

Replenishing the cyclic-di-AMP pool: regulation of diadenylate cyclase activity in bacteria

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Abstract Bacteria can sense environmental cues and alter their physiology accordingly through the use of signal transduction pathways involving second messenger nucleotides. One broadly conserved second messenger is cyclic-di-AMP (c-di-AMP) which regulates a range of processes including cell wall homeostasis, potassium uptake, DNA repair, fatty acid synthesis, biofilm formation and central metabolism in bacteria. The intracellular pool of c-di-AMP is maintained by the activities of diadenylate cyclase (DAC) and phosphodiesterase (PDE) enzymes, as well as possibly via c-di-AMP export. Whilst extracellular stimuli regulating c-di-AMP levels in bacteria are poorly understood, recent work has identified effector proteins which directly interact and alter the activity of DACs. These include the membrane bound CdaR and the phosphoglucoamine mutase GlmM which both bind directly to the membrane bound CdaA DAC and the recombination protein RadA which binds directly to the DNA binding DisA DAC. The genes encoding these multiprotein complexes are co-localised in many bacteria providing further support for their functional connection. The roles of GlmM

in peptidoglycan synthesis and RadA in Holliday junction intermediate processing suggest that c-di-AMP synthesis by DACs will be responsive to these cellular activities. In addition to these modulatory interactions, permanent dysregulation of DAC activity due to suppressor mutations can occur during selection to overcome growth defects, rapid cell lysis and osmosensitivity. DACs have also been investigated as targets for the development of new antibiotics and several small compound inhibitors have recently been identified. This review aims to provide an overview of how c-di-AMP synthesis by DACs can be regulated.

Keywords Cyclic-di-AMP · Diadenylate cyclase · Peptidoglycan · DNA · Regulation

Introduction

Nucleotide second messengers are used by a wide range of bacteria in signal transduction pathways to regulate a plethora of physiological functions through binding and affecting the activity of downstream protein and riboswitch targets (Colton and Stabb 2016; Gomelsky 2011; Kalia et al. 2013). A relative newcomer to the second messenger family is cyclic-di-AMP (c-di-AMP), which was first identified in the crystallised protein structures of DisA from *Thermotoga maritima* and *Bacillus subtilis* (Witte et al. 2008) and subsequently found to be the interferon- β stimulating compound produced by *Listeria monocytogenes* during infection (Woodward et al. 2010). In most instances this broadly conserved nucleotide is synthesised from two molecules of ATP or ADP by a single membrane bound diadenylate cyclase (DAC) enzyme called CdaA (DacA); however, some bacteria such as *B. subtilis* contain two additional DACs (DisA and CdaS) (Corrigan and Gründling 2013).

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Degradation of c-di-AMP to 5'-pApA occurs via the action of phosphodiesterases (PDE) with a DHH-DHHA1 domain (GdpP and DhhP) or a HD domain (PgpH) (Huynh and Woodward 2016). The number of c-di-AMP synthesis and degradation enzymes thus far characterised are few when compared to the cyclic-di-GMP system where dozens of diguanylate cyclase and PDE enzymes can occur in the one bacterium (Bordeleau and Burrus 2015; Römling et al. 2013). In addition to enzymatic control of the c-di-AMP level, its reduction in the cytoplasm can also be achieved possibly through active export via multidrug resistance transporters, as shown in *L. monocytogenes* (Huynh and Woodward 2016). C-di-AMP has also been identified in the supernatant of the *Lactococcus lactis gdpP* mutant but not wild-type, suggesting that this is a common phenomenon, even in non-pathogenic bacteria (Pham and Turner, unpublished data). C-di-AMP binds to and affects the function of effector proteins such as DarR, KtrA, CpaA, PstA (DarA), pyruvate carboxylase, CbpA, CbpB, NrdR and the *ydaO* riboswitch (Commichau et al. 2015). Mutations in DAC and PDE genes result in major phenotypic effects including altered resistance to heat, salt, DNA damaging agents and cell wall acting antibiotics, as well as in virulence, cell structure and growth (Commichau et al. 2015). Low and high c-di-AMP levels are detrimental for growth and in many bacteria c-di-AMP is an essential molecule when grown in rich media possibly due to a toxic increase in (p)ppGpp levels as shown for *L. monocytogenes* (Mehne et al. 2013; Whiteley et al. 2015).

Like the c-di-GMP system, environmental stimuli which regulate c-di-AMP signalling are poorly understood. Recent work has found c-di-AMP levels in *Staphylococcus aureus* increase ~4-fold in late exponential/stationary phase, corresponding with an increase in the CdaA protein level (Corrigan et al. 2015). In addition to this, the nitrogen source appears to affect the c-di-AMP level in *B. subtilis*, with cells grown in glutamate having higher c-di-AMP levels compared to cells grown in glutamine (Gundlach et al. 2015). Some intracellular signals regulating PDE activity have been identified including the stringent response regulator (pppGpp for both GdpP (Rao et al. 2010) and PgpH (Huynh et al. 2015), and heme and nitric oxide for GdpP (Rao et al. 2011; Tan et al. 2013). GdpP gene transcription is also regulated by an antisense RNA controlled by the alternative sigma factor σ^D in *B. subtilis* (Luo and Helmann 2012).

With respect to DAC regulation, it is emerging that DNA damage repair and cell wall peptidoglycan biosynthetic processes are closely connected to DAC activity. This short review will explore DAC binding effector proteins and also suppressor mutations which have been identified in DACs which affect the c-di-AMP pool. These provide either transient (reversible) or permanent (irreversible) changes in

DAC activity, respectively. We will also examine recent efforts in identifying chemical DAC inhibitors which may have therapeutic potential by blocking c-di-AMP synthesis. For more comprehensive overviews of c-di-AMP signalling or specifically c-di-AMP depletion, several excellent recent reviews are available (Commichau et al. 2015; Corrigan and Gründling 2013; Huynh and Woodward 2016).

Transient regulation of DAC enzymes

The CdaA-CdaR-GlmM protein complex

In numerous Firmicutes and Deltaproteobacteria, genes encoding CdaA, CdaR and GlmM are adjacently located (Fig. 1a). In some Clostridia, *glmM* is separated by *cdaR* by one or several genes (Fig. 1a). The dimer-forming CdaA contains three transmembrane domains with the DAC domain (Dis_N Pfam PF02457) located intracellularly, while CdaR contains one transmembrane domain and several YbbR domains (Pfam PF07949) predicted to be located extracellularly (Fig. 1b). The first crystal structure of CdaA from *L. monocytogenes* was recently reported and was found to be highly similar to other DACs DisA and CdaS (Rosenberg et al. 2015). *B. subtilis* CdaR was found to bind to CdaA using a bacterial two hybrid system (B2H) and upregulate c-di-AMP synthesis by CdaA (but not other DAC enzymes DisA and CdaS) when co-expressed in *Escherichia coli* (Mehne et al. 2013). Recent work has shown CdaA and CdaR from *L. monocytogenes* also directly interact, however, c-di-AMP measurements in *L. monocytogenes* strains with altered *cdaR* expression suggests that CdaA is more likely to be negatively regulated by CdaR (Rismondo et al. 2015). Experiments expressing different combinations of *S. aureus cdaA*, *cdaR* and *glmM* genes in *E. coli* also suggests that CdaR negatively regulates c-di-AMP synthesis by CdaA (Zhu et al. 2016). The reason for the differences in these results is not clear as yet; however, CdaR may have positive and negative regulatory activity depending upon the experimental conditions used. Alternatively, due to moderately low homology between CdaR from *B. subtilis*, *L. monocytogenes* and *S. aureus* (~30 % amino acid identity between each), variations in function may exist.

The function of the YbbR domain is not known, but it does share structural similarity to domains in ribosomal proteins which have RNA and protein binding activities (Barb et al. 2011). YbbR domains have also been identified as efficient substrates for surfactin-type phosphopantetheinyl transferases (Yin et al. 2005). Several bacteria which contain CdaA do not possess CdaR, and in some bacteria, CdaR encoding genes contain frameshift mutations rendering them non-functional (Fig. 1a), suggesting that CdaR is

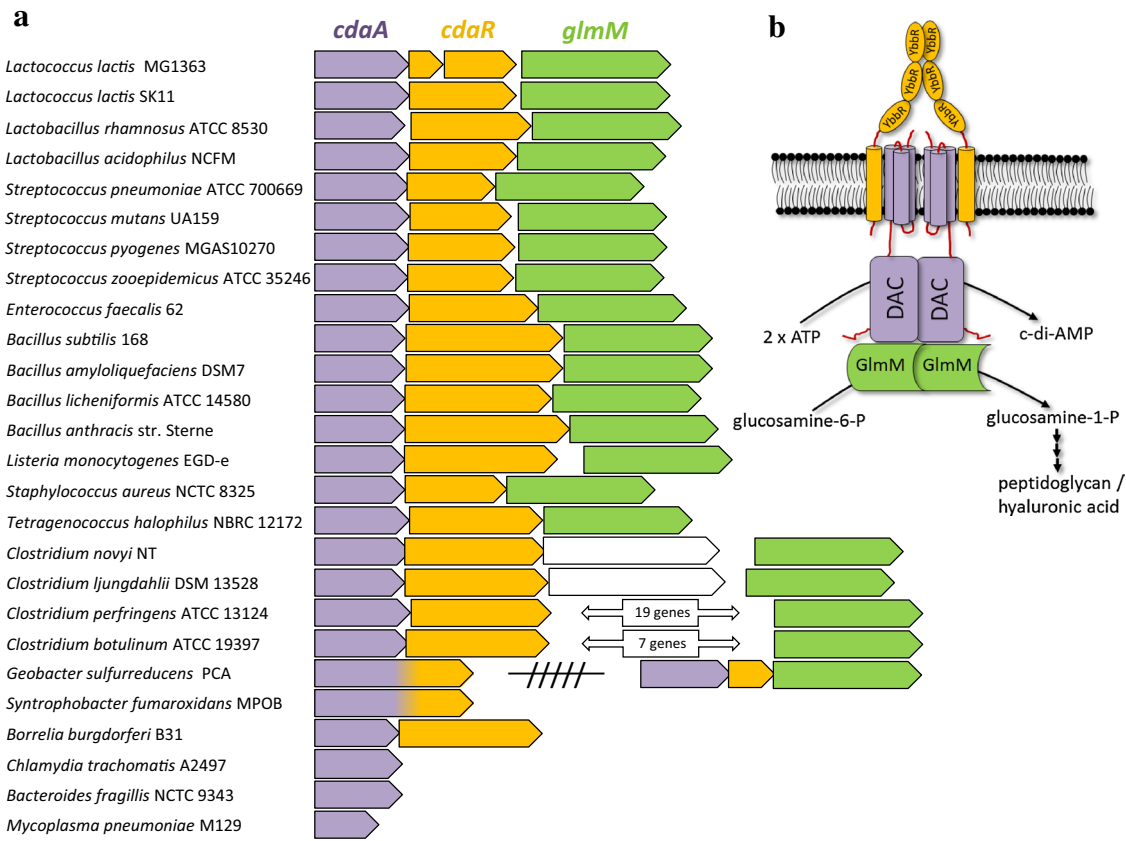


Fig. 1 Arrangement of CdaA, CdaR and GlmM encoding genes in representative bacteria and a model for the membrane-bound CdaA-CdaR-GlmM protein complex. **a** The *cdaA* (purple), *cdaR* (orange), *glmM* (green) and *cdaA-cdaR* fusion (purple and orange) genes are drawn in proportion to their size. In several bacteria the *cdaA* gene is not located next to *cdaR* and *glmM* genes, which are either not present in the genome or located elsewhere. In Clostridia, other genes

(denoted by white arrows) separate *cdaR* and *glmM*. For *Geobacter sulfurreducens* PCA, both the *cdaA-cdaR* fusion gene and a separate *cdaA* containing gene cluster exist in distant genomic locations. **b** A model showing CdaA dimer (purple) bound to the GlmM dimer (green) and their respective enzymatic reactions. The CdaR protein (orange) also interacts with CdaA and likely with another CdaR protein(s)

not essential for CdaA function in the native host. CdaR proteins from *L. monocytogenes* can interact with each other possibly via their YbbR domains (Rismondo et al. 2015) thus forming a multimeric structure on the outside of the cytoplasmic membrane (Fig. 1b). Deletion of the membrane spanning regions from CdaA and CdaR resulted in a lack of interaction between these proteins, which as predicted, suggests that the cytosolic part of CdaA does not interact with the multiple YbbR domains predicted to be extracellular (Rismondo et al. 2015). It is more likely that the interaction between CdaA and CdaR occurs via their transmembrane regions or perhaps between the YbbR domains and the short extracellular exposed N-terminus or loop regions of CdaA. There are substantial variations in the lengths of CdaR proteins between bacteria due to differing numbers of YbbR repeats (Fig. 1a). *B. subtilis* and *L. monocytogenes* CdaR contain 4 YbbR domains while *S. aureus* and some *L. lactis* CdaR contain 3 YbbR domains which may affect how stimuli are sensed or the binding

affinity of CdaR to other CdaR proteins or CdaA. In several Deltaproteobacteria, such as *Geobacter sulfurreducens* and *Syntrophobacter fumaroxidans*, *cdaA* is genetically fused to *cdaR* with a transmembrane domain encoding region separating the two (Fig. 1a). Only one or two YbbR domains exist in these CdaA-CdaR fusion proteins. Interestingly in the completely sequenced genome of *G. sulfurreducens* PCA, multiple CdaA genes exist. One (GSU1807) is 261αα in length and clustered with *cdaR* and *glmM* while the other (GSU0868) is 470αα and encodes the CdaA-CdaR fusion protein (Fig. 1a).

GlmM encodes a phosphoglucosamine mutase which forms an early step in the synthesis of UDP-N-acetylglucosamine (UDP-NAG), a building block for cell wall peptidoglycan. *B. subtilis* GlmM was identified in a screen for proteins which bind to CdaR and was subsequently shown using B2H to bind directly to CdaA and indirectly to CdaR (Gundlach et al. 2015). *L. lactis* GlmM was identified in a screen for genes which affect CdaA activity and

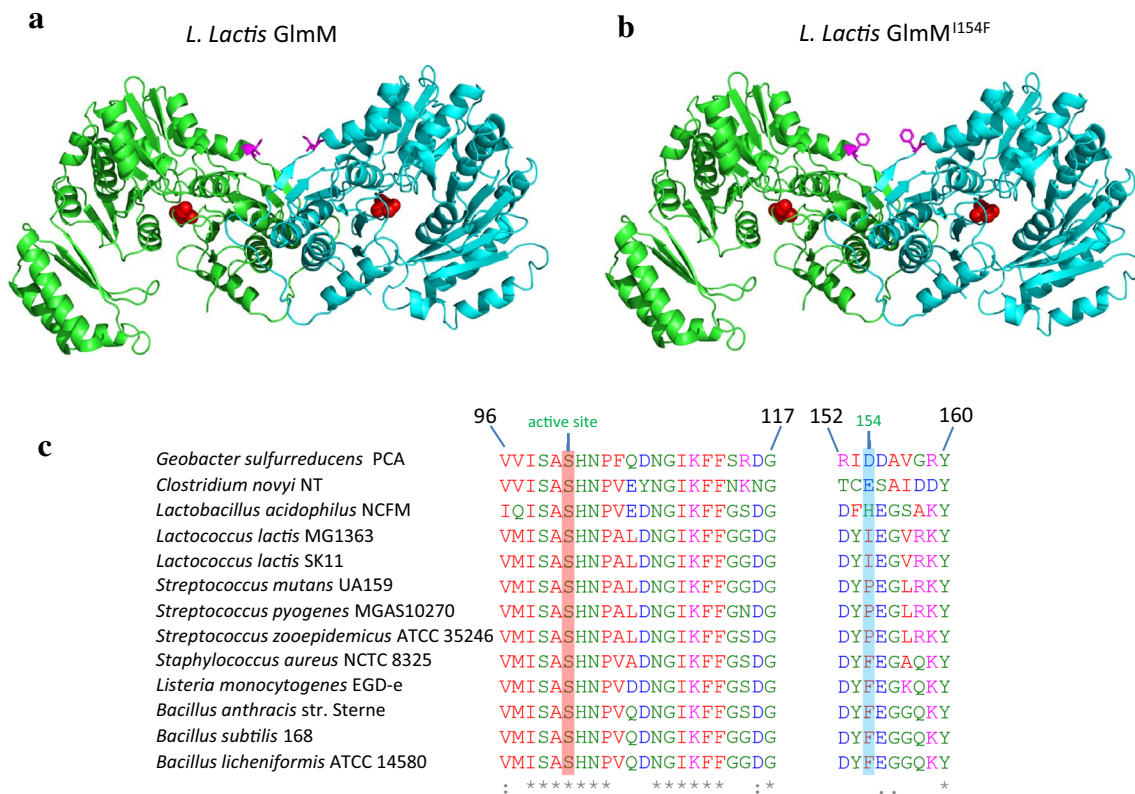


Fig. 2 Structural and sequence analysis of GlmM from *L. lactis* and other bacteria. SWISS-MODEL (Biasini et al. 2014) was used to generate a structure of wild-type *L. lactis* GlmM (a) and the I154F variant GlmM (b) which was generated by changing the I154 residue to F154 in PyMOL (Version 1.3 Schrödinger, LLC). The GlmM monomers are colored differently (blue and green) and the 154 residues are shown as pink ‘sticks’ while the serine active site residues are red

spheres. The strong conservation of the region surrounding the active site serine (residue numbering is according to *L. lactis* MG1363 GlmM) and the variability of the 154 residue are shown in (c). At the bottom of the alignment, an asterisk indicates complete residue conservation while a colon and full stop indicate 1 or 2 differing residues, respectively, across the shown proteins

osmoresistance (Zhu et al. 2016). A mutation in *L. lactis* GlmM (I154F) resulted in lower c-di-AMP in *L. lactis* and GlmM was found to be a negative regulator of CdaA activity when co-expressed in *E. coli* (Zhu et al. 2016). GlmM^{I154F} caused greater inhibition of CdaA activity than wild type GlmM, most likely a result of their stronger interaction as determined by B2H (Zhu et al. 2016). Modelling using the available *Bacillus anthracis* GlmM structure (Mehra-Chaudhary et al. 2011), shows the location of the 154 residue in *L. lactis* GlmM on a surface away from the active site serine (Fig. 2a, b). GlmM is a highly conserved protein and interestingly phenylalanine is present in the 154 position in *B. subtilis*, *S. aureus* and *L. monocytogenes* which may allow for stronger interaction with CdaA (Fig. 2c). We speculate that this surface region encompassing the 154 residue in the GlmM homodimer may form the CdaA binding interface, however, more work is needed to determine this.

It has been shown that UDP-NAG levels are elevated in a PDE (*gdpP*) mutant of *L. lactis* suggesting that the

c-di-AMP level regulates peptidoglycan precursor synthesis (Zhu et al. 2016). How CdaA affects GlmM activity, and vice versa, is not yet fully understood, however, it is in agreement with the role of c-di-AMP in regulating cell wall homeostasis and affecting phenotypes such as resistance to cell wall acting antibiotics, lysis and osmoresistance. The coordination between c-di-AMP synthesis and peptidoglycan precursor synthesis likely occurs through physical interaction of CdaA and GlmM. It is not known if this regulation occurs simply following protein–protein binding, through the two enzymes sensing each other’s activity or if either or both are responsive to c-di-AMP levels. Interestingly the step before that catalysed by GlmM involves the conversion of fructose-6-phosphate to glucosamine-6-phosphate along with the deamination of glutamine to glutamate by GlmS. Several areas of investigation have identified links between glutamate and c-di-AMP signalling (Gundlach et al. 2015; Sureka et al. 2014; Whiteley et al. 2015). In *L. monocytogenes* there is an inverse relationship between c-di-AMP and glutamate levels at least in part due

to pyruvate carboxylase activity being allosterically inhibited by c-di-AMP (Sureka et al. 2014). Therefore it would make sense that heightened flux towards UDP-NAG in high c-di-AMP cells would drive conversion of glutamine to glutamate, thus restoring the glutamate level in the cell. Interestingly, the next closest match from SWISS-Model for GlmM following sugar mutase enzymes are mammalian glutamate receptor ion channels, otherwise known as iGluRs (Timm et al. 2011). This similarity is shared in the central region of GlmM which may form an allosteric regulatory binding site for glutamate, thus providing a link between glutamate levels, cell wall precursor levels and c-di-AMP synthesis.

With their demonstrated binding in two unrelated bacteria (*B. subtilis* and *L. lactis*) and co-localisation of the encoding genes in many other bacteria, CdaA and GlmM interaction is likely to be widespread. The downstream product of the GlmM pathway, UDP-NAG, is also used as a precursor for non-peptidoglycan bacterial polymers including the hyaluronic acid (HA) capsules of group A and C streptococci (Marcellin et al. 2014). HA is a polysaccharide consisting of repeating units of glucuronic acid and N-acetylglucosamine (NAG) joined by alternating β -1,3- and β -1,4-glycosidic bonds. HA capsules are virulence factors in pathogenic streptococci providing protection against phagocytosis (Wessels et al. 1991), and are also utilised in a variety of medical applications (Marcellin et al. 2014). UDP-NAG levels and GlmM play a central role in yield and molecular weight of the biopolymer in *Streptococcus equi* subspecies *zooepidemicus* (*S. zooepidemicus*) (Chen et al. 2009, 2014). Over-expression of GlmM in *S. zooepidemicus*, resulted in a significant increase in HA yield (50 % higher than wild-type) as well as a significantly higher biomass yield (10–15 % increase) (Chen et al. 2014). The *cdaA*, *cdaR* and *glmM* genes are co-localised in *S. zooepidemicus* and *Streptococcus pyogenes* (Fig. 1a) therefore suggesting that c-di-AMP may play a role in regulating HA synthesis in these species.

The RadA-DisA protein complex

The first identified DAC, DisA, contains a RuvA-like helix–hairpin–helix (HhH) DNA binding domain linked to a DAC domain. It was the first identified as a DNA integrity scanning checkpoint protein that rapidly scans the genome for lesions, which when encountered, prevents entry into sporulation by inhibiting phosphorylation of the master regulator of sporulation Spo0A (Bejerano-Sagie et al. 2006). This allows for DNA repair to occur to improve spore viability. Structural analysis unexpectedly revealed the presence of c-di-AMP in the nucleotide binding domain of DisA and subsequent work demonstrated DAC activity by DisA is strongly inhibited by branched DNA structures, which

could be stalled replication forks or recombination intermediates (Witte et al. 2008). Exposure of sporulating *B. subtilis* cells to DNA damaging agents (nalidixic acid and mitomycin C) led to stalled DisA complexes and a resultant lower c-di-AMP level (Oppenheimer-Shaanan et al. 2011). The addition of exogenous c-di-AMP to *B. subtilis* induced higher sporulation rates, essentially overriding the DisA checkpoint process (Oppenheimer-Shaanan et al. 2011). DisA has also been shown to play a role in delaying spore outgrowth until the DNA is free of damage (Campos et al. 2014). It is not known if stalled DisA complexes recruit other proteins involved in DNA repair to specific sites or if lower c-di-AMP synthesis is a general signal to simply slow down DNA replication to allow for DNA repair to occur. DisA was originally identified as a binding partner of RacA, a protein involved in anchoring the chromosome to the cell poles (Bejerano-Sagie et al. 2006). However, their effects on each other in vivo are not yet known. Recent work has identified that DisA binds to the recombination protein RadA using B2H with proteins derived from *Mycobacterium*, *Bacillus* and *Streptomyces* (Zhang and He 2013). RadA strongly inhibited DAC activity of DisA, and similar to that found for proteins interacting with CdaA, the genes encoding RadA and DisA are located adjacent to each in the chromosome of many bacteria (Zhang and He 2013). RadA is required for efficient DNA recombination (Burghout et al. 2007) and DNA repair (Song and Sargentini 1996) and *radA* and *disA* mutants are more sensitive to DNA damaging agents (Gándara and Alonso 2015). It is possible that stalled DisA complexes recruit RadA to sites of DNA damage which leads to a further lowering of c-di-AMP levels. DisA's presence in several non-sporeforming bacteria (Corrigan and Gründling 2013) indicate that it has a broader role in DNA maintenance than just during sporulation and germination processes.

Permanent dysregulation of DAC enzymes through suppressor mutations

In addition to reversible alterations in DAC activity, bacteria can also sustain irreversible genetic mutations in their DAC encoding gene to alter their c-di-AMP level under selective pressure, thus giving them a growth/survival advantage. Whilst researchers have generated DAC knockout, conditional knockout and overexpression strains which have provided valuable insight into c-di-AMP signalling, we will only discuss naturally-occurring bacterial-initiated suppressor mutations in this section.

A single mutation (G206S) in CdaA (DacA) was acquired by a fast growing variant originating from a homogeneous and highly methicillin resistant *S. aureus* (MRSA) strain through a competitive growth experiment

(Dengler et al. 2013). The mutation led to reduced and heterogeneous resistance towards β -lactam antibiotics and a lower c-di-AMP level. Mutations in *cdaA* have also been acquired in strains which contain inactivated PDE genes, likely a consequence of toxic high c-di-AMP levels (Commichau et al. 2015). Independent *gdpP* mutations were identified in five analysed lipoteichoic acid-deficient *S. aureus* suppressors and genome sequencing revealed that two out of five of these mutants also contained new changes in *cdaA* (L193F and T236I) (Corrigan et al. 2011). These *cdaA* changes likely occurred after the *gdpP* mutation resulting in a moderate lowering of the c-di-AMP level to improve growth. Recently a *B. subtilis* strain lacking both PDEs (GdpP/PgpH) was found to lyse during prolonged incubation on agar plates (Gundlach et al. 2015). Several non-lytic suppressor mutants were isolated and examined, and several *cdaA* frameshift mutations and a nucleotide change in the *cdaA* ribosome binding site were identified (Gundlach et al. 2015). Interestingly, despite having significant phenotypic effects, these mutations did not affect the total cellular level of c-di-AMP, suggesting that a CdaA controlled local c-di-AMP pool exists, which needs to be carefully controlled to maintain cell wall homeostasis (Gundlach et al. 2015). In *L. lactis*, a screen for salt resistant suppressors from a *gdpP* mutant led to the identification of a large number of *cdaA* mutations (Zhu et al. 2016). The changes included frameshift mutations and amino acid changes throughout the protein including in the transmembrane regions and the DAC domain. In contrast to *B. subtilis*, the *L. lactis* *cdaA* suppressors contained significantly lower total c-di-AMP, likely due to CdaA being the only DAC in *L. lactis*. The location of mutations suggests that the CdaA transmembrane domains and the small extracellular exposed N-terminus and loops are important for c-di-AMP synthesis, perhaps through involvement in sensing stimuli.

The sporulation specific DAC CdaS has also been found to undergo suppressor mutations, however, in contrast to those for CdaA mentioned above, higher c-di-AMP synthesis activity was observed. These mutations were found in a *B. subtilis* strain which contained CdaS as the only DAC and it being under control of a xylose inducible promoter (Mehne et al. 2013). This strain was unable to grow in the absence of xylose, however, after extended incubation, several suppressor mutants were isolated and found to contain changes in CdaS, primarily in the two N-terminal α -helices (Mehne et al. 2013, 2014). It was found that the N-terminal α -helices act as auto-inhibitors of DAC activity possibly through affecting the oligomerisation of the CdaS subunits (Mehne et al. 2014).

Chemical inhibitors of DAC

In many bacteria, DAC activity and thus c-di-AMP has been shown to be essential for growth in rich media, important for virulence and necessary for maximal β -lactam antibiotic resistance (Commichau et al. 2015). For these reasons, there have been recent efforts to identify small compound inhibitors of DACs, which may have potential as antibiotics. Small compound and drug libraries were screened for inhibitors of DisA activity using a simple c-di-AMP measuring assay involving the fluorophore coralyne (Opoku-Temeng and Sintim 2016; Zheng et al. 2014). Bromophenol thiohydantoin and suramin were identified in these screens, for which suramin, an anti-parasitic drug exhibited potent DAC inhibitory activity (Opoku-Temeng and Sintim 2016). In another study, structural and biochemical analysis of DisA identified 3'-deoxyATP as a DAC inhibitor which traps the enzyme in the pre-reactive state (Müller et al. 2015). This compound is similar to the fungal derived cordycepin (3'-deoxy adenosine) which is phosphorylated inside the cell to 5'-mono-, di- or triphosphate forms (Tuli et al. 2013). Suramin and 3'-deoxyATP (cordycepin triphosphate) have similarly strong DAC inhibition potency (Opoku-Temeng and Sintim 2016). Suramin has been shown to sensitise *Mycobacterium smegmatis* to the DNA gyrase inhibitor ciprofloxacin (Nautiyal et al. 2014), while cordycepin has been shown to have strong antimicrobial activity against *Clostridium* spp. (Ahn et al. 2000). Cordycepin, however, was found to be inactive against *Lactobacillus* spp. (Ahn et al. 2000), despite these bacteria containing CdaA (Fig. 1a). It remains to be determined if the antimicrobial activities observed by these compounds are due to DAC inhibition or because they affect more general nucleotide or nucleic acid metabolic pathways. These compounds, however, may provide useful scaffolds for further optimisation which could generate a new class of antibiotics for controlling multi-drug resistant pathogenic bacteria.

Conclusions

Since its discovery ~8 years ago, c-di-AMP has been demonstrated to bind to a number of proteins and a riboswitch and play a critical role in a variety of important phenotypes in a wide range of bacteria. Thus far, however, only a few signals (DNA damage and cell wall peptidoglycan precursor synthesis) have been demonstrated to affect the c-di-AMP synthesis activity of DACs. There are likely to be additional internal as well as external stimuli which regulate the activity of DACs still yet to be discovered. Through a better understanding of input signals controlling the

c-di-AMP pool, we will be able to better appreciate how bacteria sense and subsequently adapt to various environmental conditions. This knowledge may also lead to new ways to manipulate the tightly controlled level of c-di-AMP to affect bacterial sensitivity to stressors or antibiotics, thus providing alternative approaches to controlling pathogenic and/or industrially used bacteria.

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