xxxxLxxxLxxQ, and the residues involved to ligand binding are shown in bold and correspond to $R_9L_{10}G_{11}$ and $L_{25}xxxL_{29}xxQ_{32}$ for the motif I, and $R_{38}L_{30}G_{40}$ and $L_{54}xxxL_{58}xxQ_{61}$ for motif II. Other residues also important to ligand binding are: R7, D108 (from the C-terminal ATPase domain), and the main chain of D41. The ligand was found as *open monomers*, similar to those found in EAL domains, **Figure 2**.	[90]

Receptor Class (Pfam/Rfam)	Organism (PDBID)	Receptor Function	Ligand Binding Site	Ref.
3′-5′ c-di-GMP				
MerR domain (PF00376)	*P. aeruginosa* (5XQL)	BrlR upregulates the expression of multidrug efflux pumps. c-di-GMP activates BrlR expression and enhances its affinity for binding DNA. BrlR has an N-terminus DNA-binding motif (HTH_MerR domain described in the Pfam as MerR domain), and a C-terminus effector-binding domain (GyrI-like domain) linked by a coiled-coil region.	There are two different c-di-GMP binding sites located at the N-terminus of the protein, mainly at the DNA binding domain of each BrlR protomer of the protein tetramer. Binding site 1 is composed of M1, R31, D35, Y40, and Y270. The binding site 2 is composed of P61, A64, R67, R70, F83, R86. The ligand was found as *closed monomers*, **Figure 2**.	[91]
Sigma54_activat (PF00158) or **AAA+_ATPase** (IPR003593)	*P. aeruginosa* (5EXX)	FleQ is a transcription regulator and a contains three domains: a central AAA+ ATPase σ(54)-interaction domain, flanked by a divergent N-terminal receiver domain and a C-terminal helix-turn-helix DNA-binding motif. FleQ binds c-di-GMP through itsAAA+ ATPase domain at a different binding site than the catalytic pocket site. FleQ regulates the expression of flagellar and exopolysaccharide biosynthesis genes in response to cellular levels of c-di-GMP.	FleQ binds c-di-GMP at the N-terminal part of the AAA+ ATPase through the $L_{142}F_{143}R_{144}S_{145}$ motif (R-switch), $E_{330}xxxR_{334}$ motif, and residues R185 and N186 of the post-Walker A motif KExxxRN. The ligand was found as *closed dimers*, **Figure 2**.	[92]

Receptor Class (Pfam/Rfam)	Organism (PDBID)	Receptor Function	Ligand Binding Site	Ref.
3′-5′ c-di-GMP				
HATPase_c (PF02518)	*C. vibrioides* (5IDM)	Cell cycle kinase CckA is a bifunctional histidine kinase/phosphatase enzyme, mediating both phosphorylation and dephosphorylation of downstream targets. CckA binds c-di-GMP and drives the cell cycle progression by swapping the CckA kinase activity into phosphatase mode.	CckA is a membrane and multidomain protein, in which a catalytically active (CA) domain binds c-di-GMP. The CA domain of cell cycle kinase CckA interacts with c-di-GMP by the residues Y514, K518, W523, I524, E550, *H551, *H552, *H553, *H554 and *H555. * H551 to H555 belong to a polyhistidine fusion tag at the C-terminus of the protein. Therefore, these histidines are not biological interactions. The ligand was found as *open monomer*, **Figure 2**.	[93]
3′-5′ cGAMP or 3′-3′ cGAMP				
STING (TMEM173, PF15009)	*N. vectensis* (5CFM)	STING regulates the induction of type I interferons via recruitment of protein kinase TBK1 and transcription factor IRF3, activating IFN-β gene transcription. cGAS-STING responds to cytosolic DNA via binding to 3’-5’cGAMP.	STING proteins interact with cGAMP at the dimer interface. In the anemone STING (PDBID 5CFM), the residues involved with the ligand interaction are: Y206, R272, F276, R278, and T303 of each protomer of the dimer. Y280 binds the ligand by a water molecule. The ligand was found as *intermediate monomer*, **Figure 2**.	[54]
c-di-GMP I Riboswitch (RF01051)	*Geobacter* (4YAZ)	Acts as a transcriptional factor, switching between RNA secondary structures when bound to cGAMP, regulating its own expression. A human c-di-GMP I Riboswitch mutant (G20A) can also bind cGAMP.	3’-5’ cGAMP riboswitches bind cGAMP (PDBID 4YAZ) through the nucleotides G8, A11, A12, U13, A14, C15, A41, A42, G74, C75, and C76. The ligand was found as *closed monomer*, **Figure 2**.	[57] [57]
	Homo sapiens (4YB1)			
2’-3′ cGAMP				

Receptor Class (Pfam/Rfam)	Organism (PDBID)	Receptor Function	Ligand Binding Site	Ref.
3′-5′ c-di-GMP				
STING (TMEM173, PF15009)	*Sus scrofa* (6A06)	STING regulates the induction of type I interferons via recruitment of protein kinase TBK1 and transcription factor IRF3, activating IFN-β gene transcription. The STING pathway plays an important role in the detection of viral and bacterial pathogens in animals.	STING proteins interact with 2’-3’ cGAMP produced by eukaryotic cGAS enzyme at the dimer interface. In the porcine STING (PDBID 6A06), the residues involved in ligand binding are: S162, Y167, I235, R232, R238, Y240, E260, and T263. The ligand was found as *closed monomer*, **Figure 2**.	[53]
	Gallus gallus (6NT7, 6NT8)			[94]
	Rattus norvegicus (5GRM)			[95]
	N. vectensis (5CFQ)			[54]
	Homo sapiens (4LOH, 4LOJ, 4KSY, 6DNK)			[46] [96] [97]
3′-5′ c-di-AMP				
STING (TMEM173, PF15009)	*Sus scrofa* (6A03, 6IYF)	STING binds eukaryotic 2’-3′ cGAMP with high affinity compared with bacterial CDNs such as c-di-GMP, c-di-AMP, and 3′-5′ cGAMP.	STING proteins interact with c-di-AMP in a different manner than c-di-GMP, but still at the same dimer interface. In the porcine STING (PDBID 6A03), the amino acids involved with the interaction are: S162, Y167, I235, R232, R238, Y240, and T263. The ligand was found as *closed monomers*, **Figure 2**.	[53]
	N. vectensis (5CFN)			[54]
	H. sapiens (6CFF and 6CY7)			[97]
	Mus moluscus (4YP1)			[98]

Receptor Class (Pfam/Rfam)	Organism (PDBID)	Receptor Function	Ligand Binding Site	Ref.
3′-5′ c-di-GMP				
Aldo-keto reductase (PF00248)	*Mus musculus* (5UXF)	RECON (reductase controlling NF-κB) is an aldo-keto reductase and a STING antagonist. It negatively regulates the NF-κB activation that induces the expression of IFN-induced genes. RECON recognizes c-di-AMP by the same site that binds the co-substrate nicotinamide. One AMP molecule (AMP1) of c-di-AMP has essentially the same position as the AMP portion of the NAD+ co-substrate, while another AMP (AMP2) presents a shifted position.	RECON binds c-di-AMP by the residues: E276, E279, N280, L219, and A253 in contact with AMP1, while Y24, Y216, Y55, and L306 are in contact with AMP2. L219, T221, and G217 are also involved in ligand binding. The ligand was found as open *monomers*, **Figure 2**.	[99]

Receptor Class (Pfam/Rfam)	Organism (PDBID)	Receptor Function	Ligand Binding Site	Ref.
3′-5′ c-di-GMP				
c-di-GMP I Riboswitch (RF01051)	*E. coli* (G20A/C92U mutant Riboswitch, 3MUV)	Bacterial c-di-AMP is involved in cell wall stress and signaling DNA damage through interactions with several protein receptors and a widespread *ydaO*-type riboswitch, one of the most common riboswitches in various bacterial species. This riboswitch is found in the vicinity of genes involved in cell wall metabolism, synthesis and transport of osmoprotectants, sporulation and other important biological processes [34][35]. A c-di-GMP I Riboswitch mutant (G20A/C92U, PDB 3MUV) can also bind c-di-AMP.		[55]
ydaO-yuaA Riboswitch (RF00379)	*T. pseudethanolicus* (4QK8 and 4QKA)		*ydaO* riboswitch (PDBID 3MUV) binds c-di-AMP molecules into two binding sites: site 1 (G5, C6, C7, G8, A45, G68, G69, A70, U71, A72, C82, C83, G107, C108, and A109) and site 2 (A9, G23, G24, A25, G26, G41, G42, U43, C88, C89, A93, G102, AND G103). The ligand was found as *closed monomers*, **Figure 2**.	[100]
	T. lienii (4QK9)			[100]
	B. subtilis (4W92 and 4W90)			[101]
	C. subterraneus (4QLM and 4QLN)			[102]
	H. sapiens (6N5K, 6N5L, 6N5N, 6N5O, 6N5P, 6N5Q, 6N5R, 6N5S and 6N5T)			[103]
Cyclic-di-AMP receptor (PF06153)	*S. aureus* (4WK1 and 4D3H)	PII-like signal transduction protein (PtsA) is a c-di-AMP receptor. PII-like proteins are associated with nitrogen metabolism using different pathways. PtsA binds c-di-AMP with a *Kd* of 0.37 μM (intracellular c-di-AMP is in μM range). Others c-di-AMP receptors bind the ligand with a *Kd* range of 0.1 to 8 μM.	PstA (PDBID 4D3H) forms trimers and binds to c-di-AMP at the interface between two molecules through interactions with the residues N24, R26, T28, A27, F36, L37, N41, G47, F99, and Q108. The ligand was found as *intermediate monomer*, **Figure 2**.	[104] [105]
	L. monocytogenes (4RWW)			[106]
	B. subtilis (4RLE)			[107]

Receptor Class (Pfam/Rfam)	Organism (PDBID)	Receptor Function	Ligand Binding Site	Ref.
3′-5′ c-di-GMP				
Pyruvate carboxylase (HMGL-like, PF00682)	*L. lactis* (5VYZ and 5VZ0)	*L. monocytogenes* pyruvate carboxylase (LmPC) or *L. lactis* pyruvate carboxylase (LIPC) are inhibited by c-di-AMP. LmPC is biotin-dependent enzyme with biotin carboxylase (BC) and carboxyltransferase (CT) activities.	LIPC forms a tetramer and each c-di-AMP molecule binds at a protein dimer interface at the carboxyltransferase (CT) domain (HMGL-like domain in the Pfam) (PDBID 5VYZ) in a binding site that is not well conserved among pyruvate carboxylases. The residues involved in the interaction are: Q712, Y715, I742, S745, G746, and Q749 from both monomers. The ligand was found as *intermediate monomers*, **Figure 2**.	[45]
	L. monocytogenes (4QSH and 4QSK)	c-di-AMP causes conformational changes in the CT dimer that may explain the molecular mechanism for its inhibitory activity.		[108]
TrkA_C (PF02080)	*S. aureus* (4YS2, 4XTT, and 5F29)	Potassium transporter A (KtrA) and Bacterial cation-proton antiporter (CpaA) are members of the RCK domain family of proteins (Regulator of conductance of K^+) and regulates the cellular potassium conductance. The C-terminal domain (RCK_C or TrkA_C) binds specifically c-di-AMP molecules (*Kd* of 43.1 nM), causing inactivation of the KtrA.	c-di-AMP binds at the RCK_C domain of KtrA in the interface of a dimer (PDBID 4XTT). The residues involved in the interaction are I163, I164, D167, I168, R169, A170, N175, I176, and P191 from both monomers. R169 and the isoleucine residues (hydrophobic pocket) are well conserved in other species. The ligand was found as *closed monomers*, **Figure 2**.	[109] [98] [110]

Receptor Class (Pfam/Rfam)	Organism (PDBID)	Receptor Function	Ligand Binding Site	Ref.
3′-5′ c-di-GMP				
CBS domain (PF00571)	*L. monocytogenes* (5KS7)	Intracellular pathogen *L. monocytogenes* synthesizes and secretes c-di-AMP during growth in culture and also in host cells. Overexpression of c-di-AMP is toxic to the cell. c-di-AMP binds to OpuC carnitine transporter at the CBS domain (*Kd* of 4.8 μM), probably inhibiting carnitine uptake. OpuC is the ATPase subunit of the transporter complex OpuCA.	c-di-AMP binds to the cystathionine β-synthase domain (CBS) of OpuC at the dimer interface. The residues involved in ligand binding are well conserved among OpuCA orthologues and are composed by the following residues: V260, V280, T282, Y342, I355, I357, R358, and A359. The ligand was found as *open monomers*, **Figure 2**.	[111]

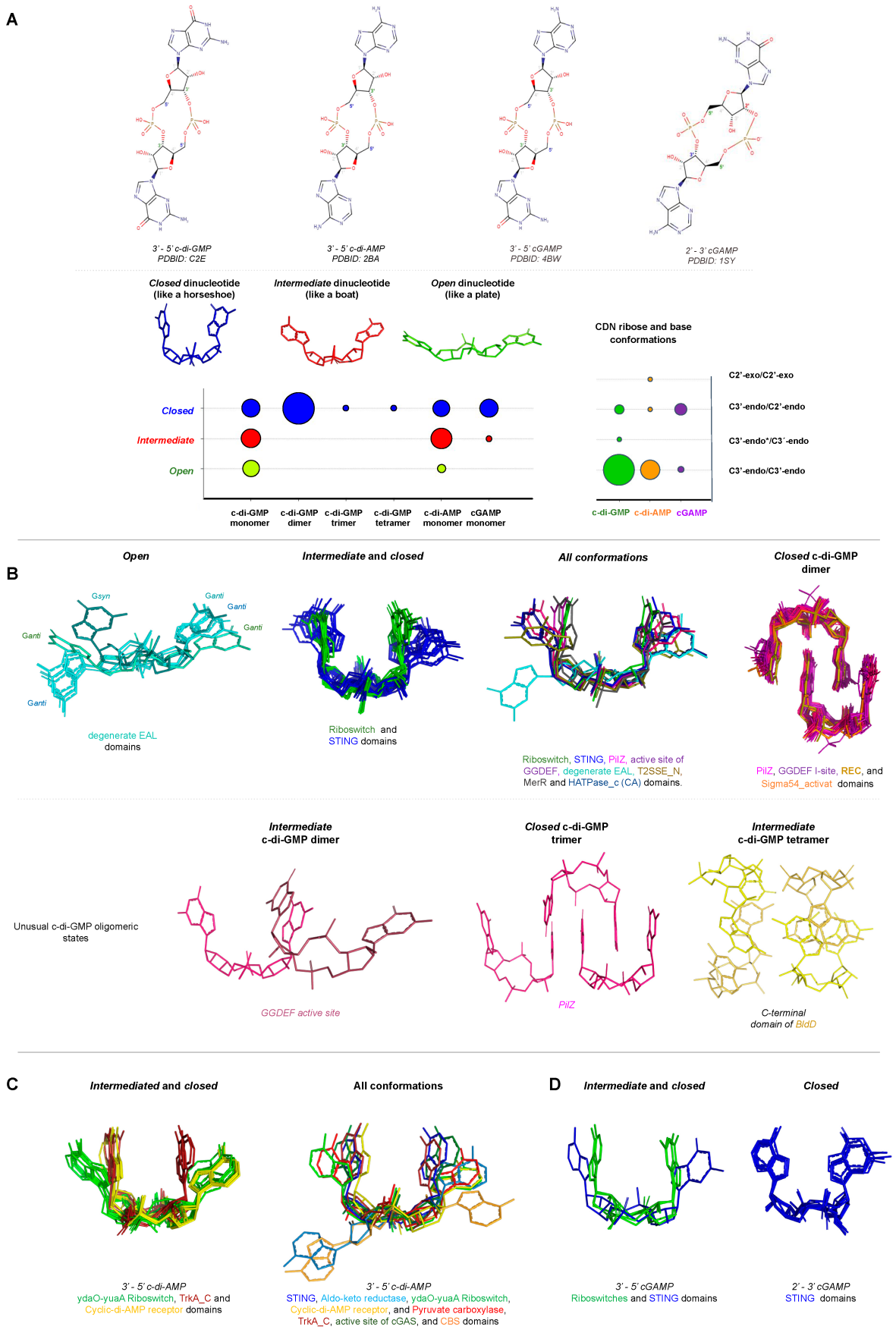

Figure 2. Diversity of cyclic dinucleotides produced by different organisms. All structures were observed within the three-dimensional protein structures deposited in the Protein Data Bank. (**A**) top panel: two-dimensional representation of different cyclic dinucleotides produced mainly by bacteria, with the exception of the 2'-3' cGAMP molecule that is produced by eukaryotic cells by cGAS enzymes. The linkages between the pentoses and the phosphates are shown in green, blue, or red, and those carbons colored in grey are not involved in the phosphate linkage cyclisation. The structures of each CDNs were initially downloaded in the SDF format at the CHEBI website [112] and edited using MarvinSketch version 19.18. The PDBID of each CDN is also described for each ligand. Bottom panel: The three main conformations of CDNs found in the protein binding pockets in respect to the base proximity are described as: 1-*closed* conformation (shaped as a horseshoe), when the two base rings are face-to-face, colored in blue; 2-*intermediate* conformation (shaped as a boat), when the bases are not in the *closed* conformation neither in the *open* conformation,

colored in red; and 3-*open* conformation (shaped as a plate), when the two base rings are far from each other in an elongated conformation, colored in lemon green. At the bottom of these structures is shown a bubble chart showing the frequency of each CDN in the *Closed*, *Intermediate*, and *Open* conformations. c-di-GMP is the only one that has been found in protein structures in different oligomeric states: as a monomer, dimers, trimers, and tetramers. The bubble chart on the right shows the ribose conformations that can be found in 3 different configurations, C3'-endo, C2'-endo, or C2'-exo, and the frequency of each of these conformation in the CDNs found in protein binding pockets. * The G*syn* conformation of the base ring in relation to the pentose is found only in one c-di-GMP structure (PDBID: 4FOK). Panels (**B**–**D**) show superpositions of different CDNs showing the heterogeneity of conformations found in the protein and riboswitch binding pockets; (**B**) top panel: different conformations for c-di-GMP found in different protein binding pockets and riboswitches shown as a superposition between them. 3'-5' c-di-GMP structures found in degenerate EAL domains are colored in cyan (PDBID: 3HV8, 4F3H, 4F48, 3PJT, 3PJU), in riboswitches are colored in green (PDBID: 3Q3Z, 3MXH, 3MUT, 3MUR, 3MUM, 3IRW, 4YB0, 3IWN), in STING proteins are colored in blue (PDBID: 4EF4, 4EMT, 6RM0, 6S86, 4F9G, 4F5D, 4F5Y, 6A04, 5CFL, 5CFP), in PilZ domains are colored in pink (dimeric c-di-GMP: PDBID: 4ZMN, 5EUH, 3BRE, 3I5C, 1W25, 2WB4, 2V0N, 3TVK, 3I5A, 3IGM, 4URG, 4URS. Trimeric c-di-GMP, PDBID: 4XRN), in the active site of GGDEF domains (monomeric c-di-GMP, PDBID: 4RT1), and in the GGDEF I-site (dimeric c-di-GMP, PDBID: 5EIY, 5EJ1, 5EJZ, 4P00, 4P02, 5KGO, 5EJL, 5VX6, 5Y4R, 5XLY, 2L74, 4RT0, 5Y6F, 5Y6G) are colored in purple, in the T2SSE_N domain is colored in brown (PDBID: 5HTL), in the HATPase_c (CA) domain is colored in blue (PDBID: 5IDM), in the REC domain is colored in yellow (PDBID: 3KLO), and in the Sigma54_activat domain is colored in orange (PDBID: 5EXX). The unusual c-di-GMP oligomeric states found in one GGDEF active site is colored in pink and brown (PDBID: 3QYY), and in the C-terminal domain of BldD is colored in yellow (PDBID: 4OAZ); (**C**) different conformations for 3'-5' c-di-AMP found in different protein binding pockets and riboswitches shown as a superposition between them. 3'-5' c-di-AMP structures found in riboswitches are colored in green (PDBID: 4QK8, 4QK9, 4W92, 4W90, 4QLM, 4QLN, 4QKA), in TrkA_C domains are colored in brown (PDBID: 4YS2, 4YP1, 5F29), in Cyclic-di-AMP receptor domains are colored in yellow (PDBID: 4WK1, 4D3H, 4RWW, 4RLE), in a STING protein is colored in dark blue (PDBID: 6IYF), in an Aldo-keto reductase domain is colored in light blue (PDBID: 5UXF), in a Pyruvate carboxylase domain is colored in red (PDBID: 5VZ0), in the active site of cGAS is colored in dark green (PDBID: 3C1Y), and in a CBS domain is colored in orange (PDBID: 5KS7); (**D**) 3'-5' cGAMP is found in *intermediate* and *closed* conformations in the ligand binding pocket of riboswitches, colored in green (PDBID: 4YAZ and 4YB1), and in a STING protein, colored in blue (PDBID: 5CFM). 2'-3' cGAMP is found in a *closed* conformation in the ligand binding pocket of STING proteins, colored in blue (PDBID: 6NT7, 6NT8, 5CFQ, 4LOH, 4LOJ, 5GRM, 4KSY, and 6A06).

4. Distribution of Proteins Containing GGDEF and DAC Domains in Bacteria

Initial reviews of the distribution of DisA homologs across bacterial clades suggested that c-di-AMP would play a more important role in Gram-positive bacteria than in Gram-negative and that, in general, bacteria would avoid allowing these two signaling networks to co-exist, so as to avoid unintended crosstalk and to easily regulate the balance of these second messengers within the cell [113][22]. Subsequent surveys on the distribution of DAC and GGDEF homologs don't support the idea that DAC homologs are rare among Gram-negative bacteria, as members of lineages such as Cyanobacteria, Spirochaetes, and Deltaproteobacteria often carry both DAC and GGDEF genes, a profile compatible with the complex lifestyles and genomes of these lineages. In addition, among Gram-positives, most members of Firmicutes and Actinobacteria, including model organisms such as *Bacillus*, *Clostridium*, *Streptomyces*, *Listeria*, and *Mycobacterium*, produce both signaling molecules and possess a wide array of GGDEF genes, following the general trend of having close to as many genomes with both DAC and GGDEF as possible (**Figure 3**). The only lineages were several of the genomes sampled that seem to have at least one DAC homolog, but no or very few and rare recognizable GGDEF homologs are Bacteroidetes and the Archaea. In both lineages, the number of genomes with both DAC and GGDEF falls below 50% of the maximum allowed, i.e., the smallest between the number of genomes carrying DAC or GGDEF. Genomic data strongly suggest that there is a tendency for bacterial cells to use both c-di-AMP and c-di-GMP signaling networks simultaneously, which would imply that both the control of their synthesis and turnover and the specificity of their sensors are carefully tuned.

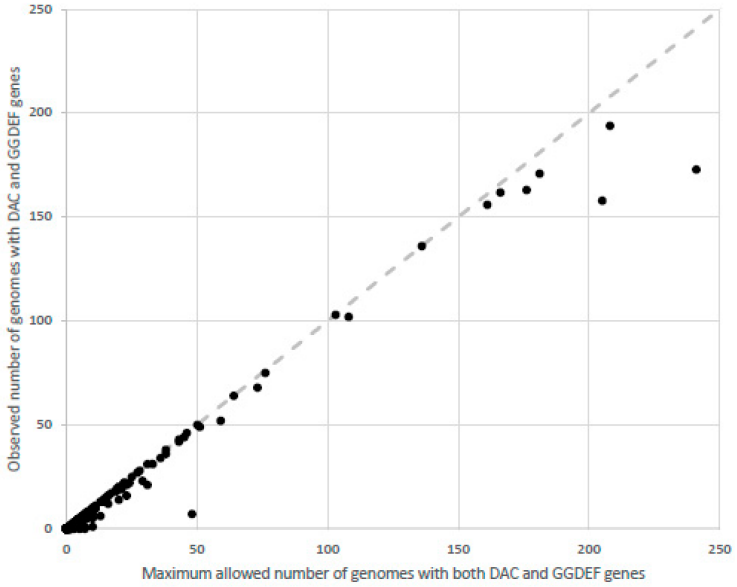

Figure 3. Lack of anti-correlation in the distribution of DAC and GGDEF genes per prokaryotic clades. Each dot represents a prokaryotic class, such as Gammaproteobacteria or Bacilli, as defined in the NCBI's Taxonomy Database. For each class, the number of genomes harboring at least one DAC and one GGDEF gene and the number of genomes harboring both was calculated. If, for a given class, we consider the number of genomes with DACs and the number of genomes with GGDEF, the smallest of these numbers is the maximum number of genomes that could, in principle, carry both genes. That number is seen on the horizontal axis while the actual number of genomes carrying both genes is on the *y*-axis. These numbers are very close to the diagonal line, indicating that, in most cases, if members of a given lineage are carrying both DAC and GGDEF, they tend to keep both genes, instead of having to choose between them.

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