Cyclic-di-AMP: another second messenger enters the fray

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Nucleotide signaling molecules contribute to the regulation of cellular pathways in all forms of life. In recent years, the discovery of new signaling molecules in bacteria and archaea and the pathways they regulate has brought insights into signaling mechanisms not only in bacterial and archaeal cells but also in eukaryotic host cells. Here, we provide an overview of the synthesis and regulation of c-di-AMP, one of the latest cyclic nucleotide second messengers to be discovered in bacteria. We will also discuss currently known receptor proteins and pathways that are directly or indirectly controlled by c-di-AMP, the domain structure of the enzymes involved in its production and degradation and the recognition of c-di-AMP by the eukaryotic host.

Nucleotides are indispensable components of all living cells as they make up DNA and RNA, and serve as important energy sources. In addition to these functions, nucleotides also play key roles in signaling in eukaryotic, bacterial and archaeal cells. In bacteria, signaling nucleotides such as cyclic adenosine monophosphate (cAMP) and guanosine tetra- or pentaphosphate (p)ppGpp have been classically linked to carbon metabolism and the stringent response, which is caused by nutrient limitation^{1, 2}. However, it has become clear that signaling nucleotides can also contribute to the regulation of multiple different pathways; for example in addition to its involvement in central carbon metabolism cAMP is also involved in the regulation of both biofilm formation and virulence gene expression in many pathogenic bacteria¹. One of the latest signaling nucleotides to be identified is cyclic diadenosine monophosphate (c-di-AMP), which after cyclic diguanosine monophosphate (c-di-GMP)³ (Box 1) is the second cyclic dinucleotide that has been shown to be produced by bacteria, but as will be described here, regulates very different processes.

c-di-AMP is produced from two molecules of ATP by diadenylate cyclase (DAC) enzymes and degraded to pApA by phosphodiesterase (PDE) enzymes (FIG. 1). It was initially discovered during a structural study on the *Thermotoga maritima* protein DisA⁴, a homologue of the *Bacillus subtilis* DNA integrity scanning protein DisA (formally Yack). This protein is a bacterial DNA damage checkpoint protein that can delay sporulation in the event of DNA damage⁵. The first report of c-di-AMP production by bacterial cells came in 2010, when it was identified as a molecule secreted into the cytosol of host cells by the intracellular bacterial pathogen *Listeria monocytogenes*⁶. Since then, it has been detected in cellular extracts from *Streptococcus pyogenes*⁷, *B. subtilis*⁸, *Chlamydia trachomatis*⁹ and *Staphylococcus aureus*¹⁰ and a DisA-type c-di-AMP synthesizing enzyme from *Mycobacterium tuberculosis* has been characterized biochemically¹¹. As previously suggested¹² and further analyzed here, c-di-AMP is likely to be widely distributed among bacteria and is also found in a subset of archaea. Based on the domain architecture, here we propose a unifying nomenclature for DAC enzymes.

The regulation of cellular pathways by c-di-AMP presumably follows the same general principles as for other signaling nucleotides, although most mechanistic details of c-di-AMP signaling are still awaiting molecular characterization. Environmental changes are sensed either directly or indirectly by the nucleotide synthesizing or degrading enzymes, leading to a change in the

cellular nucleotide concentration. At a high concentration, c-di-AMP is expected to bind to a specific set of receptor or target proteins and allosterically alter their function or the function of downstream effector proteins. In this manner c-di-AMP signaling could control specific cellular pathways (FIG. 1). Although many details of the c-di-AMP signaling network remain to be discovered, this nucleotide has now been linked to the regulation of fatty acid synthesis in *Mycobacterium smegmatis*¹³, to the growth of *S. aureus* in low potassium conditions¹⁴, to the sensing of DNA integrity in *B. subtilis*^{5, 8, 15}, and to cell wall homeostasis in multiple species^{10, 16-19}. In this Progress article, we will discuss the synthesis and regulation of c-di-AMP, the domain structure of the enzymes involved in its production and degradation and our current understanding of how this second messenger is involved in the control of diverse cellular pathways.

Synthesis of c-di-AMP

c-di-AMP is synthesized from two ATP molecules by proteins containing a DisA_N domain (Pfam02457) (FIG. 1). This domain was first identified as a c-di-AMP cyclase domain in the *T. maritima* DisA protein⁴. Based on its function as a diadenylate cyclase, DisA_N will be referred to hereafter as a DAC domain (diadenylate cyclase). In DisA proteins the N-terminal DAC domain is connected through a specific linker domain (DisA_N linker; Pfam10635) to a C-terminal helix-hairpin-helix (HhH; Pfam12826) DNA-binding domain. Eight DisA monomers form the functional octameric protein complex⁴. During crystallization studies on the *T. maritima* DisA an extra electron density was noted within the DAC domain and was identified as c-di-AMP⁴. It was further shown that in the presence of divalent cations DisA proteins synthesize c-di-AMP through the condensation of two molecules of ATP, each bound to a different subunit of the octamer^{4, 11}. The ligand-bound crystal structure of DisA revealed the presence of DGA and RHR motifs within the DAC domain⁴. These motifs are conserved within all bacterial DAC domain-containing proteins and a crucial function for these motifs in the cyclase reaction has been confirmed experimentally^{4, 11}.

The DAC domain bears no amino acid or structural similarity to the c-di-GMP cyclase GGDEF domain (Pfam00990). The observation that evolutionarily unrelated proteins have evolved for the synthesis of cyclic dinucleotides is further underscored by the recent identification of the dinucleotide cyclase DncV, which produces c-AMP-GMP (cGAMP(3'-5')) in *Vibrio cholerae*²⁰. DncV has been placed within a separate protein family, the nucleotidyl transferase superfamily (NTS), based on key amino acids it shares with 2'-5'-oligoadenyl synthases (OAS1) and poly(A) polymerases²⁰. DncV can also synthesize c-di-AMP *in vitro* and when overexpressed in *Escherichia coli*²⁰. However, only the cGAMP(3'-5') hybrid nucleotide was detected in *V. cholerae* extracts²⁰, suggesting that the c-di-AMP activity of DncV may not be physiological relevant. Currently, the DAC domain is the only *bona fide* c-di-AMP cyclase domain known. The finding that the different cyclic dinucleotides are produced by non-homologous enzymes might be somewhat surprising since it seems easier to reprogram a DGC to synthesize c-di-AMP instead of evolving a new cyclase. However, it has already been noted previously that the six different classes of prokaryotic cAMP cyclases do not have a common origin²¹. This highlights that enzymes involved in

the synthesis of the same signaling nucleotide may have evolved multiple times²¹.

To date, 1,956 DAC domain-containing sequences within 1,438 organisms can be found in the Pfam database (Table S1)²². The majority of these proteins are found in bacterial species but a small number are also present in archaea within the Euryarchaeota group (Box 2)¹². In bacteria, DAC domain proteins are most frequently found in the Gram-positive Firmicutes and within the phylum of Actinobacteria. In addition, the DAC domain is also found in Gram-negative bacteria, including the genera Bacteroidetes, Deltaproteobacteria, Cyanobacteria Chlamydiae and Fusobacteria (Table S1). Interestingly, most organisms contain only a single c-di-AMP cyclase, and organisms containing two or three DAC domains such as some *Clostridium* and *Bacillus* species are rather an exception (Table S1). This is in contrast to c-di-GMP production, where most organisms contain multiple c-di-GMP cyclases. The distribution of c-di-GMP and c-di-AMP synthesizing and degrading enzymes among bacteria is also different. The c-di-GMP turnover enzymes are especially abundant among Proteobacteria, and there not only found in deltaproteobacteria, which potentially also encode a c-di-AMP synthase, but widespread among alpha-, beta- and gamma-proteobacteria, organisms that lack a c-di-AMP signaling system²³⁻²⁵. On the other hand, c-di-GMP turnover enzymes have been reported to be absent in Bacteroidetes, Chlamydiales and Fusobacteria²⁵, which potentially encode a c-di-AMP synthesis enzyme. However the signaling systems are not mutually exclusive and several bacteria appear to contain both systems. Among the Firmicutes and Actinobacteria for instance, bacteria belonging to *Bacillus*, *Clostridium*, *Listeria*, *Mycobacterium* and *Streptomyces* spp. appear to contain both signaling systems, while other species such as *Staphylococcus*, *Streptococcus* and *Corynebacterium* no longer encode a functional c-di-GMP system. However as discussed later, the presence of degenerated GGDEF domains in *Staphylococcus* and *Streptococcus* spp. could be a remnant of the c-di-GMP system, which interestingly have now been rewired to function in the c-di-AMP signaling system.

Architecture of bacterial DAC domain proteins

DacA. Although the exact function of most DAC domain-containing proteins remains unknown, analysis of associated domains or the operon structure in which DACs are encoded can provide insights into the role of these proteins. The most abundant domain architecture for bacterial DAC proteins is one in which the DAC domain follows an N-terminal transmembrane domain (FIG. 2). Based on the nomenclature already used in several bacteria, a protein with this architecture is suggested to be referred to as DacA for diadenylate cyclase protein A (FIG. 2 and Table S1). It is plausible that the membrane domain of DacA acts as a sensory domain, directly sensing specific changes within the membrane or cell wall and adjusting c-di-AMP production accordingly. It has also been noted that the *dacA* gene is often found in an operon with a gene encoding a protein with one or multiple YbbR domains (Pfam07949)¹² (Table S1). In a few instances the YbbR domains are directly fused to DacA, and to distinguish these proteins from DacA proteins the fusions will be referred to as DacC proteins (FIG. 2). YbbR domains usually follow an N-terminal transmembrane helix and membrane topology predictions place them on the outside of the cell. It has recently been shown that YbbR (also named CdaR for cyclic di-AMP synthase A

regulator¹⁵) from *B. subtilis* physically interacts with DacA and stimulates cyclase activity¹⁵. The structures of two of the four YbbR domain-containing proteins from *Desulfitobacterium hafniense* Y51 have been solved by NMR and X-ray crystallography²⁶. It was noted that the YbbR domains assume a similar fold to the C-terminal domain of the ribosomal proteins TL5 from *Thermus thermophilus* and L25 from *Deinococcus radiodurans*^{27, 28}. It has been suggested that this domain serves to stabilize the interaction between the 5S rRNA, to which these proteins bind, and the 23S rRNA via an interaction with the 23S rRNA-binding ribosomal protein L16²⁶, highlighting that a protein with a similar fold to YbbR can bind both RNA and proteins. Therefore, it seems plausible that the extracellular YbbR domains of YbbR serve as sensory domains of a yet unknown signal and ligand in order to regulate the activity of DacA.

The *dacA* operon is frequently extended to include the glucosamine-1-phosphate mutase gene (*glmM*), which encodes a protein involved in an initial step of peptidoglycan synthesis. In *B. subtilis* this three gene *dacA*, *ybbR*, *glmM* operon is constitutively expressed from a housekeeping sigma factor SigA dependent promoter^{15, 16}. Along with the observed changes in cell wall structures in bacteria with altered c-di-AMP levels that are discussed later, this suggests a link between c-di-AMP and cell wall synthesis and homeostasis.

DisA. The second most common group of DAC domain-containing proteins are DisA-like proteins in which the DAC domain is connected through a specific linker region to a DNA binding domain (FIG. 2). Such DisA proteins are found in some Gram-negative bacteria but are mostly present in the Gram-positive bacterial spore-forming *Bacillus* and *Clostridium* spp. and in the Actinobacteria (Table S1). DisA has been assigned a function as a direct sensor of DNA integrity prior to spore formation and protein levels were shown to increase at a high cell density and at the onset of sporulation^{14, 15}. It is interesting to note that in *B. subtilis*, the *disA* gene is encoded in a large operon together with *radA*, encoding for a DNA repair protein and *clpC* and its accessory genes *mcsA* and *mcsB*, which are involved in degrading damaged proteins particular during heat stress²⁹. This large operon is preceded by SigA (housekeeping) and SigB (stationary phase) dependent promoters³⁰. Expression of *disA* is further controlled by an internal SigM dependent promoter that is activated by cell envelope stresses such as those elicited by antibiotics^{16, 30, 31}. The observation that *disA* expression is controlled by multiple promoters could suggest that DisA and hence c-di-AMP is important to ensure DNA integrity during multiple stresses and perhaps not only when bacteria enter sporulation. The function of the DisA homologues in Actinobacteria has not yet been studied. However, since *Streptomyces* spp. are also spore-forming bacteria and the slow growing *Mycobacterium* spp. enter a dormant state, it seems likely that, at least in these species, DisA and c-di-AMP ensure DNA integrity during these developmental processes.

DacB. The third most prevalent DAC domain-containing proteins are those in which a YojJ domain (Pfam10372) precedes the DAC domain (FIG. 2); these proteins are, with one exception, specific to the Bacillales (Table S1). To provide a consistent nomenclature, proteins with this domain architecture will be referred to as DacB for diadenylate cyclase protein B. The structure of the *Bacillus cereus* DacB protein (PDB number 2fb5) revealed that the N-terminal

YojJ domain forms an orthogonal bundle of long alpha helices that are followed by the DAC domain, which as expected has the same fold as the DAC domain of DisA⁴. A function as a c-di-AMP cyclase has been confirmed for the *B. subtilis* DacB protein^{15, 16}. DacB does not appear to be expressed in vegetative *B. subtilis* cells¹⁵ though its expression has been shown to increase during sporulation³². Based on these transcription data it has been suggested that *dacB* is under the control of the late forespore-specific sigma factor SigG^{15, 32} and hence c-di-AMP produced by DacB is likely to control sporulation specific processes.

Other bacterial DAC domain-containing proteins. DacA, DisA and DacB represent more than 98.7% of all bacterial DAC domain-containing proteins identified to date (FIG. 2). For the remaining proteins, the DAC domain is preceded in six cases by a phosphoenolpyruvate-dependent sugar phosphotransferase system EIIA (Pfam00359), indicating a possible link between sugar import and c-di-AMP synthesis in these organisms. In two cases the DAC domain is linked to differing tetrcopeptide repeat (TPR: Pfam00515, Pfam13414, Pfam13424) domains that are thought to mediate protein-protein interactions and in one case it is fused to a domain of unknown function (DUF4062: Pfam13271) (Table S1 and FIG. 2). To provide a consistent nomenclature, we propose to rename these proteins DacD, DacE and DacF, respectively (Table S1 and FIG. 2).

Degradation of c-di-AMP

Following the discovery of c-di-AMP synthesizing proteins, the *B. subtilis* PDE enzyme YybT, which can hydrolyze c-di-AMP, was discovered³³. This protein will be referred to as GdpP for GGDEF domain protein containing phosphodiesterase, for consistency in nomenclature with homologues in other bacteria. The *gdpP* gene is in an operon with *rplI*, the gene encoding for the ribosomal protein L9, and is also frequently located close to, if not in an operon with, the gene encoding for DnaC, a protein important for DNA replication. Expression of the c-di-AMP-specific PDE together with a ribosomal and a DNA replication protein might suggest that it is important to keep c-di-AMP levels low during active growth and replication. Recently it has been reported that *gdpP* expression itself is regulated in *B. subtilis* by an antisense transcript under the control of SigD³⁴. However, if and how this antisense regulation translates into changes in cellular c-di-AMP levels awaits further investigation. The GdpP protein contains two transmembrane helices, a degenerate PAS sensory domain (Pfam00989), a highly divergent GGDEF domain (Pfam00990), a DHH domain (Pfam01368) and a DHH-associated DHHA1 domain (Pfam02272) (FIG. 2). The PDE activity is contained within the C-terminal DHH/DHHA1 region, which efficiently degrades c-di-AMP to 5'-pApA (FIG. 1) with a Michaelis-Menten constant (*K_m*) of 1.3 +/- 0.3 μ M³³. GdpP requires Mn²⁺ for activity and mutations of predicted and conserved metal binding residues abrogate the PDE activity of the *B. subtilis* and *S. aureus* GdpP enzymes^{10, 33}. Using an *in vitro* assay, it has been shown that heme binding to the PAS domain suppresses the PDE activity of GdpP while nitric oxide (NO) coordination by heme stimulates the PDE activity³⁵. This suggests that GdpP is a heme and/or NO sensor and coordinates the degradation of c-di-AMP accordingly. GdpP also contains a highly modified GGDEF domain. GGDEF domains are typically associated with proteins involved in the synthesis of c-di-GMP, in which two GGDEF domains, each bound to one molecule of GTP, produce

c-di-GMP³⁶. However in some instances, when a GGDEF and a PDE domain are present within a single protein, such as in PdeA from *Caulobacter crescentus*, binding of GTP does not result in the synthesis of c-di-GMP but instead regulates the activity of the PDE domain³⁷. The GGDEF motif in GdpP is highly modified and produces neither c-di-GMP nor c-di-AMP³³. Instead, it binds and slowly hydrolyzes ATP to ADP³³, but the physiological relevance of this enzymatic activity remains to be determined. Binding of a non-hydrolysable ATP analogue did not affect the PDE activity³³, however mutations of several conserved amino acids within the GGDEF domain in *S. aureus* GdpP significantly reduced c-di-AMP hydrolysis¹⁰, suggesting a regulatory function for this domain. It is also interesting to note that the stringent response signaling molecule ppGpp is a strong competitive inhibitor of GdpP activity *in vitro*³³. This not only links the networks of two different signaling nucleotides but also suggests that under stringent response stress conditions cellular c-di-AMP levels will increase through a decrease in PDE activity.

GdpP-type proteins containing a PAS, GGDEF and the DHH/DHHA1 PDE domains are only found in Firmicutes and in Tenericutes (Table S1) and therefore yet to be identified proteins with different domain architectures, potentially containing a DHH/DHHA1 domain associated with a different sensory domain, must be responsible for c-di-AMP degradation in the other bacteria (FIG. 2). Potential c-di-AMP hydrolyzing enzymes of archaea are discussed in Box 2.

c-di-AMP - an essential second messenger

The first report of c-di-AMP production by bacterial cells came in 2010, when it was identified as a molecule secreted into the cytosol of host cells by the intracellular pathogen *L. monocytogenes*⁶. As much as 18 nM of c-di-AMP were detected in the supernatant of wildtype *L. monocytogenes* cultures, which increased to 53 nM upon over-expression of multidrug efflux pumps⁶. Subsequently, intracellular c-di-AMP concentrations of 1.7 μ M and 2.1 μ M have been measured in cytoplasmic extracts of *B. subtilis* and *S. aureus* cells and the concentrations increased 4- or 15-fold above wildtype levels in the respective *gdpP* mutant strains^{8, 10}. These nucleotide concentrations are in a similar range as those reported for c-di-GMP^{38, 39}. Following the identification of c-di-AMP in *L. monocytogenes* and *S. pyogenes*, attempts to delete the *dacA* gene proved unsuccessful, hinting that the cyclase, and hence c-di-AMP, is essential in these bacteria^{6, 7, 40}. These results are also in line with genome wide screens for essential genes in *Mycoplasma pulmonis*, *Mycoplasma genitalium*, *Streptococcus pneumoniae* and *S. aureus*⁴¹⁻⁴⁴, all of which reported an inability to disrupt *dacA*. Furthermore, a *B. subtilis* mutant with deletions in all three cyclases genes (*disA*, *dacA_{BS}* and *dacB_{BS}*) could not be obtained, however ectopic expression of any of the three cyclases was enough to fully rescue cells suggesting that all three proteins are active cyclases when expressed^{16, 15}. As c-di-AMP is produced by many important human pathogens these observations make the identification of the cellular pathways that are regulated by c-di-AMP of great interest with the view of possibly exploiting them to develop new therapeutic interventions.

c-di-AMP receptor proteins

TetR-family transcription factor DarR. Signaling nucleotides function by binding

to specific receptor proteins to allosterically alter their activity and thus regulate specific cellular pathways. The first c-di-AMP receptor protein reported in the literature was the *M. smegmatis* protein Ms5346, a TetR-family regulator protein that was renamed DarR for c-di-AMP receptor regulator. DarR was identified by screening a library of about 500 predicted *M. smegmatis* transcription factors for their ability to bind radiolabeled c-di-AMP¹³. Using surface plasmon resonance, a specific interaction with a K_d of 2.5 μ M was revealed between purified DarR and c-di-AMP. DarR has a two-domain architecture with an N-terminal TetR-like helix-turn-helix domain (Pfam00440), a typical DNA-binding domain, and a C-terminal QacR-like (Pfam08360) domain¹³ (FIG. 3). Proteins containing QacR-like domains are not universally present in organisms that are likely to produce c-di-AMP, however this domain is for instance present in QacR, a protein found in some *S. aureus* strains, FadR from *B. subtilis* and AcrR from *Pelotomaculum thermopropionicum*¹³. In *M. smegmatis*, DarR was shown to bind to and repress transcription of its own promoter, to control the divergently transcribed operon encoding a medium-chain fatty acyl-CoA ligase and a major facilitator family transporter, as well as to repress transcription of a gene encoding for the cold shock protein CspA (FIG. 4). Deletion of *darR* in *M. smegmatis* leads to an increase in cell size, whilst overexpression is toxic to the bacteria and causes a marked decrease in fatty acid synthesis¹³. The DNA-binding ability of DarR is enhanced *in vitro* upon interaction with c-di-AMP¹³, suggesting that changes in cellular c-di-AMP levels might affect fatty acid synthesis in *M. smegmatis*. However, additional experimental evidence is needed to unambiguously show that c-di-AMP affects membrane lipid homeostasis in *M. smegmatis* or other bacteria (FIG. 4).

RCK_C domain proteins KtrA and CpaA. Using c-di-AMP coupled beads and *S. aureus* cytoplasmic protein extracts in affinity pull down assays, the *S. aureus* protein KtrA, a cytoplasmic gating component of Ktr-type potassium transport systems, was identified as a second c-di-AMP receptor protein¹⁴. Purified KtrA protein bound c-di-AMP with a higher affinity compared to DarR and a K_d below 100 nM was measured¹⁴. KtrA forms, together with a KtrB-type membrane component, a potassium transporter that is widely distributed among bacteria^{45, 46} (FIG. 3). KtrA is a member of the RCK protein family (regulator of conductance of K^+ ; also known as the KTN (K^+ transporter, nucleotide binding protein family) and is composed of an N-terminal RCK_N (Pfam02254) and a C-terminal RCK_C domain (Pfam02080; FIG. 3). The RCK_N domain was previously shown to bind nucleotides including ATP, ADP or NAD⁺ and NADH, however c-di-AMP binds specifically to the C-terminal RCK_C domain¹⁴ (FIG. 3). It is thought that nucleotide binding to KtrA leads to conformational changes or a change in the oligomerization state that are transduced to the membrane transporter component KtrB (FIG. 4), leading to channel opening or closing. KtrA_{SA} was shown to be essential for the growth of *S. aureus* in low potassium conditions¹⁴. Furthermore, *gdpP* mutant bacteria with high levels of c-di-AMP are sensitive to salt stress and require, similar to a *ktrA* mutant strain, higher levels of potassium for growth under osmotic stress conditions^{14,47}. However, additional experiments are needed to unambiguously determine how binding of the second messenger c-di-AMP to KtrA affects the activity of the potassium transporter. These findings are analogous to findings on cAMP/cGMP-gated potassium

channels, where binding of these nucleotides leads to the opening of the channel⁴⁸.

The discovery of the RCK_C domain as a c-di-AMP receptor led to the subsequent identification of the *S. aureus* cation proton antiporter CpaA as a third c-di-AMP binding protein¹⁴ (FIG. 3, FIG. 4). Even though the RCK_C domains of KtrA and CpaA show low identity, based on structural homology models they are likely to assume a very similar fold¹⁴. As CpaA is also a predicted ion transporter it is likely to be involved in the uptake of potassium or sodium in exchange for an intracellular proton¹⁴.

The RCK_C domain is associated with potassium transporters or channels and other transporters. This domain is even more widely distributed than the DAC domain and is found in bacteria, archaea and a small subset of eukaryotic green algae where the DAC domain is absent. However, organisms that contain a DAC domain usually contain one or more proteins with an RCK_C domain, indicating that c-di-AMP might regulate potassium and perhaps other transport processes in a variety of organisms.

Histidine kinase protein KdpD. Following on from this, a further c-di-AMP target protein was identified in *S. aureus* using a whole genome screening approach¹⁴. This protein, KdpD, also has a potential role in potassium homeostasis and in addition has been implicated as a regulator of virulence and intracellular survival of a number of bacterial pathogens⁴⁹. KdpD is a histidine kinase that in many bacteria, together with its cognate response regulator KdpE, controls the expression of a second type of potassium uptake system (FIG. 3, FIG. 4). This system is, in contrast to the Ktr system, an ATP-dependent potassium uptake system and in *E. coli* consists of four membrane components KdpFABC. The two-component system KdpDE is required for the expression of *kdpFABC* at a very low potassium concentrations when the other uptake systems are no longer sufficient for adequate ion uptake⁴⁵. KdpD is a predicted membrane protein with N-terminal and C-terminal cytoplasmic portions containing multiple domains (FIG. 3). The protein contains neither an RCK_C nor a QacR-like domain and the protein region interacting with c-di-AMP still needs to be determined. Also, the role of the Kdp system in potassium uptake in *S. aureus* has not been fully elucidated and additional work is needed to understand the function of this two-component system and its interplay with cellular c-di-AMP levels.

Potassium transport is of fundamental importance in all living cells and in bacteria has essential roles in the adaptation to changes in osmolarity, controls the activity of intracellular enzymes and functions as a regulator of internal pH and in the maintenance of an appropriate membrane potential⁴⁵. The link between c-di-AMP signaling and ion homeostasis may therefore in part explain why this nucleotide is important for bacterial survival.

PII-like signal transduction protein PstA. The final c-di-AMP target protein, identified to date, is a PII-like signal transduction protein, that has been renamed PstA¹⁴. This *S. aureus* protein was again discovered using a whole genome screen¹⁴. PstA is a predicted cytoplasmic protein containing a DUF970 domain of unknown function (Pfam06153; FIG. 3). Structurally this domain resembles a ferredoxin-like alpha/beta sandwich, which is also found in the cation tolerance protein CutA1⁵⁰ and the ATP phosphoribosyltransferase HisG, the first enzyme of

the histidine pathway⁵¹. However the best characterized proteins with this structural domain are PII nitrogen regulatory proteins belonging to the GlnB superfamily. These are key signal transduction proteins that report on the nitrogen and carbon status of cells by sensing glutamine and 2-ketoglutarate levels⁵². While as yet unconfirmed, it will be interesting to discover if PstA plays a role in nitrogen regulation in *S. aureus* and how this role is affected by c-di-AMP levels.

Other cellular processes influenced by c-di-AMP levels

Cell wall homeostasis. In several recent studies alterations in cellular c-di-AMP levels have been connected to changes in the bacterial cell wall. For instance, *S. aureus* mutants lacking the essential cell wall polymer lipoteichoic acid (LTA) regained the ability to grow almost normally upon inactivation of GdpP, a mutation that leads to a 15-fold increase in intracellular c-di-AMP levels¹⁰. Furthermore, an *S. aureus* strain in which *gdpP* is mutated had increased amounts of cross-linked peptidoglycan, which was hypothesized to help bacteria compensate for the lack of LTA. Microscopic analysis of wildtype and *gdpP* mutant strains revealed a 13-22% reduction in the cell size of *S. aureus* cells with increased c-di-AMP levels¹⁰. *S. aureus*, *B. subtilis* and *L. monocytogenes* strains carrying a mutated *gdpP* display increased resistance to β -lactam antibiotics, further indicating that changes in the cell wall have occurred in strains with high c-di-AMP levels^{10, 16-19, 40}. Conversely, upon overexpression of GdpP, which decreases c-di-AMP levels, *B. subtilis* and *L. monocytogenes* become highly sensitive to cell wall targeting antibiotics^{16, 40}. Finally *Lactococcus lactis*, *L. monocytogenes* and *B. subtilis gdpP* mutant strains are more resistant to acid^{33, 40, 53} and heat stress⁴⁷.

Together these data suggest that c-di-AMP is involved in controlling *S. aureus* cell wall synthesis and cell size and in helping bacteria to cope with membrane and cell wall damage, acid as well as heat stress (FIG. 4) The molecular mechanisms responsible for these processes are not understood. We speculate that several of these phenotypes are linked to an altered ability to take up potassium under varying c-di-AMP concentrations. Potassium is important for the maintenance of an adequate membrane potential, which is known to contribute to antibiotic resistance^{54, 55}. This ion also plays a crucial role in osmoregulation, a function that has also been attributed to the cell wall⁴⁵. Lastly, potassium uptake is required for pH homeostasis, another phenotype that has been linked to differences in c-di-AMP levels³³.

Sensing of DNA damage. DisA is an octameric protein that binds to DNA as a single focus via the C-terminal HhH domain and scans the chromosome while synthesizing c-di-AMP^{4, 5} (FIG. 4). The movement of the DisA focus is dependent on the ability of the protein to synthesize c-di-AMP^{5, 8}. Upon detection of branched DNA, such as holliday junctions or stalled replication forks, DisA movement and synthesis of the second messenger c-di-AMP cease^{4, 8} (FIG. 4). This decrease in c-di-AMP levels is believed to act as a signal to stop replication until the DNA can be repaired, potentially through the action of RadA. It has been reported that DisA expression increases during late exponential phase and during sporulation⁵. This results in a 3-fold increase in c-di-AMP levels in cells that are undergoing sporulation as compared to vegetative cells⁸. When DNA

damage is sensed by DisA prior to the onset of sporulation the levels of c-di-AMP drop resulting in a delay in the onset of sporulation, which can be reversed by the exogenous addition of c-di-AMP⁸. Concurrently, expression of GdpP increases in *B. subtilis* cells that have been exposed to a DNA-damaging agent, which results again in a decline in c-di-AMP levels and a decreased sporulation rate⁸. In this way, c-di-AMP levels report on DNA integrity and in this manner helps to ensure that undamaged DNA is packed into spores in *B. subtilis*.

Induction of eukaryotic host cell response. In addition to its functions in bacterial physiology, c-di-AMP is also sensed by eukaryotic host cells and has been recently shown to elicit a type I interferon response. A type I interferon response is a well-characterized innate immune response leading to the activation of defense mechanisms against viral infection. However, more recently it has been recognized that extracellular and intracellular bacterial pathogens can also induce a type I interferon response and the activator molecules and signaling pathways responsible are beginning to emerge (FIG. 5). *L. monocytogenes* is an intracellular pathogen, which after entering a eukaryotic host cell escapes from the vacuolar compartment and resides and propagates within the cytosol. Once there, the cyclic dinucleotide c-di-AMP is secreted by the multidrug efflux pumps MdrM and MdrT, the expression of which are controlled by the regulators MarR and TetR⁵⁶⁻⁵⁸. c-di-AMP then induces, like its counterpart c-di-GMP, a type I interferon response leading to the production of IFN- β (FIG. 5)^{6, 58}. This response is dependent on the host cell helicase DDX41⁵⁹, as well as the transmembrane receptor STING^{60, 61}. Both human and mouse cell lines depleted for DDX41 or STING as well as STING-knockout mice or mice harboring a loss of function mutation, no longer elicit a type I interferon response during infection with *L. monocytogenes*^{59, 60, 62}. An interaction between STING and c-di-GMP has been reported in several studies^{61, 63-66}. This, together with the co-crystal structure of STING and c-di-GMP⁶³⁻⁶⁷, identified this protein as a receptor for c-di-GMP. The interaction between c-di-GMP and STING can be inhibited in a dose-dependent manner with c-di-AMP, suggesting that STING is also a receptor for c-di-AMP⁶¹. However, very recently a direct interaction between c-di-GMP and DDX41, more specifically its DEAD-box domain, was reported and c-di-AMP was also demonstrated to bind to this helicase⁵⁹. Based on the higher affinity of DDX41 for c-di-GMP as compared to STING, it was suggested that DDX41 is the actual sensor, not only for B-DNA but also for cyclic dinucleotides. Cyclic nucleotide-bound DDX41 then forms a complex with STING, which in this study was proposed to act as a co-receptor⁵⁹. However, it should be noted that as of yet no binding affinities between c-di-AMP and either DDX41 or STING have been determined and further work is needed to fully ascertain whether c-di-AMP is a biologically relevant ligand for either of these eukaryotic protein.

Once STING is activated, it will then interact with and activate the kinase TBK1⁶⁸. This in turn will lead to the phosphorylation of the transcription factor IRF-3 and its translocation into the nucleus and activation of type I interferon response and production of IFN- β (FIG. 5) Whether this is advantageous to *L. monocytogenes* or the host or is just a by-product of the bacterium regulating intracellular c-di-AMP levels by transporting it out of the cell remains to be determined. Regardless if beneficial or detrimental to the bacterium MDR

transporters are conserved among bacteria. Other bacteria such as *S. aureus* can also enter the host cell cytosol, therefore it seems possible that c-di-AMP is also secreted by several other bacterial pathogens, potentially leading to immune recognition/modulation. However, the matter is even more complex, as it was recently shown that following detection of foreign DNA in the cytosol, eukaryotic cells themselves can synthesize a cGAMP species^{69,70}. This eukaryotic dinucleotide, which contains a 2'-5' linkage and hence is referred to here as cGAMP(2'-5'), is distinct from the bacterial dinucleotide cGAMP(3'-5')⁷¹⁻⁷⁴. cGAMP(2'-5') can then also bind the receptor protein STING to activate a type-I IFN response (FIG. 5)⁶⁹⁻⁷⁴. These studies have also shown that the cytosolic DNA binds directly to the eukaryotic cGAMP synthase (cGAS) and stimulates enzyme activity, leading to the production of cGAMP(2'-5')^{84,72, 73}.

Once cyclic dinucleotides were recognized as immunomodulatory and immune stimulatory molecules, this prompted further research into their potential use as vaccine adjuvants. There are now several promising reports describing a beneficial effect for c-di-GMP and c-di-AMP as adjuvant for both mucosal and systemic vaccination, highlighting another potential use for these molecules⁷⁵⁻⁷⁸.

Unresolved questions Since the discovery of c-di-AMP in a structural study in 2008, its existence in several bacteria has now been confirmed experimentally. Based on the many phenotypes associated with altered c-di-AMP levels, there is no longer a doubt that this molecule plays an important role as second messenger and the first target proteins and cellular pathways controlled by it have now been identified (FIG. 4). Despite these advances, there are many outstanding questions. Firstly, in the majority of microorganisms, c-di-AMP production is only inferred based on the occurrence of DAC domain-containing proteins and remains to be confirmed. Likewise the corresponding c-di-AMP degrading enzymes need to be identified in bacteria outside the Firmicutes and in archaea. Currently only one signal, DNA damage has been directly linked to changes in c-di-AMP levels^{4, 5,8}. However, it can be expected that other signals will affect c-di-AMP synthesis especially in those organism lacking DisA homologues (FIG. 4). But the focus of most research is on identifying other c-di-AMP receptor and effector proteins. Based on the observation that most organisms contain only a single DAC enzyme, the c-di-AMP network appears to be much simpler than the c-di-GMP signaling network where organisms often contain more than twenty enzymes involved in the synthesis or hydrolysis of this second messenger. Therefore, one may not expect to find as many receptor proteins and induction signals as for c-di-GMP, however it seems likely that in addition to the targets described here, other protein will be able to bind c-di-AMP in order to explain all the observed phenotypes. This would be similar to the “simpler” cAMP and ppGpp signaling systems, which despite the limited number of turnover enzymes, function through several different effector proteins and are of very broad physiological impact^{1, 2}. Recently, novel tools such as chemically modified nucleotide analogues that can be used in combination with sensitive mass spectrometry methods for the capturing and identification of receptor proteins and a new, simple and fast binding assay suitable for high throughput screening assays, have been developed^{79, 80}. This will make the rapid discovery of

additional c-di-AMP receptor proteins possible and guarantee an exciting future for this field.

Key points

- When faced with stresses in a rapidly changing environment, bacteria must be able to sense these changes and coordinate responses rapidly. To do this they employ a number of nucleotide signaling molecules that act as second messengers to transduce signals around the cell.
- Cyclic diadenosine monophosphate (c-di-AMP) is a recently discovered signaling nucleotide that is present in many Gram-positive bacteria, a limited number of Gram-negative species and likely also some archaea.
- This signaling molecule is synthesized by cyclase enzymes, which condense two molecules of ATP to c-di-AMP using the enzymatic activity that is contained within the DisA_N domain, a domain that is distinct from other cyclic dinucleotide cyclase domains.
- c-di-AMP is degraded to the linear molecule pApA by the action of phosphodiesterases containing a DHH/DHHA1 domain architecture.
- Once synthesized, this molecule binds to a specific set of receptor or target proteins and allosterically alters their function or the function of downstream effector proteins. To date five c-di-AMP receptor proteins have been identified, namely DarR, which is involved in the regulation of fatty acid synthesis in *Mycobacterium smegmatis*, KtrA, CpaA and KdpD, which are all potentially linked to potassium or ion transport in *Staphylococcus aureus* and PstA, a receptor of unknown function.
- Although the exact pathways have yet to be fully worked out, a number of phenotypes associated with altered c-di-AMP levels in the cell have now been characterized. For instance c-di-AMP is linked to the sensing of DNA integrity in *Bacillus subtilis*, and to cell wall homeostasis in multiple species and can also induce a eukaryotic immune response when secreted by the bacterium *Listeria monocytogenes*.

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Competing interests statement

The authors declare no competing financial interests.

Box 1: c-di-GMP signaling in bacteria

Cyclic di-guanosine monophosphate (c-di-GMP) is a well-studied bacterial secondary messenger that was first discovered in 1987 by work performed in the lab of Moshe Benziman, where it was shown to activate cellulose biosynthesis in *Gluconacetobacter xylinus*⁸¹. c-di-GMP homeostasis in the cell is controlled by the opposing actions of diguanylate cyclases (DGCs) and phosphodiesterases (PDEs)^{3, 36, 82}. Signal-dependent dimerization of GGDEF domain-containing DGCs, each bound to one molecule of GTP, results in the activation of cyclase activity and the condensation of the two GTP molecules to form c-di-GMP³⁶. The dinucleotide is degraded by PDE enzymes, which are characterized by the presence of EAL or HD-GYP domains³⁶. While the vast majority of signals that regulate the activity of c-di-GMP DGCs and PDEs remain to be established, a number of environmental queues such as light⁸³, oxygen⁸⁴, nutrient starvation⁸⁵, the presence of antibiotics⁸⁶ and redox state⁸⁷ have been shown to affect their activity (reviewed in^{3, 82}). Furthermore, indirect activation of c-di-GMP synthesizing or degrading proteins by phosphotransfer systems in response to environmental changes have been described in several bacteria where GGDEF or EAL domain-containing proteins contain an N-terminal receiver (REC) domain that is phosphorylated by cognate sensor kinases^{88, 89}.

Once synthesized, c-di-GMP binds to receptor proteins, allosterically affects their activity and in this manner modulates specific cellular pathways. The phenotype most frequently associated with an increase in c-di-GMP levels is a switch from a motile to a biofilm-associated lifestyle⁸². Other outputs linked with alterations in cellular c-di-GMP levels include changes in virulence related properties and a regulation of the bacterial cell cycle^{3, 82, 90}. A large number of receptor proteins involved in controlling these phenotypes have now been identified and these fall into four different classes⁹⁰. PilZ was the first domain shown to bind c-di-GMP^{81, 91} and is present in proteins frequently involved in regulating motility and the production of cellulose and alginate^{81, 92}. A second class of c-di-GMP receptor proteins are GGDEF or EAL domain-containing proteins possessing a degenerate active site. While these proteins are no longer capable of synthesizing or degrading c-di-GMP, they can still bind the dinucleotide and thus translocate the signal within the protein^{93, 94}. A third type of c-di-GMP receptor includes transcription factors such as FleQ, a master regulator of flagellar gene expression in *Pseudomonas aeruginosa*³⁹. The final class of receptors are riboswitches, small mRNA segments present in the upstream untranslated region of some genes. Binding of c-di-GMP to this mRNA region regulates the transcription or translation of the downstream gene⁹⁵. Although much about c-di-GMP remains to be uncovered, the multiplicity of c-di-GMP DGCs and PDEs encoded in bacterial genomes, in proteobacteria in particular, highlights the importance of this secondary messenger in enabling bacteria to switch between different lifestyles depending on specific environmental cues.

Box 2: Potential c-di-AMP cyclases and hydrolases in archaea.

Based on bioinformatics analysis and the occurrence of DAC domain-containing proteins it is likely that c-di-AMP is also produced by a subset of archaea¹². Here, the DAC domain is found primarily in cytoplasmic proteins with a stand-alone DAC domain. This architecture might suggest that these cyclases cannot directly sense environmental or cellular changes and instead need a separate sensory protein. Aside from the stand-alone DAC domain proteins, the DAC domain is, in several cases, fused to a domain annotated as a pyruvate kinase C-terminal alpha/beta C domain (PK_C; Pfam02887). Pyruvate kinase enzymes are central metabolic enzymes involved in glycolysis. They are homotetrameric enzymes, which in bacteria and archaea consist of three domains (A, B, and C). While the active site is located in the interface between domains A and B, the PK_C domain can serve as the binding site for an allosteric effector⁹⁶. It is therefore plausible that the PK_C domain in these archaeal proteins serves as a small molecule-binding domain, which in turn regulates the cyclase activity. Furthermore, the cyclase domain is also found in association with PAS, polycystic kidney disease I (PKD; Pfam00801) or TPR domains. These three domains have been implicated as ligand binding or protein-protein interaction domains and therefore might serve to regulate the activity of the associated DAC domain upon ligand binding (Figure Box 2). While c-di-AMP production by archaea has yet to be confirmed experimentally, the DAC domains in these organisms have the conserved DGA amino acid signature and a conserved RH in place of the RHR motif found in active cyclases and it is therefore proposed that these enzymes are renamed DacV- DacZ (Table S1 and Figure Box 2).

No homologues of the c-di-AMP-degrading enzyme GdpP are present in archaea. However, proteins belonging to a different family of DHH/DHHA1 domain-containing proteins could serve this function. Interestingly candidates for c-di-AMP PDEs in archaea are proteins with a TrkA_N-DHH/DHHA1 domain structure. These proteins co-occur in species containing DAC domain proteins and the TrkA_N domain, which is also referred to as an RCK_N (regulator of conductance of K⁺) domain, is associated with potassium or other transporters, a cellular pathway that has been linked to the c-di-AMP signaling network in bacteria as we will discuss later.

FIGURE Box 2: Architecture of DAC domain-containing proteins and potential c-di-AMP hydrolases in archaea. Of the 65 proteins, most either do not contain any recognizable associated domain or a pyruvate kinase C-terminal alpha/beta C (PK_C) domain, which together represent 93.9% of all archaeal DAC domain-containing proteins (red box). For the remaining four archaeal proteins the DAC domain is associated twice with PAS domains and once each with extracellularly located tetrcopeptide repeat (TPR) or polycystic kidney disease I (PKD) domains. Candidates for c-di-AMP phosphodiesterases in archaea are proteins with a TrkA_N-DHH/DHHA1 domain structure. These proteins co-occur in species containing DAC domain proteins and the TrkA_N domain, otherwise known as an RCK_N domain.

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FIGURE 1: c-di-AMP production. c-di-AMP is synthesized by the condensation of two molecules of ATP by diadenylate cyclase (DAC) enzymes (blue). DacA, DisA and DacB are the three most prevalent bacterial cyclases that have been shown to be active *in vitro* or *in vivo*. c-di-AMP is then degraded to pApA by the action of phosphodiesterases (PDEs) (red). GdpP is currently the only characterized c-di-AMP PDE. Next, it is assumed that c-di-AMP binds to a specific set of receptor or target proteins, which in turn affect the activity of effector proteins and in this manner contribute to the regulation of specific cellular pathways.

FIGURE 2: Domain architecture of known and predicted bacterial c-di-AMP synthases and hydrolases. Currently 1887 bacterial DAC domain-containing sequences are listed in the Pfam database. The three most abundant ones are DacA (69.1%), DisA (24.1%) and DacB (5.5%), which together represent 98.7% of the total DAC domain-containing proteins in bacteria (red box). DisA is associated with a HhH domain, DacA with a transmembrane domain and DacB with a YojJ domain. The rarer DAC domain-containing proteins (DacC, DacD, DacE and DacF) contain DAC domains associated with a YbbR domain, a phosphoenolpyruvate-dependent sugar phosphotransferase system EIIA domain, a tetrcopeptide repeat (TPR) domain, and a domain of unknown function (DUF4062), respectively (associated domains in orange). Bacterial c-di-AMP phosphodiesterases with homology to GdpP are only found in the Firmicutes and Tenericutes. In other phyla, it is possible that the DHH and DHHA1 domains are associated with other sensory domains or that a different class of enzyme is responsible for c-di-AMP hydrolysis (red enzyme).

FIGURE 3: c-di-AMP receptor proteins. a) Schematic representation of the *M. smegmatis* c-di-AMP receptor protein DarR. DarR is a cytoplasmic transcription factor with a DNA-binding HTH domain and a C-terminal QacR-like domain. The c-di-AMP binding domain has not yet been identified. b) Schematic representation of the *S. aureus* c-di-AMP receptor protein KtrA, a cytoplasmic gating component of the Ktr-type potassium transport system. KtrA is composed of an N-terminal RCK_N domain indicated in blue and a C-terminal RCK_C domain shown in orange. The RCK_N domain is known to bind ATP, ADP, NAD⁺ and NADH, while the RCK_C domain has recently been shown to interact with c-di-AMP. Ktr-type potassium transport systems are composed of a KtrB-type membrane protein and the cytoplasmic KtrA-type gating component. Binding of c-di-AMP to the gating component KtrA has been suggested to result in changes in potassium transport activity. c) Schematic representation of *S. aureus* c-di-AMP receptor protein CpaA. CpaA is a predicted K⁺ or Na⁺ antiporter protein and consists of an N-terminal transmembrane domain (yellow), followed by a cytoplasmically located RCK_N (blue) domain and the c-di-AMP binding RCK_C (orange) domain. d) Illustration of the two-component system KdpDE, known to be involved in K⁺ uptake in *E. coli*. KdpD is a histidine sensor kinase that has been shown to bind to c-di-AMP in *S. aureus*. This protein is responsible for phosphorylating the response regulator KdpE. The cytoplasmic regions of KdpD contain multiple domains and it is not yet known which one binds c-di-AMP. e) Schematic illustration of the *S. aureus* cytoplasmic protein PstA, which binds c-di-AMP and may be involved in nitrogen regulation.

FIGURE 4: The c-di-AMP signaling network. For both *S. aureus* and *B. subtilis* environmental changes (lightening bolt) are potentially sensed by the sensory domains associated with YbbR or by other as of yet unidentified mechanisms. YbbR has been shown to interact with DacA resulting in an increase in cyclase activity and c-di-AMP synthesis¹⁵. To date four c-di-AMP receptor proteins have been identified in *S. aureus*; the potassium transporter gating component KtrA, the ion transporter CpaA, the two-component sensor kinase KdpD and the hypothetical protein PstA¹⁴. In the case of CpaA, c-di-AMP binding is thought to directly affect ion transport. KtrA interacts with the effector protein KtrB, a membrane component responsible for the uptake of potassium. It is thought that nucleotide binding to KtrA leads to conformational changes or a change in the oligomerization state that are transduced to the membrane transporter component KtrB resulting in a change in potassium uptake activity¹⁴. KdpD phosphorylates the response regulator KdpE to effect changes in the cell. The function of PstA is unknown¹⁴. Based on *in vitro* activity data it is thought that the c-di-AMP-specific phosphodiesterase GdpP senses intracellular heme through an interaction with the PAS domain, resulting in decreased PDE activity, while nitric oxide (NO) binding to heme results in an increase in PDE activity and potentially a decrease in c-di-AMP concentration³⁵. *B. subtilis* has two additional c-di-AMP cyclases, DisA and DacB. The sensory function of DisA is the best characterized. This protein binds via its associated HhH domain to DNA and upon detection of branched DNA c-di-AMP synthesis stops and cellular nucleotide levels drop^{4,5}. DacB has an associated cytoplasmic YojJ domain that may be involved in sensing as of yet unknown intracellular signals and based on expression data it has been suggested that DacB is active during sporulation³². In *M. smegmatis* c-di-AMP is synthesized by a DisA protein and has been demonstrated to bind to the transcriptional repressor DarR and increase association of DarR with the promoter regions of three genes, the cold shock protein *cspA*, a medium-chain fatty acyl-CoA ligase and a major facilitator family transporter¹³. In all cases, it is assumed that at high c-di-AMP levels the nucleotide will bind to these or other still unknown receptor proteins (orange) and in doing so allosterically modify their function. In this way, changes in c-di-AMP levels affect ion transport, cell wall homeostasis, DNA integrity and through DacB perhaps function in the sporulation processes in *B. subtilis* as well as in membrane homeostasis as suggested for *M. smegmatis*.

FIGURE 5: Induction of a type I interferon response by bacteria. Recognition of extracellular bacteria occurs through pathogen-associated molecular patterns (PAMPs) such as LPS or DNA by host pattern recognition receptors (PRRs)⁹⁷. This recognition leads to activation of the kinase TBK1 through the MyD88 or TRIF pathways⁹⁸. Activated TBK1 in turn phosphorylates IRF-3, resulting in its translocation across the nuclear membrane and induction of a type I interferon response⁶⁸. On the other hand, the intracellular bacterial pathogen *L. monocytogenes* enters eukaryotic host cells and is initially contained within a vacuolar compartment. However, *L. monocytogenes* can escape from the vacuole and enter the cytosol where some of the cyclic dinucleotide c-di-AMP pool is secreted⁶. Next, c-di-AMP is thought to bind to the eukaryotic helicase DDX41 which in turn interacts with the co-receptor STING⁵⁹. Alternatively, it has been suggested that c-di-AMP directly interact with the cytosolic portion of the receptor protein STING⁶⁰⁻⁶⁶, which upon nucleotide binding becomes activated and can then interact with and activate the kinase TBK1⁶⁸. This will again lead to phosphorylation and translocation of the transcription factor IRF-3 and activation of type I interferon response and production of IFN-β. STING is also the receptor for cyclic c-di-GMP and can additionally be activated by dsDNA and the eukaryotic cyclic dinucleotide cGAMP(2'-5')^{82,69, 70,68, 71-74, 99}. cGAMP(2'-5') is a eukaryotic cyclic dinucleotide synthesized by the cytoplasmic synthase cGAS in response to the sensing of DNA in the cell.

Table S1: Phylogenetic distribution of DAC and PDE enzymes

BACTERIA									
Associated domain Proposed protein name Number of proteins	YbbR*	DAC (1887)							PDE
		DisA_N linker/HhH		YojJ	YbbR	PTS_EIIA_2	TPR	DUF 4062	GdpP
		DacA 1304	DisA 454	DacB 104	DacC 16	DacD 6	DacE 2	DacF 1	
Phylum (number of DAC domains)**									
Cyanobacteria (65)		X							
Fusobacteria (25)			X						
Deferribacteres (3)	X	X							
Proteobacteria (95)									
<i>Deltaproteobacteria</i>	X	X			11				
Lentisphaerae (2)	X	X							
Spirochaetes (47)	X	X				1			
Synergistetes (7)	X	X							
Deinococcus-Thermus (13)	X	X							
Verrucomicrobia (8)	x	X				4			
Dictyoglomi (2)			X						
Bacteroidetes (129)	X	X							
Chlamydiae (27)		X							
Thermotogae (12)			X						
Acidobacteria (4)	x	X							
Nitrospirae (4)	x	X							
Aquificae (12)	x	X							
Actinobacteria (228)			X						
<i>Streptomyces spp.</i>			X						
<i>Corynebacterium spp.</i>			x						
<i>Mycobacterium spp.</i>			X						
<i>Propionibacterium spp.</i>			X						
<i>Actinomyces spp.</i>			X				1		
Planctomycetes (10)	x	X				1			
Chloroflexi (11)	X	X							
Firmicutes (1139)	X	X	X						
<i>Clostridium spp.</i>	X	X	X	1	1		1	1	X
<i>Lactobacillus spp.</i>	X	X							X
<i>Streptococcus spp.</i>	X	X			4				X
<i>Lactococcus spp.</i>	X	X							X
<i>Enterococcus spp.</i>	X	X							X
<i>Staphylococcus spp.</i>	X	X							X
<i>Bacillus spp.</i>	X	X	X	X					X
<i>Listeria spp.</i>	X	X							X
<i>Negativicutes</i>	X	X	X						X
<i>Erysipelotrichi</i>	X	X							X
Tenericutes (33)		X							X
ARCHAEA									
Associated domain Proposed protein name		DAC (65)					PDE		
		none	PK_C	PAS	TPR	PKD	TrkA_N/ DHH/DHHA1		
		DacZ	DacY	DacX	DacW	DacV			
Euryarchaeota (65)		49	12	2	1	1	58		

*X = number of YbbR domains is the same or greater than the number of DAC domains within the phylum; X = number of YbbR domains is at least half of the number of DAC domains within the phylum, x = number of YbbR domains is less than half of the number of DAC domains within the phylum. **All phyla containing more than 1 DAC domain-containing protein are listed with the number of DAC domains given in parenthesis. *** Abbreviations are as follows: DAC – diadenylate cyclase; PDE – phosphodiesterase; HhH – helix hairpin helix; PTS_EIIA_2 – phosphoenolpyruvate-dependent sugar phosphotransferase system EIIA; TPR – tetracopeptide repeat; PK_C – pyruvate kinase C-terminal alpha/beta C; PAS – Per Arnt Sim; PKD – polycystic kidney disease I