Coping with an Essential Poison: a Genetic Suppressor Analysis Corroborates a Key Function of c-di-AMP in Controlling Potassium Ion Homeostasis in Gram-Positive Bacteria

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ABSTRACT Cyclic di-AMP (c-di-AMP) is an important second messenger in bacteria. In most *Firmicutes*, the molecule is required for growth in complex media but also toxic upon accumulation. In an article on their current study, Zarrella and coworkers present a suppressor analysis of a *Streptococcus pneumoniae* strain that is unable to degrade c-di-AMP (T. M. Zarrella, D. W. Metzger, and G. Bai, J Bacteriol 200:e00045-18, 2018, https://doi.org/10.1128/JB.00045-18). Their study identifies new links between c-di-AMP and potassium homeostasis and supports the hypothesis that c-di-AMP serves as a second messenger to report about the intracellular potassium concentrations.

KEYWORDS second messenger, *Streptococcus pneumoniae*, potassium uptake, phosphodiesterase, diadenylate cyclase

Bacteria use second messengers in order to process environmental information. These molecules—often specific nucleotides—are synthesized in response to primary signals and are then perceived by intracellular targets, typically protein or RNA molecules (1). Among all second messengers, cyclic di-AMP (c-di-AMP) is unique due to three exceptional features. First, c-di-AMP is the only second messenger that is essential for many bacteria that produce it, including the model organism *Bacillus subtilis* and the pathogens *Staphylococcus aureus*, *Listeria monocytogenes*, and *Streptococcus pneumoniae*, as well as the genome-reduced bacterium *Mycoplasma pneumoniae* (2–8). Second, the *Firmicutes* are unable to grow under standard conditions on complex media in the absence of c-di-AMP, but its accumulation poses problems for the bacteria as well; c-di-AMP has therefore been coined an "essential poison" (9). For *B. subtilis* and *M. pneumoniae*, loss of the phosphodiesterases that degrade c-di-AMP leads to cell death (8–10). Third, c-di-AMP is the only second messenger that modulates a biological process by binding to a protein and also to an RNA molecule that controls its expression. This was discovered in *B. subtilis*, where c-di-AMP limits potassium uptake by binding to the KtrA signaling subunit of the KtrAB potassium transporter as well as to the riboswitch in the untranslated region of the *ktrAB* mRNA. Binding of c-di-AMP to the riboswitch prevents transcription beyond the riboswitch and thus reduces the expression of the potassium transporter (11, 12).

The essential role of c-di-AMP for the bacterial physiology and its requirement for the virulence of several important pathogens have attracted considerable interest in the elucidation of the reason for its essentiality and for its functions in the cell (reviewed in references 13 to 15). The data obtained so far suggest key functions of c-di-AMP in the control of central metabolism, potassium homeostasis, and osmotic adaptation (6, 15–18). However, the identification of target proteins of so far unknown function (11, 18, 19) suggests that c-di-AMP may be involved in additional processes.

The making and breaking of cyclic di-AMP involve two classes of enzymes: the

diadenylate cyclases and phosphodiesterases, respectively. The diadenylate cyclases are characterized by a conserved catalytically active domain, the so-called DAC (dia-denylate cyclase) domain (13, 14, 20). Four types of cyclases have so far been investigated. They differ by the regulatory domains that are attached to the DAC domain (8, 14). *B. subtilis* carries genes that encode three diadenylate cyclases: DisA, CdaA, and CdaS. DisA and CdaS have been implicated in the control of DNA integrity and spore germination, respectively (21, 22). CdaA is the most widespread diadenylate cyclase, and it is the only cyclase in pathogenic *Firmicutes* (14). This membrane protein consists of an N-terminal transmembrane domain and the enzymatically active DAC domain, which is flanked by coiled-coil domains on each side (9, 20). As observed for the cyclases, many bacteria possess multiple c-di-AMP-degrading phosphodiesterases. One class of phosphodiesterases is characterized by conserved DHH/DHH1 domains, whereas the enzymes of the second class possess an HD domain. *L. monocytogenes* and *B. subtilis* have a phosphodiesterase of each class (9, 23). *S. aureus* only encodes one c-di-AMP-degrading enzyme of the DHH/DHH1 family, whereas a second cytoplasmic DHH/DHH1 class protein degrades the linear dinucleotide pApA rather than c-di-AMP (24). In *S. pneumoniae*, two DHH/DHH1-type phosphodiesterases are present. One of these enzymes cleaves c-di-AMP to two molecules of AMP, whereas the second enzyme produces pApA (25). Loss of these enzymes results in the accumulation of c-di-AMP and loss of virulence of the bacteria (25, 26).

MUTATIONS AFFECTING THE MAJOR DIADENYLATE CYCLASE CdaA PREVENT c-di-AMP ACCUMULATION

As mentioned above, not only is c-di-AMP essential in Gram-positive bacteria, its accumulation is also detrimental to the cells or even toxic (3, 9). In particular, several studies have revealed an increased sensitivity to salt and other stress factors, which is often accompanied by resistance to β-lactam antibiotics (2, 25, 27–30 [see reference 15 for review]). Based on the impaired growth of strains lacking the phosphodiesterases, suppressor mutations have been identified that provide novel insights into c-di-AMP signaling. In *B. subtilis*, such strains that lack both phosphodiesterases rapidly lyse and acquire suppressor mutations that allow survival (9). An analysis of those mutants revealed that in all cases the major diadenylate cyclase CdaA was affected: frameshift mutations resulted in the expression of truncated CdaA proteins that lack the catalytically relevant DGA and RHR motifs in the DAC domain or in a deletion of the C-terminal eight amino acids of CdaA. The inactivation of CdaA in *B. subtilis* upon loss of the phosphodiesterases made DisA the only expressed diadenylate cyclase in these mutants. Simultaneous deletion of the genes encoding both phosphodiesterases and DisA provoked a mutation in the ribosomal binding site of the *cdaA* mRNA, resulting in reduced CdaA synthesis (9). Taken together, these results suggested that CdaA is the major diadenylate cyclase in *B. subtilis* and that c-di-AMP produced by this enzyme is the cause of c-di-AMP toxicity. Zhu et al. have isolated suppressor mutants that overcome the salt sensitivity of *Lactococcus lactis* strains lacking the single c-di-AMP-degrading phosphodiesterase (28). With an impressive set of more than 60 distinct mutations affecting CdaA, and thus reduced levels of c-di-AMP, this study convincingly reinforced the conclusion that c-di-AMP accumulation causes osmosensitivity in *L. lactis* (28). The current study by Zarrella et al. (31) adds another flavor to the adaptation of c-di-AMP-accumulating bacteria to stress conditions (Fig. 1). *S. pneumoniae* lacking both phosphodiesterases exhibits reduced growth on complex media and increased sensitivity to stress conditions, including osmotic and heat stresses (31). The suppressor mutants that are more resistant to heat stress carry mutations, and two distinct mutations affecting the single diadenylate cyclase CdaA are involved in this resistance: a V76G substitution at the outer side of the third transmembrane helix, and a frameshift at the C terminus-encoding end of the *cdaA* gene that results in the addition of a 32-amino-acid tail. Interestingly, a mutation corresponding to the V76G mutation was also found in the *L. lactis* suppressor screen, and mutations affecting the C terminus of the protein were detected in both *B. subtilis* and *L. lactis* (9, 28). Both mutations resulted
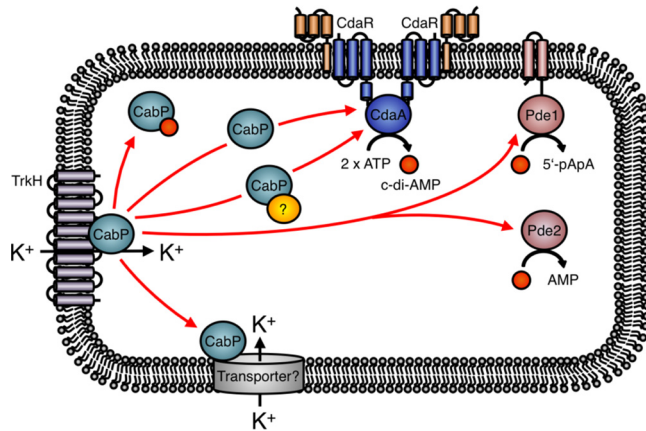
FIG 1 c-di-AMP signaling in *Streptococcus pneumoniae*. CdaR directly interacts with the diadenylate cyclase CdaA and regulates its activity (7). The red arrows indicate the possibilities of how the Trk family protein CabP modulates the cellular c-di-AMP level. The CabP-dependent control of c-di-AMP synthesis or degradation might also depend on additional factors, as indicated by the yellow circle. Moreover, CabP could control the activity of an unknown additional potassium transporter, and the changes in cellular potassium concentration could then affect the c-di-AMP levels.

in a severe reduction of the c-di-AMP concentrations and restored the wild-type levels of the nucleotide. Interestingly, one of the mutants had an additional substitution affecting the potassium transporter TrkH, but this mutation made only a minor contribution to the reduction of the c-di-AMP levels (31).

A LINK BETWEEN POTASSIUM UPTAKE AND c-di-AMP SYNTHESIS

In all organisms studied so far, at least one of the c-di-AMP target molecules is involved in potassium homeostasis, and in several more intensively studied bacteria, such as *S. aureus*, *L. monocytogenes*, and *B. subtilis*, even multiple potassium-related targets have been detected. In these three bacteria, c-di-AMP not only binds to the cytoplasmic gating subunits of potassium transporters to control their activity but also binds to proteins and RNA molecules that control the expression of potassium transporters (14). Interestingly, c-di-AMP binds KdpD, a sensor kinase of a two-component system that activates expression of the high-affinity potassium uptake system KdpABC in *S. aureus* and *L. monocytogenes*. *B. subtilis* does not possess the Kdp system and relies on the KtrAB and the newly discovered KimA transporters for high-affinity uptake of potassium (17, 32, 33). The *ktrAB* and *kimA* genes for these transporters are each under the control of a c-di-AMP-responsive riboswitch that causes premature transcription termination upon binding of the second messenger (12, 17). Thus, potassium uptake is reduced as long as the intracellular concentration of c-di-AMP is high. This connection between potassium homeostasis and c-di-AMP signaling is also reinforced by the current study of Zarrella et al. (31) (Fig. 1). First of all, a mutation in TrkH, the integral membrane subunit of the potassium transporter TrkH-CabP, contributes to the decrease in the c-di-AMP levels in one of the suppressor mutants that were isolated as a result of c-di-AMP accumulation (described above). CabP, the gating component of this transporter, has previously been identified as a target of c-di-AMP in *S. pneumoniae* (34). This protein is similar to the c-di-AMP-binding KtrA proteins from *B. subtilis* and other *Firmicutes*. Interestingly, the TrkH variant isolated by Zarrella et al. allows improved growth of the strain lacking any c-di-AMP-degrading phosphodiesterases on complex media. As c-di-AMP binding to CabP inhibits potassium uptake, the mutant TrkH protein may exhibit improved potassium transport. The subunits of the TrkH-CabP complex have different effects on the intracellular c-di-AMP levels: while a deletion of the *trkH* gene has no effect, the deletion of *cabP* results in a reduction of the c-di-AMP levels by about 50%. This finding indicates that CabP is involved in the control of c-di-AMP homeostasis, even though the current work could not yet identify the precise

mechanism. In *S. aureus*, KtrA controls the activity of two distinct membrane proteins, KtrB and KtrD. Thus, it is tempting to speculate that CabP serves as gating subunit not only for TrkH but also for a second membrane-spanning transporter. Indeed, the genome of *S. pneumoniae* also encodes the protein SPD_0429, which is most similar to KtrB. Thus, deletion of *trkH* may not be sufficient to reduce potassium uptake due to the activity of SPD_0429. Loss of CabP as the single gating subunit for both transporters would in any case result in reduced potassium uptake. In *B. subtilis*, reduced potassium levels also result in reduced c-di-AMP levels (17). Thus, the presence of a second potassium transporter subunit nicely explains the seemingly contradictory results obtained with the *trkH* and *cabP* mutants.

c-di-AMP, A KEY PLAYER IN CONTROLLING OSMOLYTE TRANSPORT IN GRAM-POSITIVE BACTERIA?

In the past few years, several studies have demonstrated that perturbations of c-di-AMP metabolism affect the integrity of the protective cell wall in Gram-positive bacteria, including streptococci (for a recent review, see reference 15). For instance, elevated cellular concentrations of c-di-AMP due to the loss of the phosphodiesterase GdpP in *L. monocytogenes*, *L. lactis*, and *B. subtilis* reduced the susceptibility of the bacteria to β-lactams (2, 28, 35). A *Streptococcus suis* mutant tolerating large amounts of lysozyme had acquired a mutation in the *cdaA* diadenylate cyclase gene, which probably alters the cellular c-di-AMP concentration (36). In contrast, a decrease of the cellular c-di-AMP levels in *B. subtilis* and *L. monocytogenes* caused cell lysis (2, 7, 17). Moreover, strains that synthesize smaller amounts of c-di-AMP are more susceptible to cell wall-targeting antibiotics (15). Therefore, it has been concluded that c-di-AMP is essential for proper cell envelope synthesis (2, 7, 35). However, today we know that the nucleotide rather indirectly affects the integrity of the cell envelope by modulating the cellular turgor, a physical variable that has to be tightly adjusted depending on the osmotic pressure of the environment (15). For *B. subtilis*, *L. monocytogenes*, and *S. aureus*, it has been shown that c-di-AMP becomes dispensable for the cell if the bacteria are cultivated in a chemically defined medium that does not lead to the excessive accumulation of osmolytes and amino acids (6, 16, 17). Thus, many c-di-AMP-related cell wall phenotypes in *Firmicutes* can be explained by the perturbation of osmolyte homeostasis.

As described above, c-di-AMP plays a crucial role in controlling the activities of potassium uptake systems in a variety of Gram-positive bacteria, including *S. pneumoniae*. Recently, it has been uncovered that c-di-AMP also controls the uptake of other osmolytes, such as the compatible solutes carnitine and glycine-betaine. c-di-AMP inhibits uptake of carnitine via the high-affinity OpuC ATP-binding cassette (ABC) transport systems in *L. monocytogenes* and *S. aureus* (29, 37). The OpuC transport system consists of the subunits OpuCA, OpuCB, OpuCC, and OpuCD (38). OpuCA is a cytoplasmic protein that contains tandem cystathionine-β-synthase (CBS) and ABC domains, of which the latter fuels the transport activity of the Opu system. c-di-AMP was shown to inhibit carnitine uptake by binding in the cleft between the two CBS domains of the OpuCA subunit (29). Thus, c-di-AMP prevents uptake of carnitine to toxic levels, as is the case for the osmolyte potassium. It is interesting to note that many bacterial ABC transporters involved in the transport of compatible solutes contain tandem CBS domains (39). In *B. subtilis* and *L. lactis*, the CBS domains in the osmoregulatory ABC transporter OpuA, which transports the osmolyte glycine-betaine, sense the ionic strength and control the transport activity (39, 40). However, it has to be tested whether c-di-AMP also controls the activity of the OpuA transport system in *B. subtilis* and *L. lactis*. Interestingly, c-di-AMP also binds to the CBS domain-containing proteins CbpA and CbpB from *L. monocytogenes* (18). The OpuCA homolog of *S. pneumoniae* (designated PotA) does not possess CBS domains, and it is therefore not involved in the c-di-AMP-dependent uptake of osmolytes. However, in future studies it will be interesting to elucidate whether c-di-AMP is also a key player of osmolyte homeostasis in *S. pneumoniae* as is the case in other *Firmicutes*. For this purpose, a

saturating suppressor screen with an *S. pneumoniae cdaA* mutant isolated in the current study could be a straightforward approach to identify novel c-di-AMP-binding proteins that are probably involved in the transport of osmoactive substances. Moreover, it is essential to elucidate how the diadenylate cyclases and the phosphodiesterases that synthesize and degrade c-di-AMP, respectively, sense the potassium concentrations and/or the cellular turgor to adjust uptake and export of osmolytes. The study by Zarrella et al. (31) reveals that the *S. pneumoniae* c-di-AMP effector protein CabP modulates the synthesis of the nucleotide, probably by controlling the activity of the diadenylate cyclase CdaA (31). As discussed above, CabP could affect the c-di-AMP pool by modulating potassium uptake via TrkH and SPD_0429. As a consequence, the alteration of the c-di-AMP concentration could be due to a change in diadenylate cyclase activity that responds to potassium. Therefore, the precise role of CabP in controlling the cellular c-di-AMP levels should be elaborated in future studies (Fig. 1).

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