

## MicroReview

# A jack of all trades: the multiple roles of the unique essential second messenger cyclic di-AMP

Fabian M. Commichau,<sup>1</sup> Achim Dickmanns,<sup>2</sup>  
Jan Gundlach,<sup>1</sup> Ralf Ficner<sup>2</sup> and Jörg Stülke<sup>1\*</sup>

<sup>1</sup>Department of General Microbiology,  
Georg-August-University Göttingen, Grisebachstr. 8,  
D-37077 Göttingen, Germany.

<sup>2</sup>Department of Molecular Structural Biology,  
Georg-August-University Göttingen,  
Justus-von-Liebig-Weg 11, D-37077 Göttingen,  
Germany.

### Summary

Second messengers are key components of many signal transduction pathways. In addition to cyclic AMP, ppGpp and cyclic di-GMP, many bacteria use also cyclic di-AMP as a second messenger. This molecule is synthesized by distinct classes of diadenylate cyclases and degraded by phosphodiesterases. The control of the intracellular c-di-AMP pool is very important since both a lack of this molecule and its accumulation can inhibit growth of the bacteria. In many firmicutes, c-di-AMP is essential, making it the only known essential second messenger. Cyclic di-AMP is implicated in a variety of functions in the cell, including cell wall metabolism, potassium homeostasis, DNA repair and the control of gene expression. To understand the molecular mechanisms behind these functions, targets of c-di-AMP have been identified and characterized. Interestingly, c-di-AMP can bind both proteins and RNA molecules. Several proteins that interact with c-di-AMP are required to control the intracellular potassium concentration. In *Bacillus subtilis*, c-di-AMP also binds a riboswitch that controls the expression of a potassium transporter. Thus, c-di-AMP is the only known second messenger that controls a biological process by interacting with both a protein and the riboswitch that regulates its expression. Moreover, in *Listeria monocytogenes* c-di-AMP con-

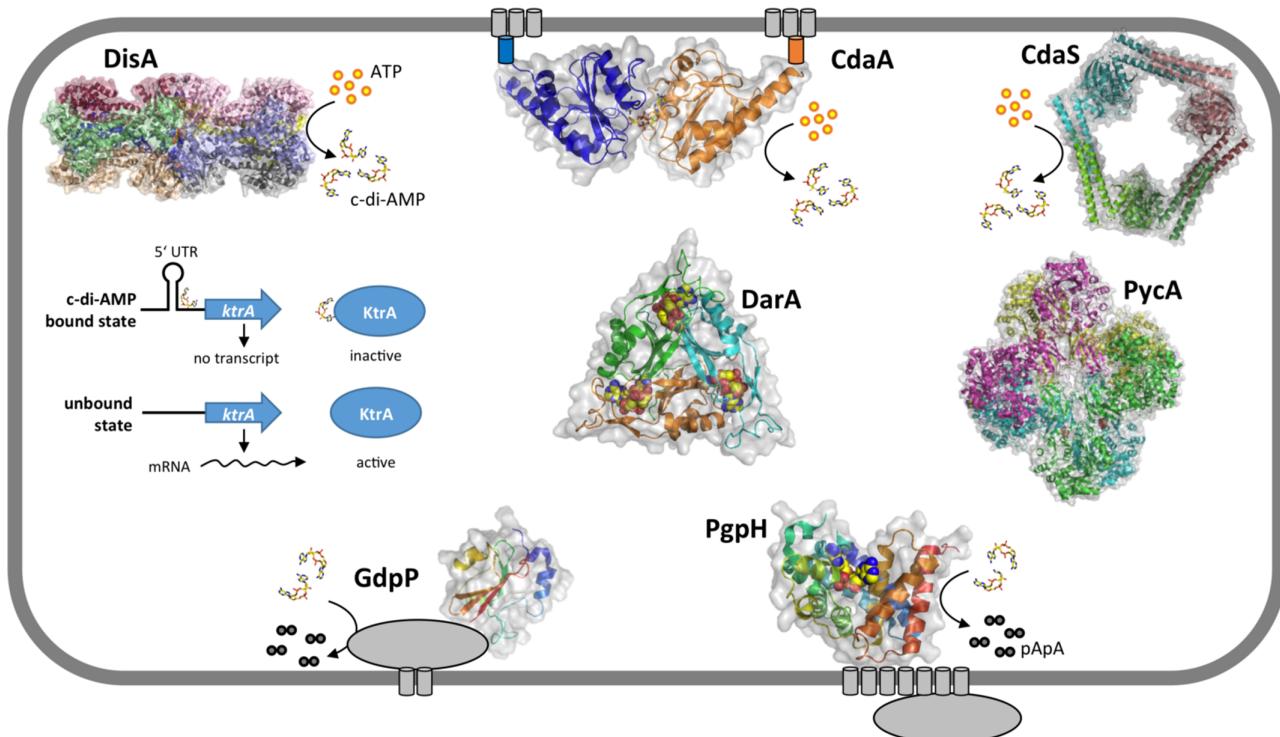
trols the activity of pyruvate carboxylase, an enzyme that is required to replenish the citric acid cycle. Here, we review the components of the c-di-AMP signaling system.

### Introduction

To recognize changes in the environment and to respond appropriately to them, all organisms rely on signal transduction pathways. These pathways include signal recognition, the transfer of the signal and the response. For the signal transfer, many cells use so-called second messengers, small molecules that can easily diffuse within the cell or between cells. In bacteria, dedicated nucleotides which are not present in DNA or RNA often serve as second messengers. The classical example for this class of molecules is cyclic AMP, which is used in signal transduction in carbon catabolite repression in enteric bacteria (Görke and Stülke, 2008). If bacteria are starved for amino acids, they respond with the synthesis of ppGpp, the so-called stringent factor. This nucleotide has a global impact on gene expression due to interactions with the RNA polymerase or with nucleotide synthesizing enzymes (Potrykus and Cashel, 2008). Many bacteria also form cyclic dinucleotides. The paradigm of this group of second messengers is c-di-GMP which is implicated in the control of lifestyles, especially in the switch between motility and biofilm formation (Hengge, 2009; Schirmer and Jenal, 2009).

A few years ago, cyclic di-AMP (c-di-AMP) was discovered as a novel second messenger, produced in bacteria and archaea (Römling, 2008; Witte *et al.*, 2008). This molecule is very interesting since it is the only essential second messenger (i.e. many bacteria that produce it are unable to live without this molecule). Moreover, accumulation of c-di-AMP is toxic for many bacteria suggesting that the homeostasis of this nucleotide has to be precisely controlled (Bai *et al.*, 2013; Mehne *et al.*, 2013; Huynh *et al.*, 2015). A variety of different phenotypes have been associated with increased or reduced levels of c-di-AMP. These observations suggest that c-di-AMP is involved in functions such as DNA repair, cell wall synthesis, potas-

Accepted 10 April, 2015. \*For correspondence. E-mail: jstuelk@gwdg.de; Tel. (+49) 551 3933781; Fax (+49) 551 3933808.



**Fig. 1.** Proteins involved in c-di-AMP signaling in the firmicutes. Models based on known structures are shown in cartoon representation, and the surfaces are depicted in transparent gray. Parts not present in the crystal structures are indicated by spheres. Transmembrane regions are indicated by gray barrels. The following structures are shown: *T. maritima* DisA (3C1Y), *L. monocytogenes* CdaA DAC domain (4RV7), *B. cereus* CdaS (PDB 2FB5), *B. subtilis* DarA (4RLE), *L. monocytogenes* PycA (4QSH), *Geobacillus thermodenitrificans* GdpP PAS domain (2M1C) and *L. monocytogenes* PgpH (4S1B). Please note that the structures are not shown to scale.

sium homeostasis, virulence and regulation of gene expression. In this MicroReview, we aim to provide an overview of recent findings in the highly dynamic field of research on c-di-AMP. We discuss the different players that contribute to the control of c-di-AMP homeostasis [diadenylate cyclases (DACs), degrading enzymes, exporters]. Moreover, we give an overview on the interactions of c-di-AMP with its different targets – both proteins and RNA. A general outline of these players is given in Fig. 1. Table 1

summarizes the proteins involved in c-di-AMP signaling and their different designations in the mainly studied organisms.

### C-di-AMP: a unique essential second messenger

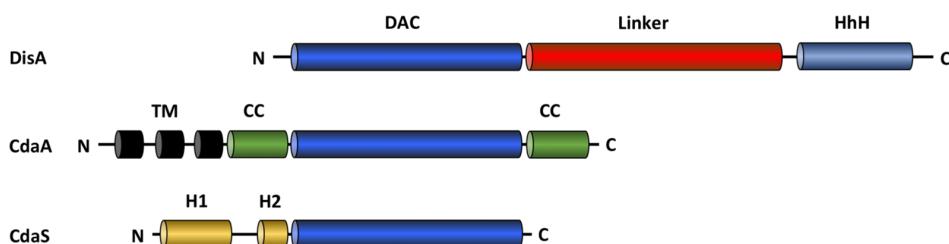
Among all known second messengers, c-di-AMP is the only that is essential for the bacteria that produce it. Essentiality of the nucleotide has been reported either

**Table 1.** Designations of the proteins involved in c-di-AMP signaling.<sup>a</sup>

Name in <i>Bacillus subtilis</i>	Function	Name in <i>Staphylococcus aureus</i>	Name in <i>Listeria monocytogenes</i>
DisA	Diadenylate cyclase	– <sup>b</sup>	–
CdaA (YbbP)	Diadenylate cyclase	DacA	DacA
CdaS (YojJ)	Diadenylate cyclase (sporulation specific)	–	–
GdpP (YybT)	Phosphodiesterase	GdpP	PdeA
YqfF	Phosphodiesterase	–	PgpH
DarA (YaaQ)	P <sub>i</sub> -like signal transduction protein	PstA	PstA
KtrA, KtrC	Potassium transporter	KtrA	KtrA

**a.** The proteins involved in c-di-AMP synthesis, degradation and sensing have got different designations in the different bacteria. Unfortunately, designations such as PstA and DacA have been used for a long time for other proteins in *B. subtilis* and other organisms. Therefore, new designations had to be used.

**b.** –, this protein is not present in this organism.



**Fig. 2.** Domain organization of the major types of diadenylate cyclases. DAC, diadenylate cyclase domain; HhH, helix-hairpin-helix domain; TM, transmembrane region; CC, coiled-coil domain; H1 and H2, inhibitory helices 1 and 2 respectively.

based on functional analyses that target the molecule directly or been inferred from the essentiality of the DAC encoding gene(s). In all firmicutes studies so far, c-di-AMP was found to be essential. These organisms include *Bacillus subtilis*, *Listeria monocytogenes*, *Staphylococcus aureus* and *Streptococcus pneumoniae* (Song *et al.*, 2005; Chaudhuri *et al.*, 2009; Woodward *et al.*, 2010; Luo and Helmann, 2012a; Mehne *et al.*, 2013; Corrigan *et al.*, 2015). Given that c-di-AMP has been implicated in cell wall synthesis and homeostasis in the firmicutes, it is rather surprising that the nucleotide is also essential in the Gram-negative bacterium *Chlamydia trachomatis*, which seems to lack peptidoglycan, and in the cell wall-less mollicutes *Mycoplasma genitalium*, *Mycoplasma pneumoniae* and *Mycoplasma pulmonis* (Glass *et al.*, 2006; French *et al.*, 2008; Barker *et al.*, 2013; Lluch-Senar *et al.*, 2015). Unfortunately, the function of c-di-AMP has not yet been studied in these bacteria. In contrast to the firmicutes, c-di-AMP was found to be dispensable in the actinobacteria *Streptomyces venezuelae* and *Mycobacterium tuberculosis* (Manzanillo *et al.*, 2012; Manikandan *et al.*, 2014; St-Onge *et al.*, 2015).

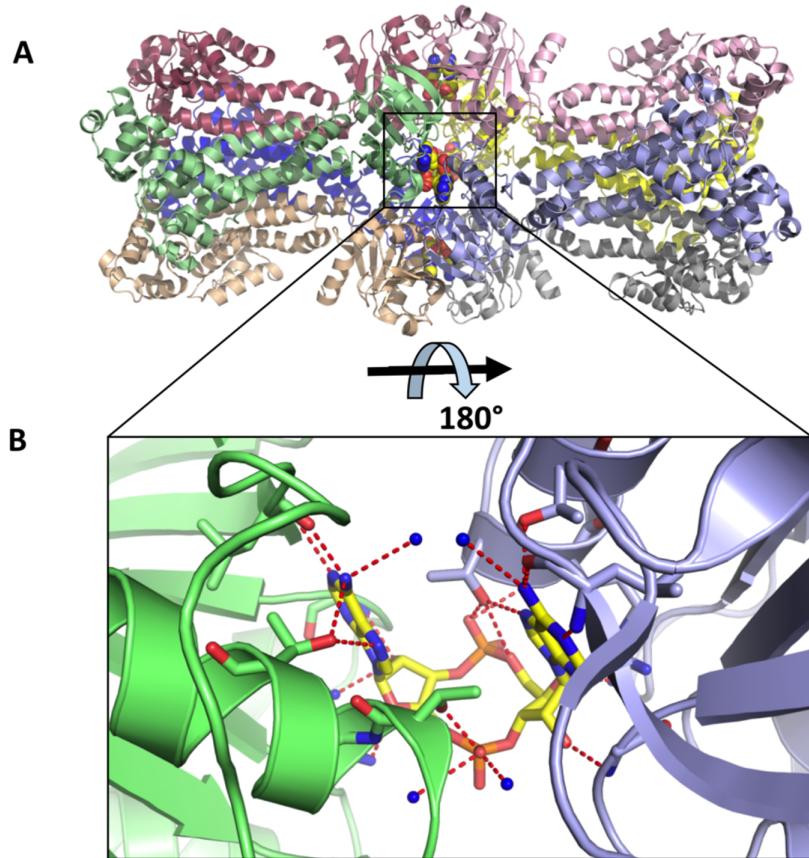
Since c-di-AMP is not known to be a part of any of the essential cellular components, the essentiality of the nucleotide in the firmicutes may have two reasons: c-di-AMP may act either as a co-factor for an essential protein or it may inhibit the activity of a toxic compound in the cells and thus have a protective function as has been shown for several essential proteins in *B. subtilis* (Commichau *et al.*, 2013). If c-di-AMP is required to stimulate the activity of an essential enzyme or process, it is tempting to speculate that this is cell wall biosynthesis. This would be in good agreement with the observation that altered levels of c-di-AMP affect the sensitivity of the cells to cell wall-active antibiotics and that the phenotypes caused by accumulation of c-di-AMP in *B. subtilis* can be suppressed by the addition of magnesium ions, which are known to rescue many mutants affected in cell wall biosynthesis and cell division (Corrigan *et al.*, 2011; Luo and Helmann, 2012a; Mehne *et al.*, 2013).

### C-di-AMP is produced by DACs

C-di-AMP and the byproduct pyrophosphate are produced from two molecules of ATP by the cyclase domain of DACs. To adjust the cellular demand for c-di-AMP and to prevent accumulation of the second messenger to toxic levels, c-di-AMP can also be degraded by c-di-AMP-specific phosphodiesterases (see below). Interestingly, some bacteria encode more than one DAC, and each enzyme seems to be devoted to a specific cellular process. This is the case for *B. subtilis* which contains the three DACs DisA, CdaA and CdaS. In contrast, most pathogenic firmicutes contain only one enzyme which is similar to CdaA, and the actinobacteria contain a DAC of the DisA type (Corrigan and Gründling, 2013). All cyclases are composed of an enzymatically active DAC domain and additional domains that may function in multimerization and in the control of enzymatic activity. Moreover, some DACs contain transmembrane helices that localize the enzymes to the membrane (see Fig. 2). Of the three most common classes of DACs that are all represented in *B. subtilis*, the DAC domains of CdaA and CdaS are closely related (about 40% identical residues), whereas the DAC domain of DisA is a more distant relative (about 19% identity to the DAC domains of CdaA and CdaS). In addition to the three architectures shown in Fig. 2, there are several proteins with distinct domain organization as reviewed recently (Corrigan and Gründling, 2013). The fact that DACs differ in their domain composition and organization suggests that each enzyme may perceive a distinct signal to adjust the activity of the DAC domain.

### DisA, a DAC involved in maintenance of DNA integrity

C-di-AMP was first identified when the so-called DNA integrity scanning protein A (DisA) from *Thermotoga maritima* was structurally and biochemically characterized (Witte *et al.*, 2008). DisA is a DNA-binding DAC that is mainly present in actinobacteria and Gram-positive spore-forming firmicutes (Corrigan and Gründling, 2013). Several lines of evidence suggest that DisA is implicated



**Fig. 3.** Overall and active site arrangement of the diadenylate cyclase DisA from *T. maritima*.

A. DisA (PDB 3C1Y) forms homo-octamers (each depicted in an individual color) made up of four homo-dimers facing each other at the site of c-di-AMP formation.  
 B. Magnification of the active site with the amino acid residues (stick models with the carbons colored according to the respective side chain and oxygen atoms in red and nitrogen atoms colored in blue) and water molecules (shown as blue spheres) involved in binding of c-di-AMP. The polar contacts are depicted as red dashed lines.

in the control of DNA integrity and DNA repair: (i) DisA is encoded in a phylogenetically conserved operon with the recombination protein RadA, and the two proteins interact physically (Zhang and He, 2013). (ii) The activity of DisA is also controlled by DNA integrity (Witte *et al.*, 2008), and (iii) inactivation of DisA affects the fidelity of DNA repair (Gándara and Alonso, 2015).

The N-terminal DAC domain of DisA that produces c-di-AMP is separated from the C-terminal helix-hairpin-helix (HhH) DNA-binding motif by a short linker domain (Fig. 2). The globularly shaped DAC domain of DisA is formed by seven  $\beta$ -sheets and five  $\alpha$ -helices (Fig. 3). A sequence alignment with DisA homologs led to the identification of the conserved DGA and RHR motifs that are located in the  $\beta$ -sheet 3 and in the  $\alpha$ -helix 5 respectively (Witte *et al.*, 2008). These motifs that are crucial for enzyme catalysis are conserved in all DACs (Witte *et al.*, 2008; Bai *et al.*, 2012; Rosenberg *et al.*, 2015). The structural characterization of DisA revealed that two DisA tetramers interact with each other through their DAC domains to form a stable octamer (Witte *et al.*, 2008). In agreement with the structural model of DisA, the face-to-face orientation of the DAC domains is a prerequisite for DACs to produce c-di-AMP (Witte *et al.*, 2008). While DisA from *B. subtilis*

uses ATP as the substrate, the enzyme from *M. tuberculosis* can generate c-di-AMP from ADP (Bai *et al.*, 2012).

Interestingly, the activity of DisA is strongly inhibited by the presence of nonstandard DNA structures such as Holliday junctions (Witte *et al.*, 2008). It was suggested that *B. subtilis* DisA rapidly scans the chromosomal DNA via its HhH DNA-binding motif for integrity. If the enzyme encounters DNA lesions, it produces less c-di-AMP, resulting in a delayed entry into sporulation until the DNA damage is repaired (Bejerano-Sagie *et al.*, 2006; Oppenheimer-Shaanan *et al.*, 2011). In addition, DisA also seems to arrest DNA replication in germinating *B. subtilis* spores until they are free of damaged DNA (Campos *et al.*, 2014). These observations suggest that the DAC is indeed involved in the control of DNA integrity. However, it is yet unclear how the inactivation of DisA leads to the recruitment of factors that repair damages on the chromosome. Recently, the RadA protein that participates in the processing of Holliday junction intermediates was shown to directly interact with DisA and to control the activity of the enzyme (Carrasco *et al.*, 2004; Zhang and He, 2013). Therefore, the recruitment of other DNA repair factors via DisA might be mediated by its interaction with RadA. However, recent evidence suggests that CdaA,

another vegetative DAC present in *B. subtilis* (see below), is also involved in DNA repair (Gándara and Alonso, 2015). Thus, based on the current knowledge, it is rather unclear whether only the activity status of DisA or changes in the cellular c-di-AMP pool *per se* report chromosomal integrity. It will be an interesting future task to uncover how the DACs and c-di-AMP contribute to recruit DNA repair proteins to DNA lesions.

### CdaA, the most abundant DAC

CdaA is the most frequent DAC that exists in a variety of bacteria (Corrigan and Gründling, 2013). Importantly, CdaA is the only DAC in many important pathogens such as *Staph. aureus*, *L. monocytogenes* or *Strep. pneumoniae*. Since the enzyme is essential for these bacteria, it might be an excellent candidate as a novel drug target in order to help overcoming multiresistance especially associated with *Staph. aureus* (Cooper and Shlaes, 2011). CdaA and the c-di-AMP produced by this enzyme are implicated in cell wall and potassium homeostasis (see below for details): (i) CdaA is encoded in a conserved operon with the enzyme GlmM that produces a building block for cell wall biosynthesis. (ii) In many bacteria with the CdaA as the only DAC, alterations in the c-di-AMP level affect cell wall synthesis and resistance to antibiotics that target cell wall synthesis. (iii) Proteins involved in potassium homeostasis are targeted by c-di-AMP in bacteria with CdaA as the only c-di-AMP synthesizing enzyme.

The involvement of CdaA in c-di-AMP production was first suggested for the Gram-positive human pathogen *L. monocytogenes*. The c-di-AMP produced and secreted by *L. monocytogenes* elicits a response of the immune system of infected host cells (Woodward *et al.*, 2010). The observation that overexpression of the *cdaA* gene resulted in an elevated response of the host cell immune system supported the hypothesis that c-di-AMP was produced by the CdaA protein in this organism. Indeed, CdaA from *Staph. aureus*, *L. monocytogenes* and *B. subtilis* are active DACs (Corrigan *et al.*, 2011; Mehne *et al.*, 2013; Rosenberg *et al.*, 2015).

The DAC CdaA contains three N-terminal  $\alpha$ -helices that form the transmembrane domain and a C-terminal DAC domain that is surrounded by two coiled-coil motifs of unknown function (Fig. 2). The structural characterization of an N-terminally truncated CdaA variant from *L. monocytogenes* lacking the transmembrane domain and the adjacent coiled-coil motif revealed that the shape of the cyclase domain is highly similar to that of DisA and the sporulation-specific DAC CdaS (Rosenberg *et al.*, 2015). A superposition of two CdaA monomers with the DAC domains of two DisA molecules results in a dimer model for CdaA with an identical arrangement of the residues in the active center (Rosenberg *et al.*, 2015). Interestingly,

neither the transmembrane domain nor the following coiled-coil motif is needed for the enzymatic activity of the *L. monocytogenes* CdaA DAC domain *in vitro* and *in vivo* (Rosenberg *et al.*, 2015). Similarly, a CdaA variant from the Gram-negative pathogenic bacterium *C. trachomatis* lacking the transmembrane domain was shown to be active (Barker *et al.*, 2013). These observations indicate that similar to the full-length proteins, the truncated variants must be oriented in a face-to-face manner, a molecular arrangement required for c-di-AMP synthesis (Rosenberg *et al.*, 2015).

In many  $\delta$ -proteobacteria and firmicutes, the *cdaA* gene is clustered with the *cdaR* and *glmM* genes encoding a regulator of CdaA activity and the phosphoglucosamine-6-phosphate mutase that generates a precursor for cell wall metabolism respectively. In *B. subtilis*, expression of the *cda-glm* operon depends on the housekeeping sigma factor  $\sigma^A$  (Mehne *et al.*, 2013). Recently, the *B. subtilis* CdaR protein was shown to physically interact with CdaA and to control the activity of the DAC (Mehne *et al.*, 2013). It will be interesting to elucidate the molecular details of the interaction between CdaR and CdaA as well as the intra- or extracellular signals that control the cyclase activity of CdaA. The conserved genomic context of the *cdaA*, *cdaR* and *glmM* genes suggests a link between c-di-AMP and cell wall homeostasis. It has indeed been shown that perturbation of the intracellular c-di-AMP pool causes alterations in cell wall composition and morphology in *B. subtilis* and *Staph. aureus* (Corrigan *et al.*, 2011; Mehne *et al.*, 2013). The connection between c-di-AMP metabolism and cell wall homeostasis is further supported by observations showing that mutant strains from *B. subtilis*, *Staph. aureus* and *L. monocytogenes* that produce different amounts of c-di-AMP had different susceptibilities to cell wall-targeting antibiotics (Luo and Helmann, 2012a; Dengler *et al.*, 2013; Witte *et al.*, 2013). It has been suggested that c-di-AMP affects the transglycosylation and transpeptidation activities of penicillin-binding proteins, which may lead to changes in cross-linking of the peptidoglycan of the cell wall (Corrigan *et al.*, 2011; Luo and Helmann, 2012a; Dengler *et al.*, 2013). The fact that c-di-AMP is implicated in cell wall-related phenotypes in *B. subtilis* and also in those pathogens that contain CdaA as the only DAC further supports the idea that the activity of CdaA is specifically related to cell wall metabolism. However, the precise function of c-di-AMP in cell wall homeostasis remains to be uncovered.

### The sporulation-specific DAC CdaS

The DAC CdaS is only present in the spore-forming *Bacillus* species and in one *Clostridium* species (Corrigan and Gründling, 2013). The corresponding *B. subtilis* *cdaS* gene is expressed exclusively during sporulation, sug-

gesting a function for this enzyme in spore development or germination (Nicolas *et al.*, 2012). Indeed, the germination efficiency of a *B. subtilis* *cdaS* mutant is about twofold reduced in comparison with that of a wild-type strain (Mehne *et al.*, 2014). Thus, CdaS is a DAC that is required for a specific developmental program in *B. subtilis* (Mehne *et al.*, 2014). However, the reason why the bacteria need a dedicated DAC during spore germination remains to be elucidated.

The structural characterization of the CdaS from *Bacillus cereus* revealed that the protein is composed of two N-terminal  $\alpha$ -helices that are attached to the C-terminal DAC domain (Fig. 2). Moreover, a model based on the CdaS structure suggests that the enzyme forms a hexamer in which the molecular arrangement of the monomers would not allow the enzyme to efficiently convert ATP to c-di-AMP (Mehne *et al.*, 2014, see Fig. 1). Indeed, the full-length CdaS enzyme is only weakly active. By contrast, mutant variants of CdaS that harbor amino exchanges in the N-terminal  $\alpha$ -helices exhibit an up to 100-fold increased activity as compared with the wild-type enzyme. Interestingly, truncated CdaS variants lacking one or both helices are enzymatically also highly active (Mehne *et al.*, 2014). These observations demonstrate that the N-terminal  $\alpha$ -helices of CdaS form an autoinhibitory domain that limits the catalytic activity of the DAC domain. It is tempting to speculate that the autoinhibitory domain of CdaS is also important for the *in vivo* control of the cyclase activity by a factor that remains to be identified.

### C-di-AMP degrading phosphodiesterases

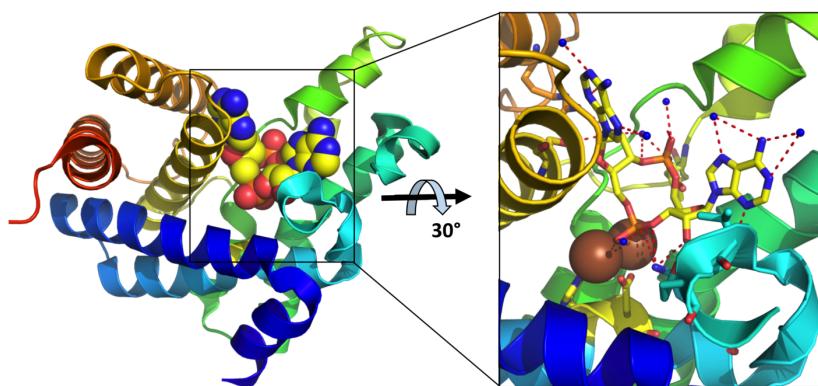
To control the cellular levels of any second messenger, not only its synthesis but also its degradation and possibly export, is an important factor. C-di-AMP is degraded by two different classes of phosphodiesterases. The enzymes of the first class contain a so-called DHH/DHHA1 domain with a catalytic Asp-His-His motif, whereas the enzymes of the second class contain an HD domain with a catalytic His-Asp motif (Rao *et al.*, 2010; Huynh *et al.*, 2015).

The GdpP phosphodiesterase was first studied since it contains a DHH/DHHA1 domain which is typically found in phosphatases and phosphodiesterases. Moreover, homologues of GdpP occur in many species that also possess DACs. Studies with a wide range of possible substrates revealed that GdpP of *B. subtilis* degrades c-di-AMP with high efficiency, whereas the activity with c-di-GMP is rather weak (Rao *et al.*, 2010). The protein consists of two N-terminal transmembrane helices suggesting that GdpP binds to the membrane, a degenerate PAS domain, a modified GGDEF domain and the DHH/DHHA1 domain. As expected, the catalytic activity of GdpP resides in the DHH/DHHA1 domain (Rao *et al.*,

2010). Phosphodiesterases containing a catalytic DHH/DHHA1 domain are present in a wide range of bacteria, including pathogenic firmicutes, the actinobacteria, but also in spirochaetes as exemplified by *Borrelia burgdorferi*. These enzymes can occur in two distinct forms: in firmicutes, the phosphodiesterases of this family usually have the same domain arrangement as described for *B. subtilis* GdpP. However, in *Strep. pneumoniae*, there is a second enzyme in addition to GdpP that consists only of the DHH/DHHA1 domain (Bai *et al.*, 2013). This short and cytoplasmic version of the enzyme is also present as the only c-di-AMP phosphodiesterase in *B. burgdorferi* and *M. tuberculosis* (Manikandan *et al.*, 2014; Ye *et al.*, 2014).

Studies of the enzymatic activity of the DHH family phosphodiesterases revealed that these enzymes require manganese for optimal activity and that they cleave c-di-AMP to the dinucleotide 5' pApA (Rao *et al.*, 2010). The multidomain phosphodiesterases (exemplified by GdpP) exhibit only this activity, whereas for two of the short enzymes, a second activity was demonstrated: These proteins can further degrade pApA to two molecules of AMP. The *M. tuberculosis* enzyme catalyzes both reactions in a sequential manner. In contrast, the short enzyme of *Strep. pneumoniae* does degrade c-di-AMP but prefers 5'pApA as the substrate indicating that c-di-AMP degradation involves the consecutive action of both phosphodiesterases in this organism (Bai *et al.*, 2013; Manikandan *et al.*, 2014).

The complex organization of GdpP and related enzymes suggests a function for the domains that are fused to the catalytically active DHH/DHHA1 domain. *In vitro*, the enzymes are active in the absence of the membrane domain. However, membrane localization has been shown to be essential for the activity of GdpP from *Streptococcus pyogenes* suggesting that the *in vitro* activity does not faithfully reflect the *in vivo* properties of GdpP (Cho and Kang, 2013). The relevance of the membrane domain for the GdpP activity has so far only been studied in *S. pyogenes*; therefore, it is not known whether the result can be generalized. The PAS (Per-Arnt-Sim) domain serves as sensory domain in many signal transduction proteins (Taylor and Zhulin, 1999). In GdpP, the PAS domain binds heme with equimolar stoichiometry, and heme binding results in inhibition of the phosphodiesterase catalytic activity. However, ferrous heme may form a complex with nitric oxide which then stimulates the enzyme activity of the DHH/DHHA1 domain (Rao *et al.*, 2011). Interestingly, the PAS domain of GdpP lacks the residues that are typically involved in the coordination of heme. Instead, this domain contains a hydrophobic pocket that may serve for heme binding (Tan *et al.*, 2013). Heme binding by GdpP may also help the cells to protect itself against the toxic effect of heme: In *Lactococcus*



**Fig. 4.** Structure of the HD domain of PgpH in complex with c-di-AMP. The monomeric HD domain of PgpH from *L. monocytogenes* (PDB 4S1B, in rainbow coloring from N- to C-terminus) is an overall helical protein binding c-di-AMP in its central depression. The box shows a magnification of the active site with the amino acid residues (stick models with the carbons colored according to the respective side chain, the other atoms colored as indicated in Fig. 3). The polar contacts are depicted as red dashed lines. The brown spheres indicate the two iron ions that are coordinated by residues below.

*lactis*, the *gdpP* mutant is much more sensitive toward heme than wild-type bacteria (Tan *et al.*, 2013). However, it is unknown whether GdpP directly sequesters toxic heme molecules or whether the control of c-di-AMP homeostasis by the phosphodiesterase activity of GdpP is responsible for the higher heme resistance of wild-type cells. Finally, GdpP contains a degenerate GGDEF domain. The GGDEF domain is usually endowed with diguanylate cyclase activity for the synthesis of c-di-GMP (Schirmer and Jenal, 2009); however, the critical residues for this activity are not conserved in GdpP and the related phosphodiesterases. Indeed, GdpP is not capable of synthesizing c-di-GMP. Instead, the GGDEF domain of GdpP binds and hydrolyzes ATP (Rao *et al.*, 2010). Inactive GGDEF domains are widespread in bacteria that produce c-di-GMP, and they are known to have a regulatory impact on adjacent domains (Christen *et al.*, 2005). However, the precise function of the GGDEF domain in GdpP still remains to be discovered.

When GdpP was first described as a c-di-AMP-specific phosphodiesterase, it was noted that much more bacterial species contain a potential DAC (based on the presence of a DAC domain) than a GdpP-type phosphodiesterase suggesting that there might be another class of enzymes involved in the degradation of c-di-AMP (Rao *et al.*, 2010). Indeed, such an alternative phosphodiesterase, PgpH, has recently been identified in *L. monocytogenes* (Huynh *et al.*, 2015). PgpH was first found in a screen for c-di-AMP-binding proteins (Sureka *et al.*, 2014). The protein binds and hydrolyzes c-di-AMP. As observed for the DHH/DHHA1-type phosphodiesterases, PgpH requires manganese ions for activity. The enzyme consists of an N-terminal extracellular domain, seven transmembrane helices and the C-terminal His-Asp (HD) domain. The catalytic activity of the protein resides in the HD domain which is present in many nucleic acid-binding and hydrolyzing domains (Aravind and Koonin, 1998). The HD domain is composed of two sub-domains, and the two active site residues Asp and His as well as the c-di-AMP molecule are located in the

depression formed by these sub-domains (Huynh *et al.*, 2015) (Fig. 4). Since *L. monocytogenes* produces not only PgpH but also PdeA, a phosphodiesterase of the GdpP type, it is possible that the two enzymes have specialized in function. This seems indeed to be the case since PgpH was found to be the major c-di-AMP degrading enzyme *in vitro*, whereas the activity of PdeA prevails when the bacteria have infected the host cells (Huynh *et al.*, 2015).

HD-type phosphodiesterases are present in a wide variety of bacteria. In particular, these phosphodiesterases seem to be the major c-di-AMP-degrading enzymes in the cyanobacteria, in the bacteroidetes, in fusobacteria and in species of the genus *Thermotoga*. In the firmicutes, these enzymes are present in many species where they coexist with phosphodiesterases of the GdpP type. This is the case in *B. subtilis*, where the phosphodiesterase GdpP and the PgpH ortholog YqfF are likely to contribute to the degradation of c-di-AMP. In contrast, groups like *Staphylococcus* or *Streptococcus* do not possess HD-type phosphodiesterases. In actinobacteria, the occurrence of HD-type phosphodiesterases is rather exceptional suggesting that these bacteria rely on GdpP-like enzymes (Huynh *et al.*, 2015).

As components of a signal transduction pathway, the expression and activity of the c-di-AMP degrading phosphodiesterases can be expected to be controlled by extra and intracellular signals. In *B. subtilis*, the expression of the *gdpP* gene encoding GdpP is controlled by a noncoding antisense RNA (Luo and Helmann, 2012b). Moreover, the activities of both classes of phosphodiesterases are controlled by the signaling nucleotide ppGpp suggesting a link between c-di-AMP-mediated signal transduction and the stringent response. This was first shown for GdpP from *B. subtilis*, which is subject to a strong competitive inhibition by ppGpp (Rao *et al.*, 2010) and was recently confirmed for GdpP from *Staph. aureus* (Corrigan *et al.*, 2015). Interestingly, in *L. lactis*, *L. monocytogenes* and in *Staph. aureus*, increased levels of c-di-AMP trigger the stringent response, resulting in increased amounts of ppGpp (Rallu *et al.*, 2000; Liu *et al.*, 2006; Corrigan *et al.*,

2015). This molecule in turn inhibits GdpP, thus keeping the concentration of c-di-AMP high. This intricate link between the two signaling pathways is also supported by the substantial overlap of transcriptional regulation upon increased c-di-AMP (*gdpP* mutant) and ppGpp (stringent response) levels in *Staph. aureus* (Corrigan et al., 2015). In bacteria with both types of phosphodiesterases, the interrelation with the stringent response would not work if one of the enzymes would still be active under starvation conditions that lead to the stringent response. Therefore, it is not surprising that also the HD-type phosphodiesterase PgpH is subject to allosteric inhibition by ppGpp (Huynh et al., 2015). This common regulation of both phosphodiesterases ensures that the stringent response signal is efficiently transmitted to the c-di-AMP signaling pathways.

### Export of c-di-AMP

The intracellular concentration of a molecule can be controlled by either degradation or secretion to the medium. Indeed, *L. monocytogenes* can export c-di-AMP. The secretion of the dinucleotide was found to trigger an immune response, the induction of interferon- $\beta$  production (Woodward et al., 2010). Four multidrug transporters (Mdr) of the major superfacilitator family are implicated in the export of c-di-AMP in *L. monocytogenes* (Kaplan Zeevi et al., 2013; Tadmor et al., 2014). Loss of all these exporters results in an increased sensitivity of the bacteria to sublethal concentrations of antibiotics that target cell wall biosynthesis due to a reduced synthesis of peptidoglycan under these conditions. However, the sensitivity of the bacteria lacking the c-di-AMP exporters to cell wall antibiotics cannot be rescued by the addition of extracellular c-di-AMP. This finding is surprising because the enzymes targeted by these antibiotics act on the extracellular penicillin-binding proteins. It has therefore been suggested that the putative c-di-AMP exporters are required for the regulation of cell wall synthesis during cell wall stress rather than for the actual assembly of peptidoglycan (Kaplan Zeevi et al., 2013). Unfortunately, the involvement of the Mdr transporters in c-di-AMP secretion has so far only been shown by indirect experiments, and the extracellular concentrations of c-di-AMP in wild-type strains as well as in bacteria lacking or overexpressing the transporters have so far not directly been determined. Moreover, the molecular mechanisms that implicate the Mdr transporters in peptidoglycan and lipoteichoic acid biosynthesis remain to be unraveled.

Interestingly, the single c-di-AMP-degrading phosphodiesterase of *B. burgdorferi* is essential (Ye et al., 2014). It is tempting to speculate that these bacteria are unable to secrete c-di-AMP and that the intracellular accumulation of the nucleotide might be toxic as has been shown

for *B. subtilis*, *L. monocytogenes* and *Strep. pneumoniae* (Bai et al., 2013; Mehne et al., 2013; Huynh et al., 2015).

### C-di-AMP-binding proteins

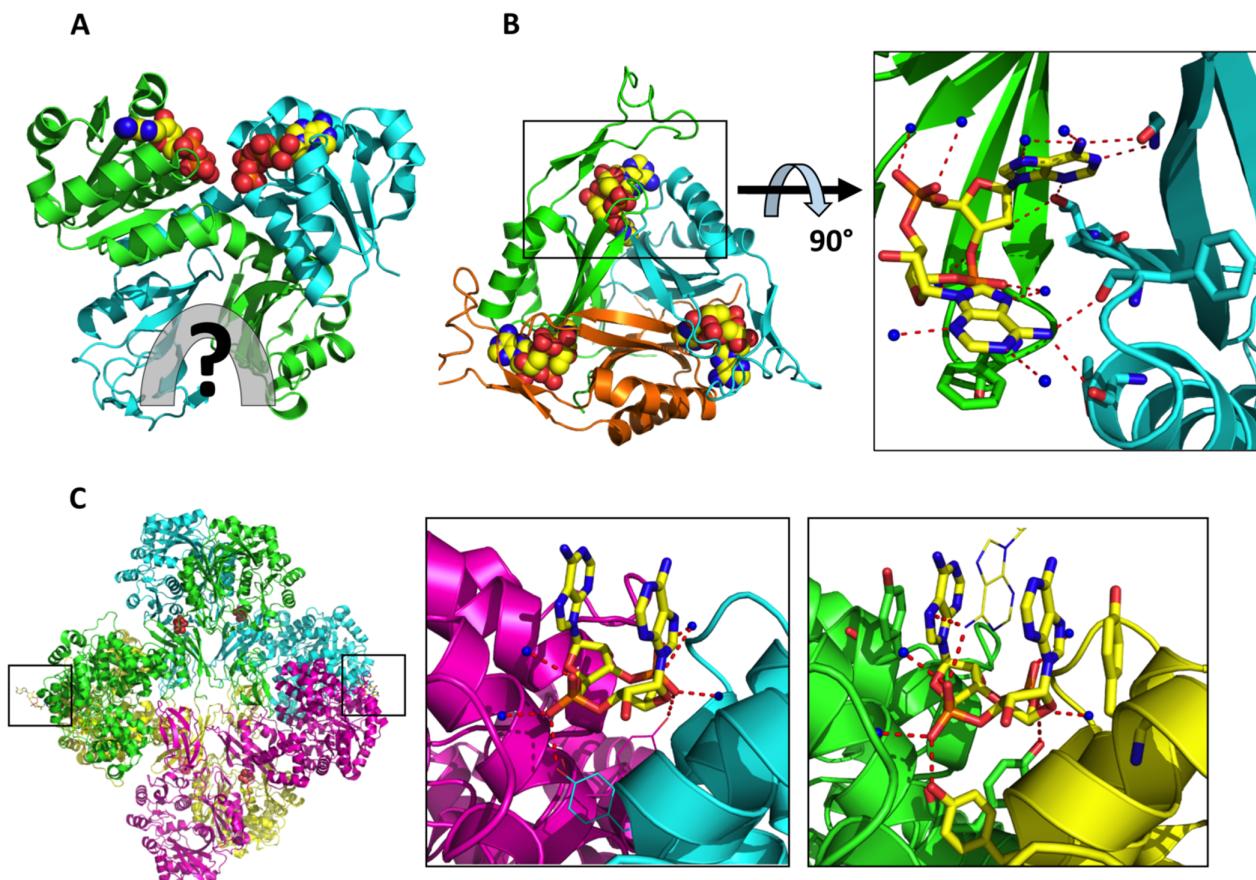
Given the importance of c-di-AMP for bacterial physiology, it is obvious that the identification of potential binding partners of the molecule has become a major issue. This search has been driven by three major expectations: (i) the identification of the target(s) that is/are causative for the essentiality of c-di-AMP; (ii) the explanation of the diverse physiological functions of c-di-AMP; and (iii) the identification of conserved protein domains that bind c-di-AMP. Indeed, in the case of c-di-GMP, two major domains interacting with the molecule have been identified: the so-called PilZ domain and degenerated GGDEF/EAL domains (Ryjenkov et al., 2006; Duerig et al., 2009; Schirmer and Jenal, 2009). However, binding of c-di-GMP is not limited to proteins containing either of these domains (Ryan et al., 2012; Tschiwri et al., 2014). Of the expectations just mentioned, one has partially come true, as we can now understand how c-di-AMP is implicated in some of its diverse functions at the molecular level.

### The *Mycobacterium smegmatis* transcription factor DarR

The first c-di-AMP-binding protein was identified in *M. smegmatis* in a global screen for transcription factors that bind the second messenger. One protein, DarR, was found in this approach (Zhang et al., 2013). C-di-AMP binds to the protein with high affinity, and the interaction is specific for this nucleotide. DarR is a so far unknown transcription factor of the TetR family. It binds to the promoter regions and thereby represses the expression of its own gene and of a divergently transcribed operon that is involved in fatty acid metabolism as well as of the *cspA* gene encoding a cold shock protein. Binding of DarR to its targets is stimulated by c-di-AMP, suggesting that it acts as a cofactor for DarR. The *darR* mutant exhibits increased cell length, a phenotype typically associated with alterations of c-di-AMP homeostasis. Interestingly, overexpression of DarR inhibits growth and is toxic at high levels. Unfortunately, these phenotypes and effects of DarR have so far not been studied in strains with altered c-di-AMP levels, so the *in vivo* confirmation for the implication of c-di-AMP in the control of gene expression via DarR is still pending. A bioinformatics analysis revealed that DarR occurs only in a few actinobacteria and thus does not seem to be a general target of c-di-AMP (Zhang et al., 2013).

### Control of potassium homeostasis by c-di-AMP

An unbiased search of c-di-AMP-binding proteins was performed with *Staph. aureus* protein extracts. For this



**Fig. 5.** Structures of c-di-AMP target proteins.

A. Structure of a KtrA in complex with ATP (PDB 4J90). Cyclic di-AMP has been shown to bind to the C-terminal region (RCK\_C domain). A possible binding site that can be proposed in analogy to the binding of c-di-AMP to its other targets is indicated by a grey arch.

B. DarA (4RLE) (left) with magnification of the binding site (right).

C. *L. monocytogenes* pyruvate carboxylase with c-di-AMP bound (4QSH). The magnifications show the two binding sites of c-di-AMP. Coloring for all panels as described in Fig. 3.

approach, c-di-AMP was coupled to magnetic beads, and proteins that bind specifically to these beads were identified (Corrigan *et al.*, 2013). This analysis uncovered one major target protein, the KtrA subunit of a high-affinity potassium transporter. KtrA forms an octameric ring and binds two membrane-spanning KtrB proteins to facilitate potassium uptake (Vieira-Pires *et al.*, 2013). KtrA is the cytoplasmic gating component of the complex. The protein consists of two so-called RCK domains (regulator of conductance of  $K^+$ ). The N-terminal RCK\_N domain binds  $NAD^+/NADH^+$  and ADP/ATP and interacts with the membrane component KtrB, whereas the RCK\_C domain is directed toward the cytoplasm (Vieira-Pires *et al.*, 2013). Binding studies with the isolated domains revealed that the RCK\_C domain is the actual target for c-di-AMP. The RCK\_C domain is made up of a five stranded  $\beta$ -sheet flanked by helices on the cytoplasmic side and a quite distant sandwich with the  $\beta$ -sheet from the other subunit resulting in an open cleft. A comparison with other known

c-di-AMP interacting motifs suggests that this pseudo-symmetric arrangement may be the binding site for the nucleotide (Vieira-Pires *et al.*, 2013) (see Fig. 5A). The physiological relevance of c-di-AMP binding to KtrA is supported by the observation that the *gdpP* mutant that accumulates c-di-AMP phenocopies the growth defect of a *ktrA* mutant at low potassium concentrations. This observation also implies that binding of c-di-AMP to KtrA at high intracellular levels of the second messenger inhibits potassium uptake. KtrA is not the only protein in *Staph. aureus* that contains an RCK\_C domain. The second protein, CpaA, a cation proton antiporter, does also bind c-di-AMP, suggesting that the RCK\_C domain functions as a regulatory domain that senses the c-di-AMP levels and causes a response in the transport of potassium ions. The interaction between the RCK\_C domain and c-di-AMP is not limited to *Staph. aureus* since the homologous KtrA proteins of *B. subtilis*, *Strep. pneumoniae* and *C. glutamicum* do also bind and respond to c-di-AMP (Corrigan *et al.*,

2013; Bai *et al.*, 2014; Oliver Goldbeck and Gerd Seibold, pers. comm.). As in *Staph. aureus*, accumulation of c-di-AMP in a mutant defective in both phosphodiesterases results in reduced growth at low potassium concentrations in *Strep. pneumoniae* (Bai *et al.*, 2014). Thus, the control of the *Staph. aureus* and *Strep. pneumoniae* KtrA proteins seems to be similar. *B. subtilis* contains five proteins containing a RCK\_C domain. These proteins are all involved in potassium and monovalent cation transport (see [http://subtiwiki.uni-goettingen.de/wiki/index.php/RCK\\_C\\_domain](http://subtiwiki.uni-goettingen.de/wiki/index.php/RCK_C_domain); Michna *et al.*, 2014). In addition, an unbiased screen using an expression library of *Staph. aureus* proteins in *E. coli* identified the sensor kinase KdpD of a potassium-responsive two-component regulatory system as an additional target of c-di-AMP (Corrigan *et al.*, 2013). Even though the physiological relevance of c-di-AMP binding has not been demonstrated for any of these proteins except KtrA, it is tempting to speculate that c-di-AMP controls potassium homeostasis at a global level. However, the fact that KtrA is inhibited by c-di-AMP indicates that this transporter is unlikely to be the essential target of c-di-AMP.

### The pyruvate carboxylase of *L. monocytogenes*

A global screen for proteins binding to c-di-AMP-coated Sepharose beads with a protein extract of *L. monocytogenes* identified the phosphodiesterases GdpP and PgpH as well as the pyruvate carboxylase, PycA, and three unknown proteins (see below) (Sureka *et al.*, 2014). The pyruvate carboxylase is an important enzyme for *L. monocytogenes* as it provides the only possibility for the bacteria to generate oxaloacetate due to an incomplete citric acid cycle. The enzyme is essential if *L. monocytogenes* grows with sugars as single carbon source but dispensable in complex media in the presence of alternative sources of aspartate and glutamate (Schär *et al.*, 2010). C-di-AMP binds the pyruvate carboxylase with high affinity ( $K_D$  of about 8  $\mu\text{M}$ ) and inhibits the activity of the enzyme. A structural investigation of *L. monocytogenes* pyruvate carboxylase revealed that each tetramer of the enzyme binds two molecules of c-di-AMP. Biochemical and crystallographic studies of the interaction of pyruvate carboxylase with c-di-AMP revealed a previously unrecognized allosteric regulatory site in 25 Å distance to the active center which is located in the interface of the carboxyltransferase domains of two subunits (Fig. 5C). Interestingly, the nucleotides are facing toward the cytoplasmic environment, raising the question of how discrimination of other cyclic dinucleotides is achieved. Binding of c-di-AMP induces large overall conformational changes that ultimately lead to inhibition of enzyme activity (Sureka *et al.*, 2014). It has been speculated that the pyruvate carboxylases of several firmicutes might be controlled by c-di-AMP;

however, the c-di-AMP binding site is only poorly conserved, and the nucleotide has no or only little inhibitory activity on the enzymes of *Enterococcus faecalis* and *Staph. aureus* respectively (Sureka *et al.*, 2014). These latter observations and the fact that the activity of the citric acid cycle has a main impact on oxaloacetate production in most firmicutes suggest that the control of pyruvate carboxylase by c-di-AMP is rather specific for *L. monocytogenes* as an organism with an incomplete citric acid cycle. As mentioned above for KtrA, the inhibition of pyruvate carboxylase by c-di-AMP as well as the limited distribution of this control among the firmicutes suggests that this enzyme is not related to the essential function of c-di-AMP.

### The $\text{P}_{II}$ -like proteins of the DarA family: a rendezvous of two paradigms of signal transduction

Among the three unknown proteins that interact with c-di-AMP in *L. monocytogenes*, one protein is highly conserved in the firmicutes. This protein was also found as a c-di-AMP binding protein in *Staph. aureus*, and it was the most prominent target of c-di-AMP in cell extracts of *B. subtilis*. The protein was designated PstA in *Staph. aureus* and *L. monocytogenes* and DarA in *B. subtilis* (Corrigan *et al.*, 2013; Sureka *et al.*, 2014; Gundlach *et al.*, 2015). Functional studies with DarA revealed that also this target protein is not implicated in the essentiality of c-di-AMP. The loss of the protein does not result in obvious phenotypic consequences during growth in minimal and complex media in *B. subtilis*, and its function has not been identified in any of the three organisms. However, the *darA* gene is in many organisms genetically linked to the *tmk* gene encoding the essential thymidylate kinase. It is therefore tempting to speculate that DarA might be implicated in the control of nucleotide biosynthesis for DNA replication. The affinity of DarA to c-di-AMP is very high ( $K_D$  values between 0.11  $\mu\text{M}$  and 1.4  $\mu\text{M}$  for the proteins of *Staph. aureus* and *L. monocytogenes* respectively) (Campeotto *et al.*, 2015; Choi *et al.*, 2015; Müller *et al.*, 2015). Interaction studies of DarA with a variety of mononucleotides and cyclic dinucleotides revealed that the protein is highly selective and does not bind to any of the mononucleotides or to cyclic di-GMP (Campeotto *et al.*, 2015; Choi *et al.*, 2015; Gundlach *et al.*, 2015; Müller *et al.*, 2015). The only molecule that binds *B. subtilis* DarA in addition to c-di-AMP is cyclic GMP-AMP. However, this molecule is synthesized in some bacteria such as *Vibrio cholerae* but not in *B. subtilis* (Davies *et al.*, 2012; Gundlach *et al.*, 2015). Therefore, this interaction is not likely to be of physiological relevance.

The determination of the crystal structure of DarA revealed that the protein is structurally similar to the  $\text{P}_{II}$  family of signal transduction proteins (Campeotto *et al.*, 2015; Choi *et al.*, 2015; Gundlach *et al.*, 2015; Müller

*et al.*, 2015). These P<sub>II</sub> proteins are highly conserved in bacteria, archaea and plants, and are involved in the regulation of nitrogen metabolism. They bind to small effector molecules such as 2-oxoglutarate and ATP and may be subject to post-translational modifications in response to the nitrogen state of the cell (Forchhammer, 2008; Huergo *et al.*, 2013). The different forms of P<sub>II</sub> proteins interact with a variety of different partners including the ammonium transporter AmtB, enzymes of nitrogen and fatty acid metabolism, and transcription factors such as the *B. subtilis* global nitrogen regulator TnrA (Heinrich *et al.*, 2006; Conroy *et al.*, 2007; Beez *et al.*, 2009; Gerhardt *et al.*, 2015; Schumacher *et al.*, 2015).

The proteins of the DarA family can be regarded as a sister group of the P<sub>II</sub> signal transducers. Interestingly, these proteins bring together signaling by small versatile regulatory proteins and second messengers, two well-established paradigms in signal transduction. The core architecture of trimeric P<sub>II</sub> proteins and DarA homologues is nearly identical: The central core of the trimer is formed by β-sheets and surrounded by two α-helices per subunit (Zeth *et al.*, 2012). The most significant alterations with respect to the P<sub>II</sub> proteins are observed in the length of the so-called B- and T-loops, which are swapped in size. The c-di-AMP molecules are bound at the interaction sites of neighboring subunits in a deep pocket (Fig. 5B). This pocket comprises in parts the highly conserved amino acid motifs identified between residues T28 and T43 (according to *B. subtilis* DarA) with the consensus sequence of TKLxxxGGFLxxGNTT and a highly conserved GGA amino acid stretch located in the C-terminal region (aa 94–96 in DarA). The binding of c-di-AMP results in a structural rearrangement of the B-loops as well as the T-loops which both become more structured upon interaction with the ligand (Campeotto *et al.*, 2015; Choi *et al.*, 2015; Müller *et al.*, 2015). This in turn may change the binding properties to other proteins downstream in the signaling cascade. The mode of binding of effector molecules and its consequences on the neighboring loop regions again resembles signal perception by P<sub>II</sub> proteins.

The proteins of the DarA family are widespread in firmicutes, and in some cases, they are found in green nonsulfur bacteria and spirochaetes. It should be noted, however, that the DarA family of proteins is absent from the actinobacteria. In contrast, some green nonsulfur bacteria such as *Sphaerotilus thermophilus* and *Thermomicrobium roseum* contain three paralogs (Campeotto *et al.*, 2015).

### C-di-AMP is a novel ligand for proteins containing a CBS domain

The screen for c-di-AMP-binding proteins in *L. monocytogenes* finally identified two unknown proteins (CbpA

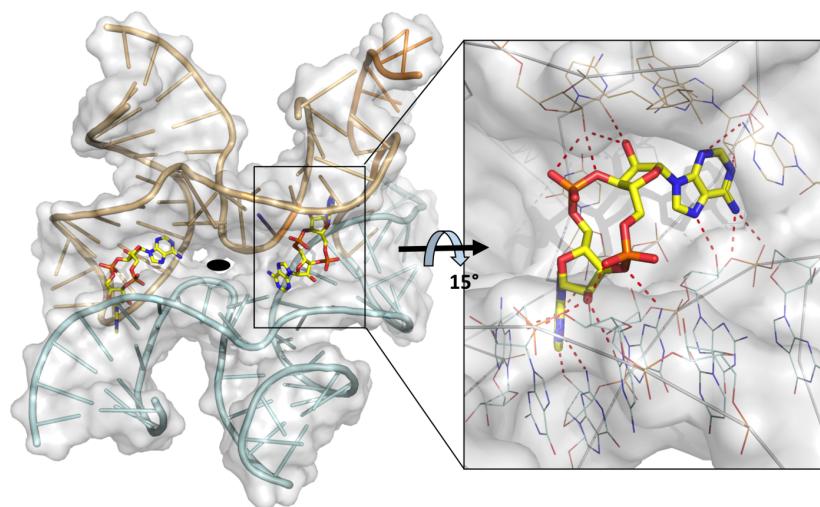
and CbpB) (Sureka *et al.*, 2014). Interestingly, both proteins contain the so-called CBS domain, which got its designation since it was first discovered in cystathione beta synthase. The CBS domain is known to bind ligands containing adenosyl groups (AMP, ATP or S-adenosylmethionine). In response to ligand binding, the CBS domain controls (usually inhibits) the activity of associated enzymatically active domains (Baykov *et al.*, 2011; Ereño-Orbea *et al.*, 2013). It is tempting to speculate that c-di-AMP might be a novel adenosyl ligand that may control the activity of CBS domain-containing proteins. Whereas CbpA is a poorly conserved protein, CbpB has a counterpart in *B. subtilis*, YkuL. However, the function of this protein has not yet been studied. It will be interesting to determine the physiological relevance of the binding of c-di-AMP to these CBS domain proteins.

### C-di-AMP-responsive riboswitches

For the second messenger c-di-GMP, it has been shown that the molecule can also bind to different classes of regulatory RNA structures, so-called riboswitches, in addition to its interactions with proteins. The c-di-GMP binding riboswitches control the expression of genes involved in virulence, motility and purine metabolism (Sudarsan *et al.*, 2008; Kim *et al.*, 2015). A detailed mutagenesis study using a c-di-GMP responsive riboswitch indicated that only very few mutations are required to switch the interaction specificity from c-di-GMP to c-di-AMP. Therefore, the existence of c-di-AMP-responsive riboswitches was proposed, and it seems possible that c-di-AMP may also control gene expression by binding to riboswitches (Smith and Strobel, 2011).

Indeed, there are several known riboswitches for which no ligand that controls their folding has been identified (Barrick *et al.*, 2004). For one of those riboswitches, the *ydaO* riboswitch, ATP has first been described to be the primary ligand (Watson and Fedor, 2012). However, the weak affinity of the *ydaO* riboswitch for ATP suggested that a different molecule might be the physiologically relevant ligand. An unbiased search for molecules that bind the *ydaO* riboswitch unequivocally identified c-di-AMP as the primary ligand, which binds to the *B. subtilis* *ydaO* riboswitch at picomolar concentrations which is far below the intracellular concentration of c-di-AMP (Nelson *et al.*, 2013).

Generally, riboswitches are composed of two parts: a ligand sensing domain and the expression platform. The recently determined structures of the ligand binding domains of c-di-AMP-responsive riboswitches from various organisms revealed two unusual features. First, the overall structure exhibits an internal pseudo-two-fold symmetry of the RNA, and second, the RNA requires two



**Fig. 6.** Structure of the c-di-AMP-binding *ydaO* riboswitch from *B. subtilis* (PDB 4W90). Left: Overall structure with the two pseudo-symmetric elements is depicted in orange and light blue respectively. C-di-AMP is indicated as described in Fig. 3. The pseudosymmetric center is indicated by a black oval. The overall surface of the RNA is shown in gray. Right: Magnification of the c-di-AMP binding site, revealing an intricate pattern of polar contacts to the nucleosides of the RNA (depicted as lines). The gray lines indicate the overall backbone of the riboswitch.

c-di-AMP molecules for a stable assembly (Fig. 6) (Gao and Serganov, 2014; Jones and Ferré-D'Amaré, 2014; 2015; Ren and Patel, 2014). The structure is composed of well-defined structural elements, namely, two similar three-way junction elements containing three to four helices each, depending on the organism. The halves are associated in opposing orientation resulting in the observed symmetry. Both the 5' and 3' ends of the RNA are directly involved in helix formation, in case of the *B. subtilis* *ydaO* riboswitch by direct base pairing (Jones and Ferré-D'Amaré, 2014) or by pairing with central stretches of the riboswitch as observed in *Thermoanaerobacter* and *Thermovirga* species (Gao and Serganov, 2014; Ren and Patel, 2014). The two c-di-AMP molecules are bound at the interface of the two halves and separated by a channel traversing the molecule in its symmetry center (Fig. 6). The conformation of the c-di-AMP molecules bound to the riboswitch differs substantially from that of c-di-AMP bound to proteins. In contrast to the U-shaped arrangement of c-di-AMP when bound to proteins (as in the DACs or in DarA) (Figs 3 and 5B), the c-di-AMP in the riboswitch is more bow shaped with the adenines further apart and tilted, a consequence of binding in the respective pockets formed most likely upon interaction. Ligand specificity is achieved by an intricate pattern of interactions between the RNA and all parts of the c-di-AMP molecule (see Fig. 6), which enable discrimination of c-di-GMP, c-GAMP and even c-di-dAMP (Ren and Patel, 2014). The main role of the two c-di-AMP molecules seems to contribute to riboswitch stability (Jones and Ferré-D'Amaré, 2014). As mentioned above, a c-di-GMP-responsive riboswitch could easily be modified to allow binding of c-di-AMP (Smith and Strobel, 2011). However, the *ydaO* riboswitch is unrelated in sequence and structure to the two distinct riboswitches that bind c-di-GMP.

The *ydaO* riboswitch is widespread in bacteria and controls the expression of genes involved in ion (mainly potassium) and amino acid transport as well as of genes involved in cell wall metabolism. Strikingly, osmoprotection and cell wall integrity seem to be unifying functions for the different genes controlled by an *ydaO* riboswitch. As just stated, this RNA motif is widespread in bacteria, but it is found only in selected organisms of the phylogenetic groups that synthesize c-di-AMP. For example, whereas essentially all firmicutes contain c-di-AMP, the *ydaO* riboswitch is present in many *Bacillus* and *Clostridium* species but absent from all members of the genera *Lactococcus*, *Listeria*, *Staphylococcus* and *Streptococcus*. Even within one genus, the riboswitch is present in some species but not in others. This is the case for the genus *Clostridium*, where the *ydaO* switch is found (e.g. in *C. acetobutylicum*, *C. tetani*, *C. botulinum* or *C. cellulolyticum* but not in *C. difficile*, *C. sticklandii* or *C. perfringens*). In addition to the firmicutes, the *ydaO* riboswitch is commonly present in the actinobacteria (including *Streptomyces coelicolor*, *M. tuberculosis* and *C. glutamicum*) as well as in some cyanobacteria (*Anabaena*, *Nostoc* and *Synechococcus*) and δ-proteobacteria (*Geobacter*, *Desulfovibrio*) (Block *et al.*, 2010). However, the biological relevance of the *ydaO* riboswitch in the latter species remains to be determined.

It is interesting to note that the *ydaO* riboswitch – as most other riboswitches – is typically located in the 5' untranslated leader regions of the controlled genes. However, in the case of several acetyltransferase genes in Firmicutes, the riboswitch resides in the putative 3' untranslated regions of the potential mRNAs. Again, the relevance of this observation is so far unknown, but it has been suggested that this putative downstream riboswitch might represent a novel expression platform architecture with a yet unknown mechanism of regulation (Block *et al.*, 2010).

In the model bacterium *B. subtilis*, the *ydaO* riboswitch is present in the 5' UTRs of the *ydaO* gene and of the *ktrAB* operon. While the precise function of the *ydaO* gene remains to be elucidated, the *ktrAB* operon encodes the two subunits of the high-affinity potassium transporter KtrAB. This latter observation is particularly striking since c-di-AMP does also bind the KtrA protein to control its activity (Corrigan *et al.*, 2013; see above). As just outlined, the essentiality of c-di-AMP might result from the stimulation of an essential biological process or, alternatively, from the inhibition of a toxic function. Since the *ydaO* gene and the *ktrAB* operon can be simultaneously deleted from the *B. subtilis* genome, the *ydaO* riboswitch is clearly not essential. Moreover, deletion of the two loci does not allow the deletion of all DAC-encoding genes. This observation indicates that the enzymes controlled by the *ydaO* riboswitch do not have a toxic activity that is prevented upon c-di-AMP binding. In conclusion, the genes controlled by the *ydaO* riboswitch are not relevant for the essentiality of this second messenger (Christina Herzberg and Jörg Stülke, unpubl. obs.).

A major issue of riboswitch regulation is the mode of action of control by ligand binding, which may result in increased or decreased expression of the controlled genes (ON or OFF switches respectively). In the case of the *B. subtilis* *ydaO* riboswitch, binding of the ligand results in premature transcription termination, and lack of either the DisA or the CdaA DAC results in increased expression of the controlled gene due to the reduced levels of c-di-AMP (Nelson *et al.*, 2013). Thus, the *ydaO* switch is a so-called genetic OFF switch. Strikingly, c-di-AMP acts in the same direction in the control of the KtrA protein activity and the *ydaO* riboswitch that controls the expression of the *ktrAB* operon. In both cases, binding of c-di-AMP has an inhibitory effect, either on protein activity or on gene expression. This makes c-di-AMP the only second messenger that controls a biological activity (potassium transport) by binding to a protein and to the corresponding mRNA.

Recently, the expression of the *S. coelicolor rpfA* gene encoding a cell wall hydrolase (the so-called resuscitation promoting factor A) that is required for spore germination was studied in detail. The expression of the *rpfA* gene and the accumulation of the RpfA enzyme are under complex control by second messengers involving cAMP, c-di-AMP and ppGpp. Transcription initiation of the gene requires cAMP and its receptor protein Crp as transcription activator. Once initiated, elongation of transcription may stop at an unusual transcriptional terminator in the 5' UTR. Strikingly, this untranslated region contains a motif similar to the *ydaO* riboswitch, and binding of c-di-AMP to this riboswitch results in transcription termination. Since c-di-AMP is essential in the firmicutes but not in actinobacteria, the effect of a deletion of the single DAC-encoding gene could be tested in *S. venezuelae*. As expected for a genetic

OFF switch, expression of the *rpfA* gene became constitutive upon loss of c-di-AMP production. This increased expression resulted from an uncontrolled read-through at the c-di-AMP-responsive riboswitch (St-Onge *et al.*, 2015). The regulation of cell wall metabolic genes by c-di-AMP may be common in the actinobacteria, since in these bacteria, the *ydaO* riboswitch is nearly exclusively linked to genes involved in cell wall metabolism (Block *et al.*, 2010).

## Open questions

The identification of different targets of c-di-AMP indicates that this second messenger is involved in a variety of cellular functions. However, one of the main questions, the reason for the essentiality of c-di-AMP, remains to be answered. Moreover, the control of c-di-AMP synthesis, especially by CdaA, is an important question for further research. Another problem to be solved is the role of c-di-AMP secretion. Does it serve an extracellular target or is the export a way of disposing off superfluous c-di-AMP? Finally, the mechanism(s) that involve c-di-AMP in DNA repair have to be uncovered. Research on this fascinating essential second messenger has been highly dynamic in the past few years; however, we can look forward to new exciting discoveries.

## Acknowledgements

We are grateful to Christina Herzberg, Jonathan Rosenberg and all other members of the Commichau and Stülke labs for fruitful discussions. Oliver Goldbeck and Gerd Seibold are acknowledged for sharing results prior to publication. We wish to thank Karl Forchhammer for helpful comments on the manuscript. Work in the authors' labs was supported by grants of the Deutsche Forschungsgemeinschaft (to J.S.) and the Fonds der Chemischen Industrie (to F.M.C.).

## References

- Aravind, L., and Koonin, E.V. (1998) The HD domain defines a new superfamily of metal-dependent phosphohydrolases. *Trends Biochem Sci* **23**: 469–472.
- Bai, Y., Yang, J., Zhou, X., Ding, X., Eisele, L.E., and Bai, G. (2012) *Mycobacterium tuberculosis* Rv3586 (DacA) is a diadenylate cyclase that converts ATP or ADP into c-di-AMP. *PLoS ONE* **7**: e35207.
- Bai, Y., Yang, J., Eisele, L.E., Underwood, A.J., Koestler, B.J., Waters, C.M., *et al.* (2013) Two DHH subfamily 1 proteins in *Streptococcus pneumoniae* possess cyclic di-AMP phosphodiesterase activity and affect bacterial growth and virulence. *J Bacteriol* **195**: 5123–5132.
- Bai, Y., Yang, J., Zarella, T.M., Zhang, Y., Metzger, D.W., and Bai, G. (2014) Cyclic di-AMP impairs potassium uptake mediated by a cyclic di-AMP binding protein in *Streptococcus pneumoniae*. *J Bacteriol* **196**: 614–623.
- Barker, J.R., Koestler, B.J., Carpenter, V.K., Burdette, D.L.,

- Waters, C.M., Vance, R.E., and Valdivia, R.H. (2013) STING-dependent recognition of cyclic di-AMP mediates type I interferon responses during *Chlamydia trachomatis* infection. *MBio* **4**: e00018-13.
- Barrick, J.E., Corbino, K.A., Winkler, W.C., Nahvi, A., Mandal, M., Collins, J., et al. (2004) New RNA motifs suggest an extended scope for riboswitches in bacterial genetic control. *Proc Natl Acad Sci USA* **101**: 6421–6426.
- Baykov, A.A., Tuominen, H.K., and Lahti, R. (2011) The CBS domain: a protein module with an emerging prominent role in regulation. *ACS Chem Biol* **6**: 1156–1163.
- Beez, S., Fokina, O., Herrmann, C., and Forchhammer, K. (2009) N-acetyl-L-glutamate kinase (NAGK) from oxygenic phototrophs: P(II) signal transduction across domains of life reveals novel insights in NAGK control. *J Mol Biol* **389**: 748–758.
- Bejerano-Sagie, M., Oppenheimer-Shaanan, Y., Berlatzky, I., Rouvinski, A., Meyerovich, M., and Ben-Yehuda, S. (2006) A checkpoint protein scans the chromosome for damage at the start of sporulation in *Bacillus subtilis*. *Cell* **125**: 679–690.
- Block, K.F., Hammond, M.C., and Breaker, R.R. (2010) Evidence for widespread gene control function by the *ydaO* riboswitch candidate. *J Bacteriol* **192**: 3983–3989.
- Campeotto, I., Zhang, Y., Mladenov, M.G., Freemont, P.S., and Gründling, A. (2015) Complex structure and biochemical characterization of the *Staphylococcus aureus* cyclic diadenylate monophosphate (c-di-AMP)-binding protein PstA, the founding member of a new signal transduction protein family. *J Biol Chem* **290**: 2888–2901.
- Campos, S.S., Ibarra-Rodriguez, J.R., Barajas-Ornelas, R.C., Ramírez-Guadiana, F.H., Obregón-Herrera, A., Setlow, P., and Pedraza-Reyes, M. (2014) Interaction of apurinic/apyrimidinic endonucleases Nfo and ExoA with the DNA integrity scanning protein DisA in the processing of oxidative DNA damage during *Bacillus subtilis* spore outgrowth. *J Bacteriol* **196**: 568–578.
- Carrasco, B., Cozar, M.C., Lurz, R., Alonso, J.C., and Ayora, S. (2004) Genetic recombination in *Bacillus subtilis* 168: contribution of Holliday junction processing functions in chromosome segregation. *J Bacteriol* **186**: 5557–5566.
- Chaudhuri, R.R., Allen, A.G., Owen, P.J., Shalom, G., Stone, K., Harrison, M., et al. (2009) Comprehensive identification of essential *Staphylococcus aureus* genes using transposon-mediated differential hybridization (TMDH). *BMC Genomics* **10**: 291.
- Cho, K.H., and Kang, S.O. (2013) *Streptococcus pyogenes* c-di-AMP phosphodiesterase, GdpP, influences SpeB processing and virulence. *PLoS ONE* **8**: e69425.
- Choi, P.H., Sureka, K., Woodward, J.J., and Tong, L. (2015) Molecular basis of the recognition of cyclic-di-AMP by PSTA, a PII-like signal transduction protein. *Microbiologyopen* **4**: 361–374.
- Christen, M., Christen, B., Folcher, M., Schauerte, A., and Jenal, U. (2005) Identification and characterization of a cyclic di-GMP-specific phosphodiesterase and its allosteric control by GTP. *J Biol Chem* **280**: 30829–30837.
- Commichau, F.M., Pietack, N., and Stülke, J. (2013) Essential genes in *Bacillus subtilis*: a re-evaluation after ten years. *Mol Biosyst* **9**: 1068–1075.
- Conroy, M.J., Durand, A., Lupo, D., Li, X.D., Bullough, P.A., Winkler, F.K., and Merrick, M. (2007) The crystal structure of the *Escherichia coli* AmtB-GlnK complex reveals how GlnK regulates the ammonia channel. *Proc Natl Acad Sci USA* **104**: 1213–1218.
- Cooper, M.A., and Shlaes, D. (2011) Fix the antibiotics pipeline. *Nature* **472**: 32.
- Corrigan, R.M., and Gründling, A. (2013) Cyclic di-AMP: another second messenger enters the fray. *Nat Rev Microbiol* **11**: 513–524.
- Corrigan, R.M., Abbott, J.C., Burhenne, H., Kaever, V., and Gründling, A. (2011) c-di-AMP is a new second messenger in *Staphylococcus aureus* with a role in controlling cell size and envelope stress. *PLoS Pathog* **7**: e1002217.
- Corrigan, R.M., Campeotto, I., Jeganathan, T., Roelofs, K.G., Lee, V.T., and Gründling, A. (2013) Systematic identification of conserved bacterial c-di-AMP receptor proteins. *Proc Natl Acad Sci USA* **110**: 9084–9089.
- Corrigan, R.M., Bowman, L., Willis, A.R., Kaever, V., and Gründling, A. (2015) Crosstalk between two nucleotide-signaling pathways in *Staphylococcus aureus*. *J Biol Chem* **290**: 5826–5839.
- Davies, B.W., Bogard, R.W., Young, T.S., and Mekalanos, J.J. (2012) Coordinated regulation of accessory genetic elements produces cyclic dinucleotides for *V. cholerae* virulence. *Cell* **149**: 358–370.
- Dengler, V., McCallum, N., Kiefer, P., Christen, P., Patrignani, A., Vorholt, J.A., et al. (2013) Mutation in the c-di-AMP cyclase *dacA* affects fitness and resistance of methicillin resistant *Staphylococcus aureus*. *PLoS ONE* **8**: e73512.
- Duerig, A., Abel, S., Folcher, M., Nicollier, M., Schwede, T., Amiot, N., et al. (2009) Second messenger-mediated spatiotemporal control of protein degradation regulates bacterial cell cycle progression. *Genes Dev* **23**: 93–104.
- Ereño-Orbea, J., Oyenarte, I., and Martínez-Cruz, L.A. (2013) CBS domains: ligand binding sites and conformational variability. *Arch Biochem Biophys* **540**: 70–81.
- Forchhammer, K. (2008) P(II) signal transducers: novel functional and structural insights. *Trends Microbiol* **16**: 65–72.
- French, C.T., Lao, P., Loraine, A.E., Matthews, B.T., Yu, H., and Dybvig, K. (2008) Large-scale transposon mutagenesis of *Mycoplasma pulmonis*. *Mol Microbiol* **69**: 67–76.
- Gándara, C., and Alonso, J.C. (2015) DisA and c-di-AMP act at the intersection between DNA-damage response and stress homeostasis in exponentially growing *Bacillus subtilis* cells. *DNA Repair (Amst)* **27**: 1–8.
- Gao, A., and Serganov, A. (2014) Structural insights into recognition of c-di-AMP by the *ydaO* riboswitch. *Nat Chem Biol* **10**: 787–792.
- Gerhardt, E.C., Rodrigues, T.E., Müller-Santos, M., Pedrosa, F.O., Souza, E.M., Forchhammer, K., and Huergo, L.F. (2015) The bacterial signal transduction protein GlnB regulates the committed step in fatty acid biosynthesis by acting as a dissociable regulatory subunit of acetyl-CoA carboxylase. *Mol Microbiol* **95**: 1025–1036.
- Glass, J.I., Assad-Garcia, N., Alperovich, N., Yoosheph, S., Lewis, M.R., Maruf, M., et al. (2006) Essential genes of a minimal bacterium. *Proc Natl Acad Sci USA* **103**: 425–430.
- Görke, B., and Stülke, J. (2008) Carbon catabolite repression in bacteria: many ways to make most out of nutrients. *Nat Rev Microbiol* **6**: 613–624.
- Gundlach, J., Dickmanns, A., Schröder-Tittmann, K.,

- Neumann, P., Kaesler, J., Kampf, J., et al. (2015) Identification, characterization and structure analysis of the cyclic di-AMP-binding PII-like signal transduction protein DarA. *J Biol Chem* **290**: 3069–3080.
- Heinrich, A., Woyda, K., Brauburger, K., Meiss, G., Detsch, C., Stölke, J., and Forchhammer, K. (2006) Interaction of the membrane-bound GlnK-AmtB complex with the master regulator of nitrogen metabolism TnrA in *Bacillus subtilis*. *J Biol Chem* **281**: 34909–34917.
- Hengge, R. (2009) Principles of c-di-GMP signaling in bacteria. *Nat Rev Microbiol* **7**: 263–273.
- Huergo, L.F., Chandra, G., and Merrick, M. (2013) P(II) signal transduction proteins: nitrogen regulation and beyond. *FEMS Microbiol Rev* **37**: 251–283.
- Huynh, T.N., Luo, S., Pensinger, D., Sauer, J.D., Tong, L., and Woodward, J.J. (2015) An HD-domain phosphodiesterase mediates cooperative hydrolysis of c-di-AMP to affect bacterial growth and virulence. *Proc Natl Acad Sci USA* **112**: E747–E756.
- Jones, C.P., and Ferré-D'Amare, A.R. (2014) Crystal structure of a c-di-AMP riboswitch reveals an internally pseudodimeric RNA. *EMBO J* **33**: 2692–2703.
- Jones, C.P., and Ferré-D'Amare, A.R. (2015) RNA quaternary structure and global symmetry. *Trends Biochem Sci* **40**: 211–220.
- Kaplan Zeevi, M., Shafir, N.S., Shaham, S., Friedman, S., Sigal, N., Paz, R.N., et al. (2013) *Listeria monocytogenes* multidrug resistance transporters and cyclic di-AMP, which contribute to type I interferon induction, play a role in cell wall stress. *J Bacteriol* **195**: 5250–5261.
- Kim, P.B., Nelson, J.W., and Breaker, R.R. (2015) An ancient riboswitch class in bacteria regulates purine biosynthesis and one-carbon metabolism. *Mol Cell* **57**: 317–328.
- Liu, S., Bayles, D.O., Mason, T.M., and Wilkinson, B.J. (2006) A cold-sensitive *Listeria monocytogenes* mutant has a transposon insertion in a gene encoding a putative membrane protein and shows altered (p)ppGpp levels. *Appl Environ Microbiol* **72**: 3955–3959.
- Lluch-Senar, M., Delgado, J., Chen, W.H., Lloréns-Rico, V., O'Reilly, F.J., Wodke, J.A.H., et al. (2015) Defining a minimal cell: essentiality of small ORFs and ncRNAs in a genome-reduced bacterium. *Mol Syst Biol* **11**: 780.
- Luo, Y., and Helmann, J.D. (2012a) Analysis of the role of *Bacillus subtilis*  $\sigma^M$  in  $\beta$ -lactam resistance reveals an essential role for c-di-AMP in peptidoglycan homeostasis. *Mol Microbiol* **83**: 623–639.
- Luo, Y., and Helmann, J.D. (2012b) A  $\sigma^D$ -dependent anti-sense transcript modulates expression of the cyclic-di-AMP hydrolase GdpP in *Bacillus subtilis*. *Microbiology* **158**: 2732–2741.
- Manikandan, K., Sabareesh, V., Singh, N., Saigal, K., Mechold, U., and Sinha, K.M. (2014) Two-step synthesis and hydrolysis of cyclic di-AMP in *Mycobacterium tuberculosis*. *PLoS ONE* **9**: e86096.
- Manzanillo, P.S., Shiloh, M.U., Portnoy, D.A., and Cox, J.S. (2012) *Mycobacterium tuberculosis* activates the DNA-dependent cytosolic surveillance pathway within macrophages. *Cell Host Microbe* **11**: 469–480.
- Mehne, F.M.P., Gunka, K., Eilers, H., Herzberg, C., Kaever, V., and Stölke, J. (2013) Cyclic-di-AMP homeostasis in *Bacillus subtilis*: both lack and high-level accumulation of the nucleotide are detrimental for cell growth. *J Biol Chem* **288**: 2004–2017.
- Mehne, F.M.P., Schröder-Tittmann, K., Eijlander, R.T., Herzberg, C., Hewitt, L., Kaever, V., et al. (2014) Control of the diadenylate cyclase CdaS in *Bacillus subtilis*: an auto-inhibitory domain limits c-di-AMP production. *J Biol Chem* **289**: 21098–21107.
- Michna, R.H., Commichau, F.M., Tödter, D., Zschiedrich, C.P., and Stölke, J. (2014) SubtWiki – a database for the model organism *Bacillus subtilis* that links pathway, interaction and expression information. *Nucleic Acids Res* **42**: D692–D698.
- Müller, M., Hopfner, K.P., and Witte, G. (2015) c-di-AMP recognition by *Staphylococcus aureus* PstA. *FEBS Lett* **589**: 45–51.
- Nelson, J.W., Sudarsan, N., Furukawa, K., Weingerg, Z., Wang, J.X., and Breaker, R.R. (2013) Riboswitches in eubacteria sense the second messenger cyclic di-AMP. *Nat Chem Biol* **9**: 834–839.
- Nicolas, P., Mäder, U., Dervyn, E., Rochat, T., Leduc, A., Pigeonneau, N., et al. (2012) Condition-dependent transcriptome reveals high-level regulatory architecture in *Bacillus subtilis*. *Science* **335**: 1103–1106.
- Oppenheimer-Shaanan, Y., Wexselblatt, E., Katzhaendler, J., Yavin, E., and Ben-Yehuda, S. (2011) c-di-AMP reports DNA integrity during sporulation in *Bacillus subtilis*. *EMBO Rep* **12**: 594–601.
- Potrykus, K., and Cashel, M. (2008) (p)ppGpp: still magical? *Annu Rev Microbiol* **62**: 35–51.
- Rallu, F., Gruss, A., Ehrlich, S.D., and Maguin, E. (2000) Acid- and multistress-resistant mutants of *Lactococcus lactis*: identification of intracellular stress signals. *Mol Microbiol* **35**: 517–528.
- Rao, F., See, R.Y., Zhang, D., Toh, D.C., Ji, Q., and Liang, Z.X. (2010) YybT is a signaling protein that contains a cyclic dinucleotide phosphodiesterase domain and a GGDEF domain with ATPase activity. *J Biol Chem* **285**: 473–482.
- Rao, F., Ji, Q., Soehano, I., and Liang, Z.X. (2011) Unusual heme-binding PAS domain from YybT family proteins. *J Bacteriol* **193**: 1543–1551.
- Ren, A., and Patel, D.J. (2014) c-di-AMP binds the ydaO riboswitch in two pseudo-symmetry-related pockets. *Nat Chem Biol* **10**: 780–786.
- Rosenberg, J., Neumann, P., Arens, J., Gunka, K., Kaever, V., Dickmanns, A., et al. (2015) Biochemical and structural analysis of the essential diadenylate cyclase CdaA from *Listeria monocytogenes*. *J Biol Chem* **290**: 6596–6606.
- Römling, U. (2008) Great times for small molecules: c-di-AMP, a second messenger candidate in bacteria and archaea. *Sci Signal* **1**: pe39.
- Ryan, R.P., Tolker-Nielsen, T., and Dow, J.M. (2012) When the PilZ domain don't work: effectors for cyclic di-GMP action in bacteria. *Trends Microbiol* **20**: 235–242.
- Ryjenkov, D.A., Simm, R., Römling, U., and Gomelsky, M. (2006) The PilZ domain is a receptor for the second messenger c-di-GMP: the PilZ domain protein YcgR controls motility in enterobacteria. *J Biol Chem* **281**: 30310–30314.
- Schär, J., Stoll, R., Schauer, K., Loeffler, D., Eylert, E., Joseph, B., et al. (2010) Pyruvate carboxylase plays a crucial role in carbon metabolism of extra- and intracellular-

- larly replicating *Listeria monocytogenes*. *J Bacteriol* **192**: 1774–1784.
- Schirmer, T., and Jenal, U. (2009) Structural and mechanistic determinants of c-di-GMP signalling. *Nat Rev Microbiol* **7**: 724–735.
- Schumacher, M.A., Chinnan, N.B., Cuthbert, B., Tonthat, N.K., and Whitfill, T. (2015) Structures of regulatory machinery reveal novel molecular mechanisms controlling *B. subtilis* nitrogen homeostasis. *Genes Dev* **29**: 451–464.
- Smith, K.D., and Strobel, S.A. (2011) Interactions of the c-di-GMP riboswitch with its second messenger ligand. *Biochem Soc Trans* **39**: 647–651.
- Song, J.H., Ko, K.S., Lee, J.Y., Baek, J.Y., Oh, W.S., Yoon, H.S., et al. (2005) Identification of essential genes in *Streptococcus pneumoniae* by allelic replacement mutagenesis. *Mol Cells* **19**: 365–374.
- St-Onge, R.J., Haiser, H.J., Yousef, M.R., Sherwood, E., Tschorri, N., Al-Bassam, M., and Elliot, M.A. (2015) Nucleotide second messenger-mediated regulation of a muralytic enzyme in *Streptomyces*. *Mol Microbiol* **96**: 779–795.
- Sudarsan, N., Lee, E.R., Weinberg, Z., Moy, R.H., Kim, J.N., Link, K.H., and Breaker, R.R. (2008) Riboswitches in eubacteria sense the second messenger cyclic di-GMP. *Science* **321**: 411–413.
- Sureka, K., Choi, P.H., Precit, M., Delince, M., Pensinger, D.A., Huynh, T.N., et al. (2014) The cyclic dinucleotide c-di-AMP is an allosteric regulator of metabolic enzyme function. *Cell* **158**: 1389–1401.
- Tadmor, K., Pozniak, Y., Golani, T.B., Lobel, L., Brenner, M., Sigal, N., and Herskovits, A.A. (2014) *Listeria monocytogenes* MDR transporters are involved in LTA synthesis and triggering of innate immunity during infection. *Front Cell Infect Microbiol* **4**: 16.
- Tan, E., Rao, F., Pasunooti, S., Pham, T.H., Soehano, I., Turner, M.S., et al. (2013) Solution structure of the PAS domain of a thermophilic YybT protein reveals a potential ligand binding site. *J Biol Chem* **288**: 11949–11959.
- Taylor, B.L., and Zhulin, I.B. (1999) PAS domains: internal sensors of oxygen, redox potential, and light. *Microbiol Mol Biol Rev* **63**: 479–506.
- Tschowri, N., Schumacher, M.A., Schlimpert, S., Chinnan, N.B., Findlay, K.C., Brennan, R.G., and Buttner, M.J. (2014) Tetrameric c-di-GMP mediates effective transcription factor dimerization to control *Streptomyces* development. *Cell* **158**: 1136–1147.
- Vieira-Pires, R.S., Szollosi, A., and Morais-Cabral, J.H. (2013) The structure of the KtrAB potassium transporter. *Nature* **496**: 323–328.
- Watson, P.Y., and Fedor, M.J. (2012) The ydaO motif is an ATP-sensing riboswitch in *Bacillus subtilis*. *Nat Chem Biol* **8**: 963–965.
- Witte, C.W., Whiteley, A.T., Burke, T.P., Sauer, J.D., Portnoy, D.A., and Woodward, J.J. (2013) Cyclic di-AMP is critical for *Listeria monocytogenes* growth, cell wall homeostasis, and establishment of infection. *MBio* **4**: e00282-13.
- Witte, G., Hartung, S., Büttner, K., and Hopfner, K.P. (2008) Structural biochemistry of a bacterial checkpoint protein reveals diadenylate cyclase activity regulated by DNA recombination intermediates. *Mol Cell* **30**: 167–178.
- Woodward, J.J., Iavarone, A.T., and Portnoy, D.A. (2010) c-di-AMP secreted by intracellular *Listeria monocytogenes* activates a host type I immune response. *Science* **328**: 1703–1705.
- Ye, M., Zhang, J.J., Fang, X., Lawlis, G.B., Troxell, B., Zhou, Y., et al. (2014) DhhP, a cyclic di-AMP phosphodiesterase of *Borrelia burgdorferi*, is essential for cell growth and virulence. *Infect Immun* **82**: 1840–1849.
- Zeth, K., Fokina, O., and Forchhammer, K. (2012) An engineered PII protein variant that senses a novel ligand: atomic resolution structure of the complex with citrate. *Acta Crystallogr D Biol Crystallogr* **68**: 901–908.
- Zhang, L., and He, Z.G. (2013) Radiation-sensitive gene A (RadA) targets DisA, DNA integrity scanning protein A, to negatively affect cyclic di-AMP synthesis activity in *Mycobacterium smegmatis*. *J Biol Chem* **288**: 22426–22436.
- Zhang, L., Li, W., and He, Z.G. (2013) DarR, a TetR-like transcription factor, is a cyclic di-AMP-responsive respressor in *Mycobacterium smegmatis*. *J Biol Chem* **288**: 3085–3096.