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Short Review

Cyclic di-AMP, a multifaceted regulator of central metabolism and osmolyte homeostasis in *Listeria monocytogenes*

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Abstract

Cyclic di-AMP is an emerging second messenger that is synthesized by many archaea and bacteria, including the Gram-positive pathogenic bacterium *Listeria monocytogenes*. *Listeria monocytogenes* played a crucial role in elucidating the essential function of c-di-AMP, thereby becoming a model system for studying c-di-AMP metabolism and the influence of the nucleotide on cell physiology. c-di-AMP is synthesized by a diadenylate cyclase and degraded by two phosphodiesterases. To date, eight c-di-AMP receptor proteins have been identified in *L. monocytogenes*, including one that indirectly controls the uptake of osmotically active peptides and thus the cellular turgor. The functions of two c-di-AMP-receptor proteins still need to be elucidated. Here, we provide an overview of c-di-AMP signalling in *L. monocytogenes* and highlight the main differences compared to the other established model systems in which c-di-AMP metabolism is investigated. Moreover, we discuss the most important questions that need to be answered to fully understand the role of c-di-AMP in osmoregulation and in the control of central metabolism.

Keywords: Osmolyte, osmoregulation, CodY, essential gene, second messenger, turgor

Introduction

Cyclic di-AMP was first discovered during structural and functional characterization of the DNA integrity scanning protein A (DisA) homologs from *Thermotoga maritima* and *Bacillus subtilis* (Witte et al. 2008). DisA has previously been shown to be a non-specific DNA-binding protein that detects chromosomal damage in *B. subtilis* (Bejerano-Sagie et al. 2006). Shortly thereafter, the observation that the overproduction of the multidrug resistance (MDR) transporter MdrM in *Listeria monocytogenes* correlated with increased activation of the mammalian innate immune system led to the identification of c-di-AMP in this organism (Fig. 1) (Crimmins et al. 2008, Woodward et al. 2010). The single c-di-AMP synthesizing enzyme CdaA in *L. monocytogenes* is essential for growth under standard cultivation conditions (Woodward et al. 2010). Next, c-di-AMP was shown to be produced by many bacteria among them *B. subtilis, Chlamydia trachomatis Lactococcus lactis*, *Mycobacterium tuberculosis, Mycoplasma pneumoniae, Staphylococcus aureus* and *Streptococcus pneumoniae* (Corrigan et al. 2011, Luo and Helmann 2012, Bai et al. 2013, Barker et al. 2013, Mehne et al. 2013, Manikandan et al. 2014, Zhu et al. 2016, Blötz et al. 2017). Albeit less well studied, it has also been shown that archaea synthesize c-di-AMP (Kellenberger et al. 2015, Braun et al. 2019, Braun et al. 2021). Although the groups of organisms are phylogenetically distantly related, an important function of c-di-AMP in bacteria and archaea is the control of osmolyte homeostasis (see below).

In addition to the cyclases DisA and CdaA, the cyclases CdaS, CdaM and CdaZ have been described (Corrigan and Gründling 2013, Commichau et al. 2019, Stülke and Krüger 2020). While the diadenylate cyclases of the CdaA- and CdaM-type are membrane-bound enzymes, the remaining three cyclases are soluble. Interestingly, bacteria such as *Clostridioides difficile* and *B. subtilis* produce two (DisA and CdaA) and three (CdaA, DisA and CdaS) diadenylate cyclases, respectively (Luo and Helmann 2012, Mehne et al. 2013, Oberkampf et al 2022). Since both, *B. subtilis* and *C. difficile* are spore forming bacteria, it was hypothesized that c-di-AMP plays a role in the developmental process of sporulation. Indeed, for *B. subtilis* it has been observed that the cyclases DisA and CdaS are involved in the initiation of spore formation and germination, respectively (Bejerano-Sagie et al. 2006, Mehne et al. 2014). The diadenylate cyclase of the CdaA-type is the most widespread cyclase that has been well studied biochemically and structurally (Rosenberg et al. 2015, Heidemann et al. 2019). Since CdaA is the only diadenylate cyclase in many pathogenic bacteria like *S. aureus, S. pneumoniae* and *L. monocytogenes*, the essential enzyme is an excellent target for novel antibiotics.

Bacterial secretion of c-di-AMP via the MDR transporter MdrA, including MdrC, MdrM, MdrL and MdrT, is considered as one possibility to decrease the intracellular c-di-AMP concentration (Fig. 1) (Crimmins et al. 2008, Woodward et al. 2010, Schwartz et al. 2012, Yamamoto et al. 2012, Kaplan Zeevi et al. 2013, Tadmor et al. 2014). Bacteria synthesizing c-di-AMP also possess specific phosphodiesterases that degrade the second messenger. So far, five different types of phosphodiesterases have been identified and characterized (Pham et al. 2016, Stülke and Krüger 2020). The GdpP-

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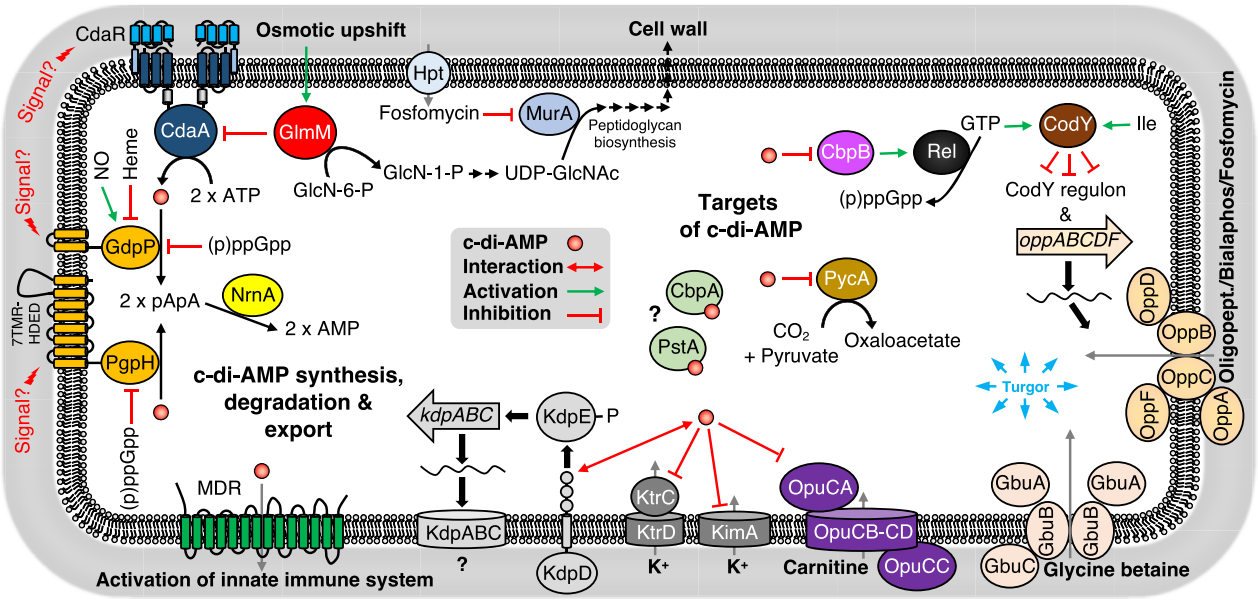

Figure 1. Schematic illustration of c-di-AMP signalling in *L. monocytogenes* (modified from Wang et al. 2022). CdaR and GlmM modulate the activity of CdaA. c-di-AMP is secreted by multidrug efflux pumps (MDRs) and degraded by the phosphodiesterases GdpP and PgpH to 5′-pApA. The nanoRNase NrnA converts 5′pApA to AMP. The uptake of potassium and carnitine is inhibited by c-di-AMP that also interacts with the sensor kinase of the putative *kdpABC* potassium transporter genes. c-di-AMP indirectly controls the CodY-dependent expression of the *opp* oligopeptide transporter genes via CbpB-dependent regulation of Rel activity. The Opp system is involved in the uptake of bialaphos and fosfomycin of which the latter is also imported by the hexose phosphate transporter Hpt. Fosfomycin inhibits the UDP-N-acetylglucosamine 1-carboxyvinyltransferase MurA. The activity of the PycA pyruvate carboxylase is allosterically regulated by c-di-AMP. The functions of the c-di-AMP receptor proteins CbpA and PstA are unknown.

and PgpH-type phosphodiesterases localize at the membrane, the DhhP- and AtaC-type phosphodiesterases are soluble enzymes (Commichau et al. 2019, Latoscha et al. 2020) and CdnP-type phosphodiesterases are exposed to the cell surface (see below) (Andrade et al. 2016). Since c-di-AMP controls the uptake and efflux of osmolytes in bacteria depending on the environmental osmolarity, the adjustment of the cellular c-di-AMP concentration by synthesis, secretion or degradation is crucial for the viability of the cell (Corrigan et al. 2013, Nelson et al. 2013, Bai et al. 2014, Chin et al. 2015, Moscoso et al. 2015, Huynh et al. 2016, Schuster et al. 2016, Gundlach et al. 2017, Devaux et al. 2018a, Rubin et al. 2018, Zeden et al. 2018, Quintana et al. 2019, Wang et al. 2019, Krüger et al. 2020, Sikkema et al. 2020, Cereija et al. 2021, Pham et al. 2021). In fact, c-di-AMP is a key factor essential for osmoregulation in many bacteria and archaea (Commichau et al. 2018, Braun et al. 2019). c-di-AMP is also involved in the control of central metabolism (Sureka et al. 2014, Choi et al. 2017, Whiteley et al. 2017), glycogen metabolism (Selim et al. 2021), DNA damage repair caused by hydrogen peroxide (Gándara and Alonso 2015), cell wall metabolism (Witte et al. 2013, St Onge and Elliot 2017, Massa et al. 2020), biofilm formation (Du et al. 2014, Gundlach et al. 2016, Peng et al. 2016, Townsley et al. 2018, Fahmi et al. 2019, The et al. 2019, Faozia et al. 2021, Rorvik et al. 2021, Wang et al. 2022) and in genetic competence (Zarrella et al. 2020). Some bacteria like the human pathogen *Streptococcus agalactiae* produce the extracellular phosphodiesterase CdnP that is attached to the cell envelope (Andrade et al. 2016). CdnP is required to decrease the activation of the innate immune system by c-di-AMP that is secreted by the bacteria to the environment (Andrade et al. 2016, Devaux et al. 2018b).

Since the discovery of c-di-AMP, many targets have been identified that are bound and regulated by the nucleotide (He et al. 2020, Yin et al. 2020). For instance, c-di-AMP inhibits and activates potassium uptake and efflux systems, respectively, in bacteria (Stülke and Krüger 2020). c-di-AMP also controls the expression of genes encoding osmolyte transporters by binding to a conserved riboswitch and to DNA-binding transcription factors (Block et al. 2010, Nelson et al. 2013, Gao and Serganov 2014, Ren and Patel 2014, Gundlach et al. 2017, Devaux et al. 2018a, Pham et al. 2018, Wang et al. 2019, Bandera et al. 2021, Oberkampf et al 2022). Recently, it was shown that the apo form of the c-di-AMP-receptor protein DarB in *B. subtilis* directly binds to the pyruvate carboxylase and the (p)ppGpp-synthetase/hydrolase, thereby controlling the flux through central carbon metabolism and stringent response, respectively (Krüger et al. 2021a, 2022). While for many c-di-AMP targets it has been elucidated how the nucleotide influences their activity, there are still targets whose function are unknown, including the P_{II}-like signal transduction protein DarA in *B. subtilis* (PstA in *L. monocytogenes* and *S. aureus*) and *L. monocytogenes* CbpA (Campeotto et al. 2015, Choi et al. 2015, Gundlach et al. 2015a, Müller et al. 2015).

We are interested in c-di-AMP metabolism in the human pathogen *L. monocytogenes*. Along with *B. subtilis*, *S. aureus* and several other bacteria as well as archaea, *L. monocytogenes* has become an important model system for studying c-di-AMP metabolism and the impact of the nucleotide on cell physiology. Here we provide an overview of c-di-AMP signalling in *L. monocytogenes* and discuss issues that need to be addressed to fully understand the physiological functions of c-di-AMP.

c-di-AMP synthesizing and degrading enzymes in *L. Monocytogenes*

In contrast to *B. subtilis* and *C. difficile*, *L. monocytogenes* only synthesizes the CdaA-type diadenylate cyclase, which is encoded by the essential *cdaA* gene in the conserved *cdaR-cdaA-glmM* module that also codes for the essential phosphoglucosamine mutase GlmM and the regulatory CdaR protein (Rismondo et al. 2016, Gibhardt et al. 2020, Fischer et al. 2022). CdaA is inserted into the mem-

brane via three N-terminally located hydrophobic helices that are not required for *in vitro* activity of the cyclase (Fig. 1) (Rosenberg et al. 2015, Rismondo et al. 2016). The c-di-AMP-synthesizing diadenylate cyclase domain, which is surrounded by two coiled coil (CC) motifs is located at the C-terminus of CdaA (Rosenberg et al. 2015). In all diadenylate cyclases known so far, the catalytic domain is fused to other protein domains that control c-di-AMP synthesis (Corrigan and Gründling 2013, Commichau et al. 2019). For the sporulation-specific diadenylate cyclase CdaS in *B. subtilis* it has been shown that the two N-terminally-located helices are important for negative control of the diadenylate cyclase domain (Mehne et al. 2013). It is tempting to speculate that also the N-terminal transmembrane helices and CC motifs are required for sensing yet unknown stimuli to control the diadenylate cyclase domain of CdaA *in vivo* (see below). The N-terminally truncated *L. monocytogenes* diadenylate cyclase CdaA lacking the transmembrane helices has been structurally characterized (Rosenberg et al. 2015, Heidemann et al. 2019). Like in DisA, the synthesis of c-di-AMP from two molecules of ATP requires the formation of CdaA dimers for proper arrangement of the important residues of the diadenylate cyclase domain in a face-to-face fashion (Rosenberg et al. 2015, Heidemann et al. 2019). It has been suggested that the full-length CdaA enzyme from *S. aureus* forms multimers consisting of two interacting dimers (Tosi et al. 2019). However, the formation of CdaA multimers *in vivo* has yet to be demonstrated. In contrast to other diadenylate cyclases from *Bacillus thuringiensis*, *M. tuberculosis* and *T. maritima* that require magnesium ions for enzyme catalysis (Witte et al. 2008, Bai et al. 2012, Zheng et al. 2013), CdaA from *L. monocytogenes* depends on manganese ions (Rosenberg et al. 2015, Heidemann et al. 2019). It would be interesting to elucidate whether the full-length CdaA protein also depends on manganese for c-di-AMP synthesis.

Listeria monocytogenes secretes c-di-AMP via MDR transporters MdrA, MdrC, MdrM, MdrL, and MdrT, (Crimmins et al. 2008, Woodward et al. 2010, Schwartz et al. 2012, Yamamoto et al. 2012, Kaplan Zeevi et al. 2013, Tadmor et al. 2014, Huynh and Woodward 2016) and the nucleotide can be degraded by GdpP and PgpH to 5′-pApA (Fig. 1) (Kaplan Zeevi et al. 2013, Witte et al. 2013, Huynh et al. 2015, Hyunh and Woodward 2016, Massa et al. 2020, Wang et al. 2022). The linear nucleotide 5′-pApA is further degraded by NrnA in *L. monocytogenes* (Gall et al. 2022). The phosphodiesterase GdpP (**G**GDEF domain protein-containing phosphodiesterase; PdeA in *L. monocytogenes*) is present in many Firmicutes (Rallu et al. 2000, Commichau et al. 2019). GdpP-type phosphodiesterases contain two N-terminal transmembrane helices, a PAS (Per-Arnt-Sim) domain, a degenerate GGDEF domain and a C-terminal DHH and DHHA1 domains (Hyunh and Woodward 2016, Commichau et al. 2019). So far, it has been shown that *b*-type heme binds to the PAS domain and inhibits the ATPase activity of the phosphodiesterase GdpP and nitric oxide stimulates the enzyme (Fig. 1) (Rao et al. 2010, 2011, Tan et al. 2013). The GdpP-type phosphodiesterases are also inhibited in a competitive manner by the bacterial alarmone (p)ppGpp (Rao et al. 2010, Corrigan et al. 2015; Bowman et al. 2016; Wang et al. 2017). The (p)ppGpp-dependent inhibition of GdpP must occur because the lack of c-di-AMP results in the activation of Rel (Fig. 1) (Peterson et al. 2020, Krüger et al. 2021a) (see below). The phosphodiesterase of the PgpH-type is also a membrane-bound enzyme and contains eight transmembrane helices (Fig. 1) (Liu et al. 2006, Huynh et al. 2015, Hyunh and Woodward 2016). An extracellular seven-transmembrane helix-HDED (7TMR-HDED) domain is located between the transmembrane helices 1 and 2 and the metal-dependent HD phosphohydrolase domain is present in the cytosol (Fig. 1A). As shown for GdpP, (p)ppGpp also inhibits PgpH (Huynh et al. 2015). For both phosphodiesterases of *L. monocytogenes* it will be interesting to identify the extracellular signals that are sensed by the enzymes (see below).

The essential function of c-di-AMP in *L. Monocytogenes*

As mentioned above, c-di-AMP is essential for bacteria like *B. subtilis*, *L. monocytogenes*, and *S. aureus* (Whiteley et al. 2015, Gundlach et al. 2017, Zeden et al. 2018). A genetic approach in combination with a suppressor analysis revealed that a c-di-AMP-free *L. monocytogenes* strain is viable on complex medium if the Opp and Gbu transport systems for the uptake of peptides and glycine betaine, respectively, were inactivated (Whiteley et al. 2015). The same study also identified the link between c-di-AMP metabolism and (p)ppGpp synthesis because some *cdaA* suppressor mutants accumulated mutations in the *rel* gene that reduced the (p)ppGpp synthetase activity of the encoded (p)ppGpp-synthetase/hydrolase. Moreover, among several other genes that were affected in the *cdaA* suppressor mutants, some suppressors had acquired mutations in the *pycA* pyruvate carboxylase gene and in the *cbpB* and *pstA* genes encoding the c-di-AMP receptor proteins CbpB and PstA, respectively (Whiteley et al. 2015). It has been suggested that the reduced conversion of GTP to (p)ppGpp by Rel stimulates the GTP-responsive transcriptional regulator CodY to represses the *opp* genes, which in turn would prevent the influx of oligopeptides to toxic levels (Fig. 1) (Stenz et al. 2011, Whiteley et al. 2015, Wang et al. 2022). Thus, c-di-AMP is required by *L. monocytogenes* to adjust the cellular turgor that is influenced by amino acids and peptides (Fig. 1) (Maria-Rosario et al. 1995, Commichau et al. 2018). This idea is supported by the finding that c-di-AMP also binds to and inhibits the ATPase subunit of the importer OpuC that transports the osmolyte carnitine (Huynh et al. 2016). Moreover, the *L. monocytogenes cdaA* mutant shows a strong lytic phenotype, which can be partially rescued by enhanced peptidoglycan biosynthesis that stabilizes the cell envelope (Wang et al. 2022). Recently, the underlying molecular mechanism of c-di-AMP-dependent control of (p)ppGpp synthesis has been elucidated in *L. monocytogenes* (Peterson et al. 2020). During growth in rich medium, c-di-AMP binds to and hinders CbpB from activating the synthesis of (p)ppGpp by Rel and a high GTP pool allows the CodY-dependent control of oligopeptide uptake. (Fig. 1). Thus, an essential function of c-di-AMP in *L. monocytogenes* is the control of the Opp system-mediated uptake of oligopeptides, which serve as a source of nutrients and influence the turgor of the cell (Maria-Rosario et al. 1995, Borezee et al. 2000, Commichau et al. 2018). The Opp system is also involved in the uptake of the epoxid antibiotic fosfomycin that targets the essential MurA enzyme (Kahan et al. 1974, Chekan et al. 2016), which catalyzes the committing reaction in peptidoglycan synthesis by (Fig. 1) (Wang et al. 2022). Thus, the promiscuous Opp system contributes together with the sugar phosphate permease Hpt to fosfomycin uptake in *L. monocytogenes* (Scortti et al. 2006, 2018).

Like in *L. monocytogenes*, c-di-AMP also plays a central role in the regulation of the cellular turgor in other bacteria (Commichau et al. 2018, Stülke and Krüger 2020). In *B. subtilis*, an essential function of c-di-AMP in *B. subtilis* is the control of the uptake of potassium ions *via* high- and low affinity potassium transport systems (Gundlach et al. 2017). Moreover, amino acids such as histidine, which is converted to glutamate, and glutamate itself were found to be toxic for a c-di-AMP *B. subtilis* mu-

Table 1. Confirmed interactions between CdaR, CdaA, GlmM in Firmicutes.

Organism	Analyzed protein pair	Interaction/methods	Effect on c-di-AMP synthesis	References
B. subtilis	CdaR-CdaA	Yes/BTH[a] and activity assay	Activation, *in vivo* (tested in *Escherichia coli*)	Mehne et al. 2013, Gundlach et al. 2015a
	GlmM-CdaA	Yes/BTH assay, SPINE[b], FPLC[c], SAXS[d] and structure analysis, activity assay	Inhibition, *in vitro*	Gundlach et al. 2015b, Pathania et al. 2021
L. monocytogenes	CdaR-CdaA	Yes/BTH assay, activity assay	Inhibition, *in vivo* (tested in *E. coli* and *L. monocytogenes*)	Rismondo et al. 2016, Gibhardt et al. 2020
	GlmM-CdaA	Yes/BTH assay, pulldown assay, SEC-MALS[e], ITC[f], activity assay	Inhibition, *in vivo* (tested in *E. coli* and in *L. monocytogenes*) and *in vitro*	Gibhardt et al. 2020
L. lactis	CdaR-CdaA	Activity assay	No effect[h]	Zhu et al., 2015
	GlmM-CdaA	Yes/BTH assay,	Inhibition *in vivo* (tested in *E. coli* and *L. lactis*)	Zhu et al., 2015
S. aureus	CdaR-CdaA	Activity assay	Inhibition *in vivo* (tested in *E. coli*)	Zhu et al., 2015
	GlmM-CdaA	SAXS[d], SEC[g], pulldown assay, activity assay	Inhibition *in vivo* (tested in *E. coli*) and *in vitro*	Tosi et al. 2019

[a]Bacterial two-hybrid assay.
[b]Strep-protein interaction experiment, *in vivo* crosslinking in combination with a pulldown assay.
[c]Fast protein liquid chromatography.
[d]Small-angle X-ray scattering.
[e]Size-exclusion chromatography and multiangle light scattering.
[f]Isothermal titration calorimetry.
[g]Size-exclusion chromatography
[h]*cdaR* is a pseudogene in the *L. lactis* strain MG1363 (Zhu et al. 2016).

tant (Kimhi and Magasanik 1970, Krüger et al. 2021b, Meißner et al. 2022). Furthermore, suppressor mutants of the c-di-AMP-free *B. subtilis* mutant that grew in rich medium could only be isolated when one of the two c-di-AMP receptor proteins DarA (PstA in *L. monocytogenes* and *S. aureus*) or DarB (CbpB in *L. monocytogenes*) were absent (Krüger et al. 2021b). Interestingly, in *B. subtilis*, apo-DarB activates the tricarboxylic acid cycle (TCA)-replenishing pyruvate carboxylase PycA and, like in *L. monocytogenes*, also the (p)ppGpp-synthetase/hydrolase Rel (Krüger et al. 2021a, 2022). In *L. monocytogenes*, c-di-AMP directly binds to and activates PycA (Sureka et al. 2014). Thus, bacteria need to couple central metabolism with osmoregulation for growth in the presence of osmolytes such as potassium, amino acids and peptides (Whiteley et al. 2015, 2017, Krüger et al. 2022, Wang et al. 2022). This hypothesis is supported by observations that the uncontrolled uptake of osmolytes is also detrimental for growth c-di-AMP-free mutants of *C. difficile*, *S. aureus* and *Streptococcus agalactiae* (Devaux et al. 2018a, Zeden et al. 2018, 2020, Oberkampf et al. 2022).

It is interesting to note that the genome of *L. monocytogenes* carries the *ktrCD, kimA* and *kdpABC* potassium transport system genes as well as the *kdpDE* two-component signaling system genes whose products are involved in the expression control of the *kdpABC* operon in bacteria like *S. aureus* (Moscoso et al. 2015, Gibhardt et al. 2019). The *L. monocytogenes* KtrCD and KimA potassium transporters were shown to mediate uptake of potassium ions when heterologously expressed in *Escherichia coli* (Gibhardt et al. 2019). Moreover, c-di-AMP binds to both transporters and inhibits their transport activity (Gibhardt et al. 2019). However, in contrast to *B. subtilis* and other bacteria, the growth of a c-di-AMP-free *L. monocytogenes* mutant was only slightly inhibited by high amounts of extracellular potassium ions (Gibhardt et al. 2019). Moreover, suppressors derived from the c-di-AMP-free *L. monocytogenes* strain rarely accumulate mutations in potassium transporter genes (Whiteley et al. 2015, Wang et al. 2022). Thus, the physiological impact of the c-di-AMP-dependent regulation of potassium uptake seems to be less pronounced than in bacteria like *B. subtilis* (Gundlach et al. 2017, Devaux et al. 2018a, Oberkampf et al. 2022), and bacteria as well as archaea have evolved species-specific signaling systems of osmoregulation in which c-di-AMP serves as a key component.

Phenotypes of mutants of *L. Monocytogenes* and related bacteria with defects in c-di-AMP metabolism

Several studies revealed that the lack and the accumulation of c-di-AMP can be detrimental for the cell. However, many phenotypes related to perturbation of c-di-AMP metabolism are now better understood such as the inverse correlation between the cellular c-di-AMP levels and susceptibility of cell wall-targeting antibiotics (Luo and Helmann 2012, Witte et al. 2013, Whiteley et al. 2017, Commichau et al. 2018, Wang et al. 2022). It is now obvious that the cellular turgor likely increases in the absence of c-di-AMP due to the uncontrolled influx of osmolytes such as potassium ions, glycine betaine, carnitine, amino acids and peptides (Commichau et al. 2018, Stülke and Krüger 2020). Due to the turgor increase, the cell wall likely gets overstrained and thus more susceptible to cell wall-targeting antibiotics (Luo and Helmann 2012,

Witte et al. 2013, Commichau et al. 2018, Pham et al. 2018, Zeden et al. 2018). By contrast, a drop in turgor due to reduced osmolyte uptake makes the cell less susceptible to antibiotics because the cell wall does not have to be as stable. Thus, in many cases, perturbation of c-di-AMP metabolism indirectly affects the integrity of the cell envelope. However, a direct link between c-di-AMP and cell wall metabolism has been identified in *Streptomyces coelicolor* (St Onge and Elliot 2017). Moreover, a recent study revealed that a *L. monocytogenes gdpP pgpH* mutant, which is unable to degrade c-di-AMP was impaired in peptidoglycan muropeptide and D-alanine-D-alanine synthesis (Massa et al. 2020). Since it was found that potassium stimulates the activity of the D-alanine-D-alanine ligase Dld, it was suggested that the reduced uptake of potassium due to c-di-AMP accumulation is responsible for the observed phenotype (Massa et al. 2020). Thus, in addition to the characterized potassium transporters KtrCD and KimA, probably yet unknown routes for potassium uptake such as the Kdp system exist in *L. monocytogenes* that are regulated by c-di-AMP.

Prior to the discovery of c-di-AMP, it was observed that the Opp oligopeptide transport system is required for growth of *L. monocytogenes* in rich medium at low temperatures (Borezee et al. 2000). Moreover, a *L. monocytogenes* transposon mutant that likely does not produce C-terminally located HD phosphohydrolase domain of the phosphodiesterase PgpH showed a cold-sensitive growth phenotype (Liu et al. 2006). Since it is now known that c-di-AMP indirectly controls the expression of the *opp* genes via CbpB and Rel, the temperature-dependent growth defect of the *pgpH* mutant might be caused by a lower abundance of the Opp transport system (Fig. 1). The surprising observation that this mutant synthesized slightly more (p)ppGpp is inconsistent with the model that c-di-AMP inhibits the formation of the alarmone by Rel (Fig. 1) (Peterson et al. 2020, Krüger et al. 2021a). However, this needs to be further investigated because the experimental design did not allow a precise quantification of the (p)ppGpp levels. In *L. lactis* it has been observed that spontaneous mutants, which grew better at elevated temperatures (37.5°C), had inactivated the *gdpP* phosphodiesterase gene (Smith et al. 2012). Thus, the link between the temperature sensitivity and c-di-AMP metabolism is still unclear.

Recently, it has been observed that isoleucine is toxic for a c-di-AMP-free *L. monocytogenes* mutant (Wang et al. 2022). The toxicity of isoleucine can be abolished by mutations in *codY* that alter the DNA-binding activity of the isoleucine- and GTP-responsive CodY protein (Wang et al. 2022). One of the isolated isoleucine-tolerant *cdaA* suppressor mutants acquired a mutation in the *lmo2419* gene encoding the ATP-binding subunit of a putative amino acid ABC transporter. According to Uniprot, Lmo2419 is part of a putative methionine transporter. It needs to be elucidated whether the putative amino acid ABC transporter is involved in the uptake of isoleucine, methionine and other amino acids.

c-di-AMP targets of unknown function in *L. Monocytogenes*

In *L. monocytogenes*, there are still at least two c-di-AMP receptor proteins of unknown function, among them the P_{II}-like PstA protein (DarA in *B. subtilis*) that has been biochemically and structurally characterized (Fig. 1) (Sureka et al. 2014, Choi et al. 2015, Gundlach et al. 2015a). The fact that some *L. monocytogenes cdaA* suppressor mutants, which gained the ability to grow in rich medium (Whiteley et al. 2017), acquired mutations in the *pstA* gene indicates that, like the CbpB protein, also PstA only exerts its function in the absence of c-di-AMP. This also holds true for *B. subtilis* because suppressor mutants of a c-di-AMP-free mutant that grew in rich medium could only be isolated when the *darA* gene was deleted (Krüger et al. 2021b). Potential interaction partners of PstA (or DarA) could therefore perhaps be identified *via* an *in vivo* crosslinking approach when the *cdaA* cells are transferred from minimal medium to rich medium.

Much less is known about the c-di-AMP receptor protein CbpA, which is a poorly conserved protein and has no counterpart in *B. subtilis* and *S. aureus* (Fig. 1). Like CbpB, CbpA contains a CBS (cystathione-beta-synthase) domain that was first defined for the cystathione beta synthase (Baykov et al. 2011, Ereno-Orbea et al. 2013). CbpA also contains an ACT (aspartate kinase-chorismate mutase-tyrA (prephrenate dehydrogenase) (ACT) domain (http://www.uniprot.org)). The ACT domain serves as a regulatory module for small ligand-dependent allosteric regulation of enzymes that are often involved in amin acid and purine biosynthesis (Liberles et al. 2005, Grant 2006). Structural analysis of CbpA allowed the construction of a sensor to assess the intracellular concentrations of c-di-AMP (Pollock et al. 2021).

Recently, it has been observed that a *L. monocytogenes* mutant that constitutively expresses the virulence genes depends on CbpA and two other proteins to cope with oxidative stress (Mains et al. 2021). However, the fact that the *cbpA* gene did not appear in any previous suppressor analysis makes it difficult to define a meaningful experimental approach for finding its function.

Control of c-di-AMP Synthesis in *L. Monocytogenes*

It is unequivocally clear that c-di-AMP plays a crucial role in the adaptation of bacteria and archaea to the osmolarity of the environment. However, it is not yet understood how the cell senses the environmental osmolarity to adjust the intracellular c-di-AMP levels that control osmolyte uptake and export, and thus the turgor. It has previously been shown that the activity of CdaA is modulated by the membrane-attached CdaR protein that directly interacts with the diadenylate cyclase (Fig. 1) (Table 1). However, it is unclear whether CdaR activates or inhibits CdaA. Since CdaR is exposed to the cell surface and gets into contact with the peptidoglycan layer, the protein might sense cell wall damage or the tension of the cell envelope and adjust the turgor of the cell by controlling the CdaA-dependent c-di-AMP synthesis. Recently, it has been demonstrated that intrinsically disordered proteins like the membrane-anchored cell surface-exposed anti-σ factor RsgI, which controls SigI activity, are required for cell wall homeostasis in *B. subtilis* (Brunet et al. 2022). When cell wall biosynthesis is impaired, RsgI, which is constitutively processed into an ectodomain and a membrane part by proteolytic cleavage, triggers activation of the SigI regulon (Brunet et al. 2022). It is tempting to speculate that CdaR senses the integrity of the cell wall like RsgI and transmits the signal to CdaA.

As mentioned above, the *cdaR*, *cdaA* and *glmM* genes are part of the *cdaRA-glmM* module that is conserved in many bacteria synthesizing c-di-AMP (Corrigan and Gründling 2013, Pham et al. 2016). Like CdaR, also GlmM interacts with and inhibits CdaA in *B. subtilis*, *L. monocytogenes*, *L. lactis*, and *S. aureus* (Fig. 1) (Table 1). Structural characterization of a complex consisting of the soluble C-terminal CdaA domain and GlmM from *B. subtilis* revealed that GlmM prevents the formation of active head-to-head CdaA oligomers (Pathania et al. 2021). Moreover, in *L. monocytogenes*, the GlmM-dependent inhibition is triggered during growth of the bacteria in a hyperosmotic environment (Gibhardt et al. 2020). It has been suggested that the cell volume changes in response to an osmotic up- or downshifts could result in a transient change of the

cellular GlmM concentration, which would facilitate the interaction with CdaA and thus the control of c-di-AMP synthesis (Gibhardt et al. 2020). However, this hypothesis needs to be verified. In contrast to *L. monocytogenes*, *B. subtilis* synthesizes the two vegetative diadenylate cyclases CdaA and DisA as well as the sporulation specific enzyme CdaS (Luo and Helmann 2012, Mehne et al. 2013). Interestingly, *B. subtilis* only needs a single c-di-AMP-producing enzyme for growth in rich medium (Mehne et al. 2013, 2014). This indicates that CdaA, DisA and a hyperactive variant of CdaS can functionally replace each other, and that the synthesis of c-di-AMP does not have to take place at the membrane. For *L. monocytogenes* it has been shown that the native diadenylate cyclase CdaA can be replaced by DisA from *B. subtilis* (Witte et al. 2013). Thus, like CdaA, also the soluble diadenylate cyclase DisA and CdaS may respond to changes in environmental osmolarity. In the case of DisA, one might hypothesize that changes in the viscosity of the cytoplasm depending on the osmolarity of the environment facilitate the inhibitory interaction between the diadenylate cyclase and DNA, and thus c-di-AMP production. However, also this idea needs to be verified.

Open questions

As mentioned above, the cellular functions of the c-di-AMP receptor proteins PstA and CbpA must be identified. The importance of c-di-AMP for osmolyte homeostasis and central metabolism only becomes apparent when the functions of all targets are known. The most exciting question how the cell 'senses' the environmental osmolarity to adjust the cellular turgor using c-di-AMP remains to be addressed. The identification of the signals that are sensed by the diadenylate cyclases and the phosphodiesterase may provide an answer to this important question. Moreover, the link between c-di-AMP metabolism and cell wall homeostasis needs to be investigated.

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