Sustained sensing in potassium homeostasis: Cyclic di-AMP controls potassium uptake by KimA at the levels of expression and activity

Received for publication, April 8, 2019, and in revised form, May 1, 2019 Published, Papers in Press, May 6, 2019, DOI 10.1074/jbc.RA119.008774

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Edited by Roger J. Colbran

The signaling nucleotide cyclic di-AMP (c-di-AMP) is the only known essential second messenger in bacteria. Recently, c-di-AMP has been identified as being essential for controlling potassium uptake in the model organism *Bacillus subtilis* and several other bacteria. A *B. subtilis* strain lacking c-di-AMP is not viable at high potassium concentrations, unless the bacteria acquire suppressor mutations. In this study, we isolated such suppressor mutants and found mutations that reduced the activities of the potassium transporters KtrCD and KimA. Although c-di-AMP–mediated control of KtrCD has previously been demonstrated, it is unknown how c-di-AMP affects KimA activity. Using the DRaCALA screening assay, we tested for any interactions of KimA and other potential target proteins in *B. subtilis* with c-di-AMP. This assay identified KimA, as well as the K^+/H^+ antiporter KhtT, the potassium exporter CpaA (YjbQ), the osmoprotectant transporter subunit OpuCA, the primary Mg^{2+} importer MgtE, and DarB (YkuL), a protein of unknown function, as *bona fide* c-di-AMP–binding proteins. Further, binding of c-di-AMP to KimA inhibited potassium uptake. Our results indicate that c-di-AMP controls KimA-mediated potassium transport at both *kimA* gene expression and KimA activity levels. Moreover, the discovery that potassium exporters are c-di-AMP targets indicates that this second messenger controls potassium homeostasis in *B. subtilis* at a global level by binding to riboswitches and to different classes of transport proteins involved in potassium uptake and export.

The essential signaling nucleotide c-di-AMP[2] is a recently discovered second messenger that is produced by many bacteria and some archaea (1, 2). The reasons for the essentiality of this dinucleotide have long remained elusive. Recent studies with the Gram-positive bacteria *Listeria monocytogenes*, *Bacillus subtilis*, *Staphylococcus aureus*, and *Streptococcus agalactiae* revealed that c-di-AMP becomes dispensable, if the bacteria are cultivated on strictly controlled minimal media (3–6). In the Gram-positive model organism *B. subtilis*, c-di-AMP is dispensable only at low potassium concentrations in minimal medium (4).

Binding assays to search for target proteins of the molecule revealed that c-di-AMP binds several different proteins, with the majority being involved in potassium and compatible solute uptake (see Ref. 7 for review). c-di-AMP binds to the conserved RCK_C (regulator of conductance of K^+) domains in the gating components of potassium channels (6, 8–12). Moreover, c-di-AMP binds and inhibits the unrelated Kup potassium transporters in *Lactococcus lactis* (13). Additionally, c-di-AMP controls the expression of different potassium uptake systems. It binds to the KdpD sensor kinase that controls the expression of the *S. aureus* and *L. monocytogenes* Kdp potassium transport systems and to the two copies of the c-di-AMP (formerly *ydaO*) riboswitch that controls the expression of the high-affinity potassium uptake systems KtrAB and KimA in *B. subtilis* and other bacteria (4, 14, 15). This makes c-di-AMP the only known second messenger that controls a single biological process by binding both to a protein and to the corresponding mRNA. Similarly, the uptake of osmoprotectants is regulated at levels both of gene expression and protein activity. c-di-AMP binds to the RCK_C domain of the transcription repressor BusR, which controls the expression of the *busAB* operon for the transport of compatible solutes in lactic acid bacteria (6, 16). Moreover, c-di-AMP binding to the regulatory CBS domain of the ATP-binding subunit directly inhibits osmoprotectant uptake systems in *S. aureus* and *L. monocytogenes* (17, 18).

Recently, the concept of sustained sensing has been proposed for second messengers that control a biological process by binding multiple targets (19). The control of potassium and compatible solute transport nicely fits this concept. In addition to these processes, c-di-AMP controls the entry to the citric acid cycle by binding to the pyruvate carboxylase of *L. monocy-*

This work was supported by Deutsche Forschungsgemeinschaft via Priority Program Grant SPP1879 (to J. S.) and by National Institutes of Health Grant AI133670 (to V. T. L.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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[2] The abbreviations used are: c-di-AMP, cyclic di-AMP; CBS, cystathionine β-synthase; DRaCALA, differential radial capillary action of ligand assay; RCK, regulating conductance of K^+; IPTG, isopropyl β-D-thiogalactopyranoside.

togenes and *L. lactis* (9, 20). Finally, DarA, a PII-like protein of unknown function binds c-di-AMP in *B. subtilis*, *S. aureus*, and *L. monocytogenes* (8, 9, 21), and CbpB, an unknown protein consisting of two CBS domains, were identified as c-di-AMP–binding protein in *L. monocytogenes* (9).

In *B. subtilis*, c-di-AMP is essential for potassium homeostasis (4). However, only very few target molecules of the dinucleotide are known for this model organism. In a first screen for c-di-AMP targets in *B. subtilis*, only one protein could be identified: the PII-like signal transduction protein DarA showed high and specific affinity for c-di-AMP, although its function has remained enigmatic (21). The other known c-di-AMP target proteins of *B. subtilis* are the RCK_C domains of the peripheral membrane proteins KtrA and KtrC, which are the regulatory subunits of the KtrAB and KtrCD complexes, respectively (8, 12, 22). For KtrA and KtrC from different organisms, it has been shown that upon binding of c-di-AMP the complexes are inhibited, most likely because of conformational changes within the regulatory subunits (10, 12, 23).

As mentioned above, the control of potassium homeostasis is a key function of c-di-AMP, and this function is crucial for the essentiality of the second messenger. Thus, it is tempting to speculate that c-di-AMP might regulate further potassium transport systems. In addition to the high- and low-affinity potassium uptake systems KtrAB and KtrCD (24), respectively, recently the high-affinity potassium importer KimA was discovered (4, 25). The expression of both high-affinity uptake systems is controlled by a c-di-AMP–responsive riboswitch (4, 15), but it has remained elusive whether KimA is also controlled on the protein level. Much less is known about the potassium export systems of *B. subtilis*. The KhtSTU complex and the YugO proteins are suggested to be potassium exporters (26, 27). In addition, the YjbQ protein is similar to the *S. aureus* potassium exporter CpaA, which was also shown to bind c-di-AMP via its RCK_C domain (28).

In general, RCK_C and CBS domains appear to contain conserved c-di-AMP–binding sites (8, 9, 17, 18, 28). *B. subtilis* encodes five proteins containing an RCK_C domain and 16 proteins with CBS domains (29), many of which are potential potassium and osmolyte transporters, respectively. To get a more comprehensive understanding of c-di-AMP–mediated signaling in this bacterium, we have tested the potential binding of the second messenger to these proteins, as well as to the newly discovered potassium importer KimA. Our results support the idea that c-di-AMP is responsible for the global control of potassium homeostasis, by controlling both its uptake and its export.

Results

Isolation of suppressor mutants that allow growth of a strain lacking c-di-AMP at 20 mM KCl

A *B. subtilis* strain lacking all three diadenylate cyclases is unable to grow at potassium concentrations of 5 mM or higher. However, the acquisition of suppressor mutations at 5 mM potassium that affect the cation exporter NhaK and result in enhanced potassium secretion prevents the toxic effect of potassium (4). However, those suppressor mutants were not viable at a potassium concentration of 20 mM. To identify the growth-limiting problem, we sought to isolate suppressor mutants that tolerate this potassium concentration. For this purpose, we plated the c-di-AMP–free strain GP2222 (4) on MSSM (modified sodium spizizen minimal) medium containing 20 mM KCl. After 4 days at 37 °C, two colonies could be isolated. Both strains (GP2737 and GP2738) were subjected to whole genome sequencing to identify the responsible mutations. Both strains carried point mutations affecting the potassium transporter KimA (Trp-520 replaced by Gly) and KtrCD's regulatory subunit KtrC (Ala-73 replaced by Val). Moreover, both strains carried a point mutation in FlhA, which is part of the flagellar type III export apparatus. Finally, one of the mutants (GP2737) carried a frameshift mutation in *odhA*, which results in loss of 2-oxoglutarate dehydrogenase activity as indicated by the reddish color of the colonies (25).

To study the role of the mutations affecting the potassium uptake systems, we first attempted to delete the *kimA* and *ktrC* genes in the c-di-AMP–free strain GP2222. Although a control experiment (deletion of the *nhaK* gene) was successful, the deletion of *kimA* or *ktrC* was not possible. This observation suggests that the point mutations did not completely prevent the KimA and KtrC activities. The mutation in KtrC affects a highly conserved residue in the RCK_N domain. Similarly, the Trp-520 residue is conserved in the KimA proteins of *B. subtilis*, *L. monocytogenes*, *S. aureus*, and *Mycobacterium tuberculosis*. The conservation of the substituted amino acids suggests that the mutant proteins have reduced activity.

Because no detailed information on the activity of KimA is available, we decided to analyze the activity of the mutant protein in a heterologous complementation assay using *Escherichia coli* LB2003 (30). This strain is deficient in the three major endogenous potassium uptake systems Trk, Kup, and Kdp and is therefore not able to grow at low potassium concentrations without complementation using a gene encoding a potassium transporter. For the IPTG-inducible expression of the WT and mutant KimA variants, we used plasmids pGP2913 and pGP2993, respectively. Accordingly, *E. coli* LB2003 was transformed with these plasmids or the empty vector control pWH844 (31), and growth was assessed in minimal medium supplemented with increasing KCl concentrations (0.001, 0.01, 0.02, 0.04, 0.06, 0.1, 0.2, 0.5, 10, and 50 mM KCl) (Fig. 1). Although 50 mM KCl was required for growth of the strain carrying the empty vector, expression of WT KimA (Ref. 4 and this study), as well as of the KimA variant W520G, allowed growth at much lower KCl concentrations. The determination of the growth rates at different potassium concentrations allowed fitting according to the Monod equation, an equation describing the growth of cultures, which is based on the Michaelis–Menten equation (32). This revealed the maximum specific growth rate μ_{max} (h^{-1}) and the substrate concentration that supports the half-maximal growth rate K_S (mM KCl) of the WT and mutant KimA proteins. The mean K_S values of three independent biological replicates are as follows: WT KimA had a K_S of 0.029 ± 0.0039 mM for potassium and a μ_{max} of 0.68 ± 0.005 h^{-1}. In contrast, the mutant KimA protein had an apparent K_S for potassium of 0.233 ± 0.021 mM and a μ_{max} of 0.84 ± 0.038 h^{-1}. This 8-fold reduction of the apparent affinity for

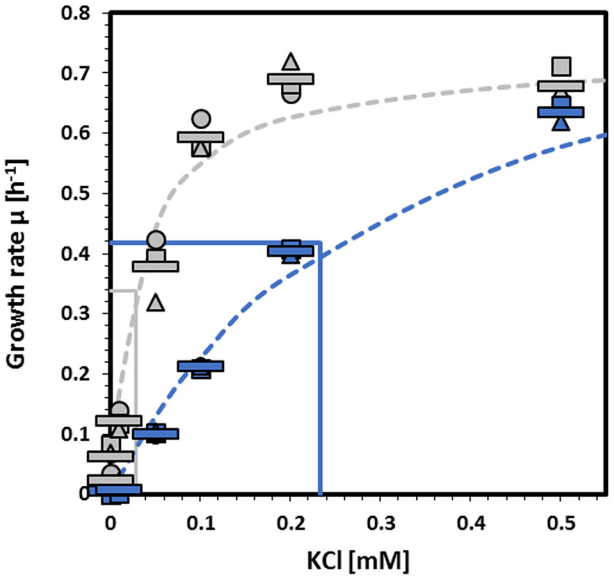

Figure 1. Activity of KimA variant proteins in a heterologous complementation assay. *E. coli* LB2003 was transformed with pGP2913 (WT KimA, *gray*) and pGP2993 (KimA-W520G, *blue*), respectively, and growth at different potassium concentrations was assessed over 24 h. The growth rate μ was determined and plotted against the potassium concentrations. *Dashed lines* show the ideal progression of μ over the different potassium concentrations according to the Monod equation, and *continuous lines* show the determined K_S. *Circles*, *squares*, and *triangles* represent independent biological replicates. The *bars* indicate the means of the replicates.

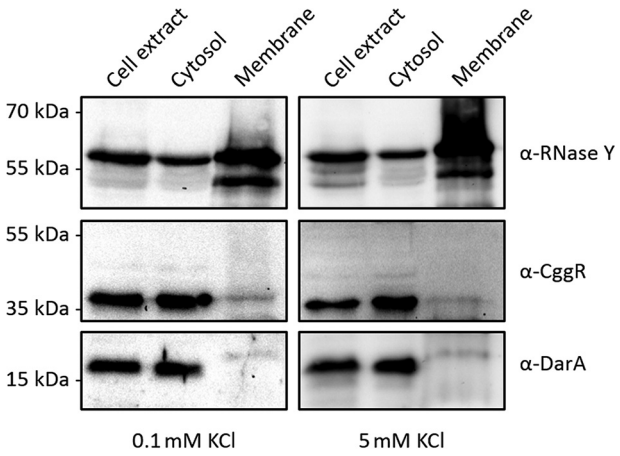

Figure 2. The c-di-AMP–binding protein DarA is a cytoplasmic protein. *B. subtilis* 168 was cultivated in MSSM minimal medium containing 0.1 or 5 mM KCl. Crude extracts were separated by ultracentrifugation to obtain cytosolic and membrane fractions. The presence of DarA was tested using antibodies recognizing DarA. To check for successful separation, the cytosolic and membrane fraction were tested with the specific antibodies recognizing CggR and Rny, respectively.

potassium perfectly fits with the conclusion that the suppressor mutant is impaired in potassium uptake without completely preventing it.

Analysis of a possible interaction between KimA and the PII-like c-di-AMP–binding protein DarA

Often, transporters for molecules that may be toxic upon accumulation are controlled at the levels of expression and of transporter activity to avoid intoxication of the cells. This has been described for the ammonium transporter AmtB, which is only expressed under conditions of ammonium limitation and additionally inhibited by binding of the PII protein GlnK if the ammonium concentrations suddenly increase (33, 34). The expression of the high-affinity potassium transporters KimA and KtrAB is controlled by a c-di-AMP–responsive riboswitch (4, 15). In addition, KtrAB is inhibited upon binding of c-di-AMP, the second messenger that transduces the information on potassium accumulation (4, 8). For the novel potassium transporter KimA, it is not known whether and how the activity of this protein is controlled. Following the concept of sustained sensing, we considered the possibility that the activity of KimA might be controlled by the PII-like c-di-AMP–binding protein DarA (21), because KimA does not contain one of the known c-di-AMP–binding domains. To test this hypothesis, we first analyzed the localization of the DarA protein. The PII protein GlnK is found in the cytoplasm at low ammonium concentrations but is recruited to the membrane via AmtB under conditions of ammonium excess (34). To study whether the localization of DarA depends on potassium availability, we raised antibodies against the protein to facilitate its detection. *B. subtilis* was cultivated in MSSM minimal medium containing 0.1 or 5 mM of KCl, and the proteins were separated into cytoplasmic and membrane-bound fractions. The fractions were then assayed for the presence of DarA. The cytoplasmic protein CggR and the membrane protein RNase Y served as controls (35, 36). Although the localization of the control proteins was observed as expected, we found DarA to be present exclusively in the cytoplasm (Fig. 2). This result suggests that DarA does not bind to the potassium transporter KimA to control its activity. To confirm this observation, we determined the cellular potassium concentrations in the WT strain *B. subtilis* 168 and the isogenic *darA* mutant GP1712 (21) by inductively coupled plasma optical emission spectrometry. Both strains had very similar intracellular potassium concentrations of 5.1 ± 0.4 and 4.8 ± 0.3 μg K^+ ml^{-1} $A_{600}{}^{-1}$, respectively. Taken together, these results strongly suggest that the activity of KimA is not controlled by the c-di-AMP–binding protein DarA.

Identification of c-di-AMP target proteins

Because our localization studies did not support the idea that the activity of KimA is controlled by DarA, we considered the possibility that c-di-AMP might directly bind and control KimA. Additionally, we aimed at identifying further targets that directly bind c-di-AMP in *B. subtilis* to detail our understanding of its regulatory function. For this broader approach, we focused on the five proteins containing RCK_C domains and the 16 proteins containing CBS domains encoded in *B. subtilis* (29) (Table 1). Interestingly, many of these proteins are implicated in ion or compatible solute transport.

We used *E. coli* cells as expression system because c-di-AMP is not synthesized by this bacterium. The 22 selected genes were cloned into the expression vector pWH844 (31). After checking expression of all genes, using strain *E. coli* BL21 as a host, the lysates of strains carrying the corresponding plasmids were assessed for a possible interaction with c-di-AMP *in vitro* using the differential radial capillary action of ligand assay (DRaCALA) (37) (see "Experimental procedures").

In an initial screen for c-di-AMP binding, eight target proteins could be identified (Fig. 3, *A* and *B*). Among these pro-

teins, the known c-di-AMP–binding protein KtrA served as positive control (8, 12). In addition to KtrA, three other proteins containing an RCK_C domain were identified as c-di-AMP–binding proteins. KtrC, the regulatory subunit of the low-affinity potassium transporter KtrCD, the cytoplasmic subunit KhtT of the K^+/H^+ antiporter KhtSTU and YjbQ, a putative cation exporter, showed strong binding to c-di-AMP. Only YrvC, a protein of unknown function, showed nonsignificant binding. Of the proteins containing a CBS domain, the Mg^{2+} transporter MgtE, the ATP-binding protein OpuCA of the compatible solute transporter OpuC and YkuL, a protein of unknown function, showed increased binding affinity toward the di-nucleotide (Fig. 3*A*). Importantly, the novel potassium transporter KimA, which possesses none of the conserved binding motifs, also bound c-di-AMP (Fig. 3*B*). Strikingly, among the c-di-AMP–binding proteins identified in this screen, YkuL is the only c-di-AMP receptor protein that is not associated to the cell membrane. Binding of OpuCA and YkuL to c-di-AMP is in excellent agreement with previously published results (9, 17, 18). Because YkuL is composed of two reiterated CBS domains and thus seems to have a regulatory function, we refer to this protein as DarB (c-di-AMP receptor protein B, in analogy to DarA) (21). The specificity of c-di-AMP binding to the identified proteins was confirmed by competition experiments with cold ATP or c-di-AMP from overnight grown cultures (Fig. 3, *C* and *D*). For all tested proteins, ATP was unable to block c-di-AMP binding, whereas cold c-di-AMP outcompeted the radioactively labeled c-di-AMP. This demonstrates the specificity of c-di-AMP binding and indicates that these proteins are true targets of the second messenger.

In total, we identified six novel c-di-AMP receptor proteins in *B. subtilis*. These novel target proteins are involved in potas-

Table 1
Expression plasmids used in the DRaCALA assay

Plasmid	Protein	Function[a]	Domain
pGP2594	KtrA	Peripheral membrane component K^+ transporter	RCK_C
pGP2906	KhtT	K^+/H^+ antiporter	RCK_C
pGP2907	KtrC	Peripheral membrane component K^+ transporter	RCK_C
pGP2908	YrvC	Unknown	RCK_C
pGP2908	YjbQ/CpaA	Unknown	RCK_C
pGP2913	KimA	K^+ transporter	[b]
pGP2922	AcuB	Unknown	CBS
pGP2923	CcpN	Transcriptional repressor	CBS
pGP2924	OpuAA	Glycine betaine ABC transporter (ATP-binding protein)	CBS
pGP2927	MgtE	Primary Mg^{2+} transporter	CBS
pGP2928	YhdT	Unknown	CBS
pGP2929	YkuL/DarB	Unknown	CBS
pGP2930	YhcV	Unknown, forespore-specific sporulation protein	CBS
pGP2931	YhdP	Potential Mg^{2+} efflux pump	CBS
pGP2932	YrkA	Unknown	CBS
pGP2933	YtoI	Unknown, similar to transcriptional regulator (GntR family)	CBS
pGP2934	YlbB	Unknown, putative oxidoreductase	CBS
pGP2935	YqhB	General stress protein	CBS
pGP2936	YugS	Unknown	CBS
pGP2937	GuaB	Biosynthesis of GMP	CBS
pGP2938	OpuBA	Choline ABC transporter (ATP-binding protein)	CBS
pGP2939	OpuCA	Glycine betaine/carnitine/choline ABC transporter (ABC-binding protein)	CBS

[a] Protein functions have been retrieved from the SubtiWiki database (29).
[b] For KimA, no conserved c-di-AMP–binding domain has been identified so far.

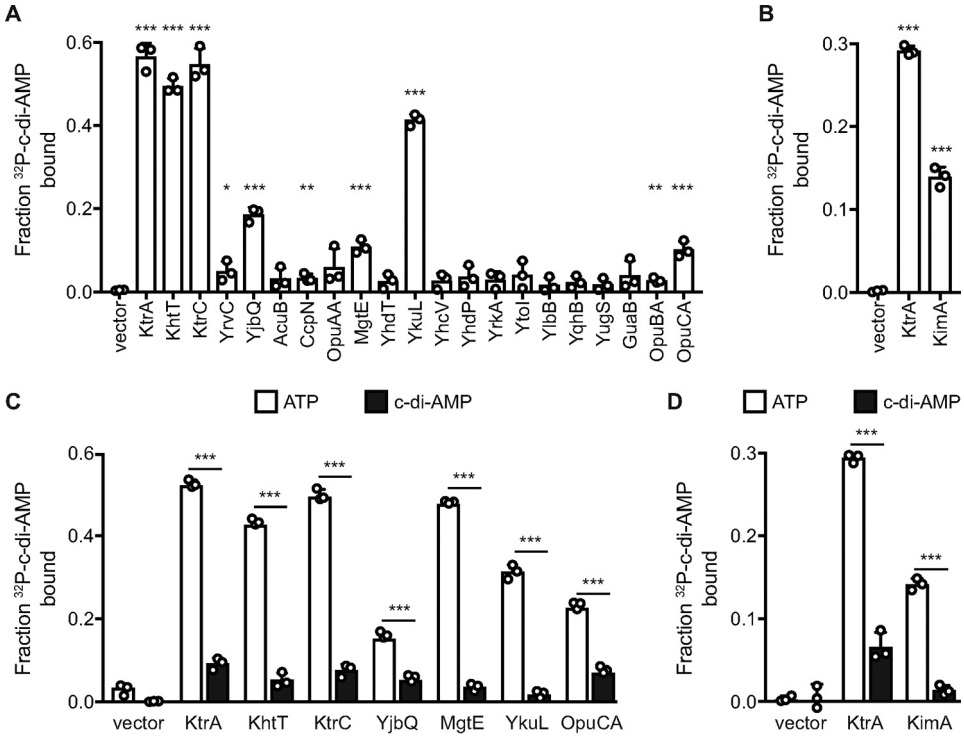

Figure 3. A subset of c-di-AMP–binding proteins overexpressed in *E. coli* whole cell lysates determined by DRaCALA. *A* and *B*, fraction bound of radiolabeled [^{32}P]c-di-AMP is shown for lysates from *E. coli* induced for the expression of the indicated genes in presence of nonspecific ATP competitor at 100 μM (*A*) or 200 μM (*B*). *C* and *D*, induced lysates overexpressing the indicated gene are tested for specificity of c-di-AMP binding to [^{32}P]c-di-AMP by competition assays. *C*, competitors are 100 μM ATP (*open bars*) or 100 μM ATP and 100 μM c-di-AMP (*closed bars*). *D*, competitors are 200 μM ATP (*open bars*) or 200 μM ATP and 100 μM c-di-AMP (*closed bars*). Significance of binding was determined using unpaired *t* test between vector-only control and indicated gene (*A* and *B*) or between ATP and c-di-AMP competitors (*C* and *D*). *p* values of <0.05, <0.01, and <0.001 are indicated by *, **, and ***, respectively.

sium uptake and export and also in magnesium and osmolyte uptake. The fact that c-di-AMP does not bind specifically to proteins involved in one homeostatic process suggests that control of potassium homeostasis is not the only function of the essential second messenger (5).

c-di-AMP inhibits potassium transport by KimA

The specific interaction of KimA with c-di-AMP suggested a functional role for c-di-AMP in the control of KimA activity. Therefore, we analyzed the impact of this second messenger nucleotide on the potassium transport activity of KimA by a growth complementation assay. For this purpose, a co-expression system producing the c-di-AMP–synthesizing diadenylate cyclase CdaA and the potassium transporter KimA was established in the potassium transporter-deficient mutant *E. coli* LB2003 (30). Importantly, *E. coli* lacks c-di-AMP–synthesizing enzymes and is unable to produce this second messenger (38, 39, 40). The growth phenotype upon co-expression of *cdaA* from *L. monocytogenes*, ($CdaA^{Lmo}$) and *kimA* genes reflects the effect of c-di-AMP binding on KimA. The co-expression of KimA with an inactive diadenylate cyclase, $CdaA^{Lmo*}$(D171N) (38), served as a negative control. Similarly, the growth phenotype of LB2003 expressing *kimA* and *cdaA* alone were analyzed.

Growth curves of LB2003 transformed with the respective plasmids and induced with 0.002% arabinose were recorded at 0.1, 0.2, 0.5, 1, 3, 10, and 30 mM KCl in phosphate-buffered minimal medium for 24 h (Fig. 4). LB2003 harboring pBP370 ($CdaA^{Lmo}$) and pBP373 ($CdaA^{Lmo*}$) (13) only grew at 30 mM KCl, whereas LB2003 producing KimA (plasmid pB24C3H-KimA) showed growth complementation at all tested potassium concentrations. KimA thus facilitates the uptake of potassium, whereas the expression of *cdaA* has no general inhibitory effect, which is in agreement with previous observations (4, 13). A very similar growth behavior to LB2003 expressing *kimA* was observed upon co-production of KimA (pB24C3H-KimA) and the inactive $CdaA^{Lmo*}$ (pBP373). However, the co-expression KimA (pB24C3H-KimA) with active $CdaA^{Lmo}$ (pBP370) abolished cell growth below 30 mM KCl. Thus, we can conclude that the binding of c-di-AMP produced by $CdaA^{Lmo}$ to KimA inhibits its potassium transport activity.

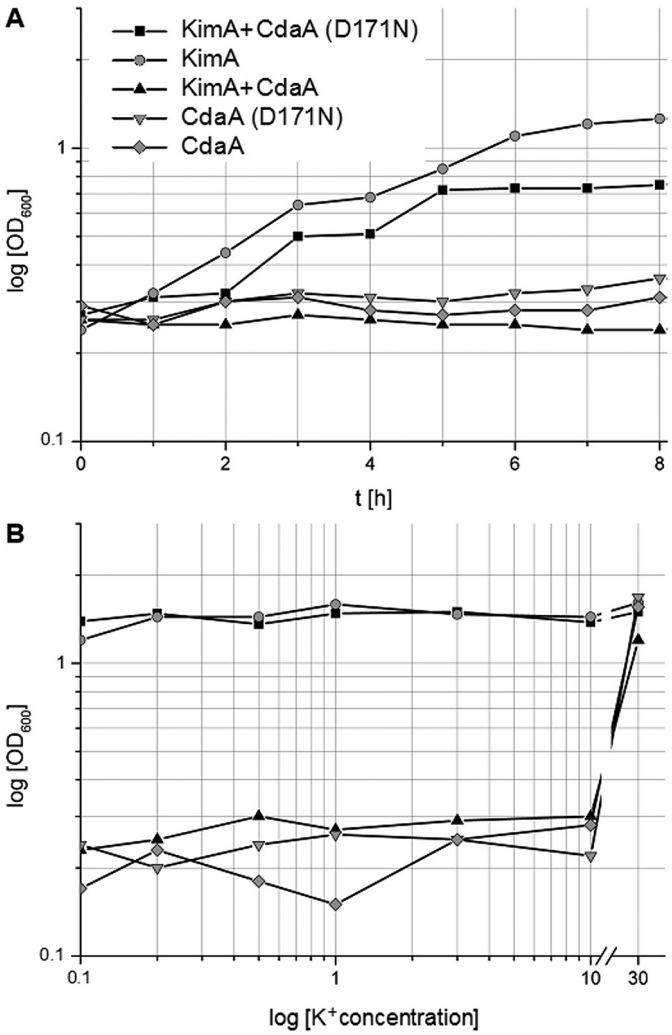

Figure 4. CdaA-dependent inhibition of KimA-mediated growth. *A*, growth curves of *E. coli* LB2003 expressing KimA and/or variants of CdaA in K minimal medium containing 3 mM KCl. *B*, A_{600} of *E. coli* LB2003 expressing KimA and/or variants of CdaA grown for 24 h in minimal medium with potassium concentrations ranging from 0.1 to 30 mM.

Discussion

The second messenger c-di-AMP is essential in many bacteria, including the Gram-positive model organism *B. subtilis* and the closely related pathogenic bacteria *L. monocytogenes* and *S. aureus*. Previous pulldown experiments revealed several target proteins for the two pathogenic bacteria (8, 9), but for *B. subtilis* the unknown c-di-AMP receptor DarA and the potassium channel subunits KtrA and KtrC were the only target proteins that had been identified (12, 21). None of the identified c-di-AMP target proteins was essential for cell viability, and therefore the essential function of the second messenger has long remained elusive (41).

In this study, we tested c-di-AMP binding for the recently discovered potassium transporter KimA, as well as for proteins containing specific domains, either the RCK_C or CBS domain. These protein domains have been shown to bind directly to c-di-AMP (9). Of the 22 tested proteins, we identified six novel c-di-AMP targets, *i.e.* the potassium transporter KimA, the RCK_C subunit (KhtT) of a potassium/proton antiporter KhtSTU (27), the primary magnesium importer MgtE (42), the glycine-betaine transporter subunit OpuCA (43), and the two proteins of unknown function DarB and YjbQ. Binding of c-di-AMP to DarB, OpuCA, and YjbQ homologs has been reported previously for *L. monocytogenes* (9), whereas KimA, KhtT, and MgtE have not been previously identified as c-di-AMP targets. The common denominator between KimA, KhtT, YjbQ, MgtE, and OpuCA is that all five proteins are transmembrane proteins. The control of potassium import has been previously linked to the essentiality of c-di-AMP in *B. subtilis* (4). The here-identified c-di-AMP–binding potassium export systems Kht and YjbQ suggest that c-di-AMP plays a more general role in controlling potassium uptake and release. Moreover, it is tempting to speculate that the control of magnesium and osmolyte homeostasis are additional essential functions of c-di-AMP. Indeed, an implication of c-di-AMP signaling in osmoregulation is a common theme in many bacteria (see Ref. 7 for review). In *L. monocytogenes*, the essentiality of the second mes-

senger is also not linked to the control of one specific protein but to the general control of the stringent response factor (p)ppGpp (3).

Importantly, this study identified the potassium transporter KimA as an additional target of c-di-AMP. Because potassium is both essential and toxic for the cells (4, 44), the control of its homeostasis is of utmost importance for the bacteria. A strain lacking c-di-AMP can grow at elevated potassium concentrations only if potassium ions can be more efficiently exported (4) or if the uptake of potassium is reduced by mutations affecting conserved residues in the transporters KtrC and KimA (this work). This observation already suggests that the KimA activity is reduced in the presence of c-di-AMP and that a corresponding reduction must be achieved in the absence of c-di-AMP by the acquisition of a mutation. Because the mechanism of KimA control by c-di-AMP had been unknown prior to this study, we hypothesized that the KimA activity might be controlled by the c-di-AMP– binding PII-like protein DarA, in analogy to the control of the ammonium transporter AmtB by the PII protein GlnK (33, 34). However, our experiments did not support this possibility and suggested that KimA might be subject to direct control by c-di-AMP. This idea was confirmed by the detection of binding of c-di-AMP to KimA and by the observation that this interaction inhibits the potassium uptake activity of KimA.

KimA represents a completely novel group of c-di-AMP–binding proteins that does not contain any of the previously identified domains that bind the second messenger. Moreover, KimA is the prototype of the fourth class of proteins that are involved in the control of potassium homeostasis and that are controlled by c-di-AMP in Gram-positive bacteria. So far, the potassium transporters containing an RCK_C domain have been studied in several bacteria, and we have recently identified the KupA and KupB proteins as novel targets of c-di-AMP in *L. lactis* (13). In all cases, these potassium transporters are inhibited by c-di-AMP. Interestingly, a *L. lactis* strain that accumulates c-di-AMP was extremely sensitive to salt stress, but the acquisition of a mutation in KupB restored growth. In this case, the variant KupB protein had an increased activity to compensate for the strong inhibition upon c-di-AMP accumulation (16). Thus, accumulation of c-di-AMP had just the opposite effect for the mutational adaptation of the potassium transporter as observed here for lack of c-di-AMP. KimA adds a third protein family to the list of c-di-AMP inhibited potassium transporters.

Interestingly, c-di-AMP does not only bind to proteins involved in potassium uptake but also to a riboswitch that controls the expression of the high-affinity KtrAB and KimA transporters in *B. subtilis*. Binding of c-di-AMP to the riboswitch prevents expression beyond the riboswitch and does thus inhibit expression of the transporter genes (4, 15). Moreover, c-di-AMP binds to the sensor kinase KdpD, which is required for the expression of the high-affinity potassium transport system KdpFABC in *S. aureus*. Again, binding of c-di-AMP inhibits the activity of the protein and does thus prevent expression of the transporter (14). In conclusion, c-di-AMP governs potassium uptake by direct binding to and inhibition of potassium uptake systems and by controlling the expression of the high-affinity transporters.

In addition to KimA, we have identified the KhtT and YjbQ proteins as novel targets of c-di-AMP. Although KhtT has been demonstrated to be a subunit of a potassium/proton antiporter in *B. subtilis* (27), less is known on YjbQ. The corresponding *S. aureus* protein CpaA (cation/proton antiporter A) has also been shown to bind c-di-AMP (8). This protein is a potassium exporter, and interestingly, its activity is stimulated by c-di-AMP (28). Based on the 52% amino acid identity between the proteins from both organisms and on the common regulation by c-di-AMP, we rename *B. subtilis* YjbQ to CpaA. It is interesting to mention that in screens for c-di-AMP–free strains that are viable at increased potassium concentrations, we never found mutations that affected the potassium exporters KhtSTU or CpaA. In contrast, variants of the NhaK cation exporter with increased specificity for potassium were selected at 5 mM potassium (4), whereas mutations resulting in reduced potassium uptake were found at 20 mM potassium (this work). These findings support the idea that the potassium exporters are inactive unless they bind and become activated by c-di-AMP. Obviously, mutations that overcome this requirement are rather unlikely.

Taken together, the results from our suppressor screens, the biochemical investigation of *S. aureus* CpaA, as well as the physiological logics all converge to support the idea that c-di-AMP inhibits potassium uptake at the levels of transporter expression and activity and stimulates potassium export by activating the potassium/proton antiporters (Fig. 5). This extends the idea that the control of potassium homeostasis by controlling potassium uptake is an essential function of c-di-AMP to the export of this cation. The binding of multiple potassium transporters, as well as the dual control of both potassium transporter expression and activity as observed for KtrAB and KimA (Refs. 4, 8, 12, and 15, and this work), makes c-di-AMP–mediated signaling on potassium homeostasis a paradigm for the concept of sustained sensing (19). It is amazing that different classes of (functionally different) proteins and even of a riboswitch have evolved to be controlled by a single second messenger, c-di-AMP. Thus, c-di-AMP is the major effector of potassium uptake in the firmicutes. Further work will be required to understand the roles of the distinct potassium export systems and to unravel the functions of the so far uncharacterized c-di-AMP– binding proteins, DarA and DarB.

Experimental procedures

Strains, media, and growth conditions

E. coli DH5α (45) was used for cloning and for the expression of recombinant proteins. *E. coli* LB2003 (30) was used to assay potassium transporter activity. All *B. subtilis* strains used in this study are derivatives of the laboratory strain 168. *B. subtilis* was grown in LB or in sporulation medium (45, 46). *E. coli* was cultivated in MSSM medium (4) or in modified M9 medium in which KH_2PO_4 was replaced by NaH_2PO_4 and 50 mM KCl was added. The media were supplemented with ampicillin (100 μg/ml), kanamycin (10 and 50 μg/ml for *B. subtilis* and *E. coli*, respectively), chloramphenicol (5 μg/ml), tetracyclin (12.5 μg/ml), spectinomycin (150 μg/ml), or erythromycin and lincomycin (2 and 25 μg/ml, respectively) if required.

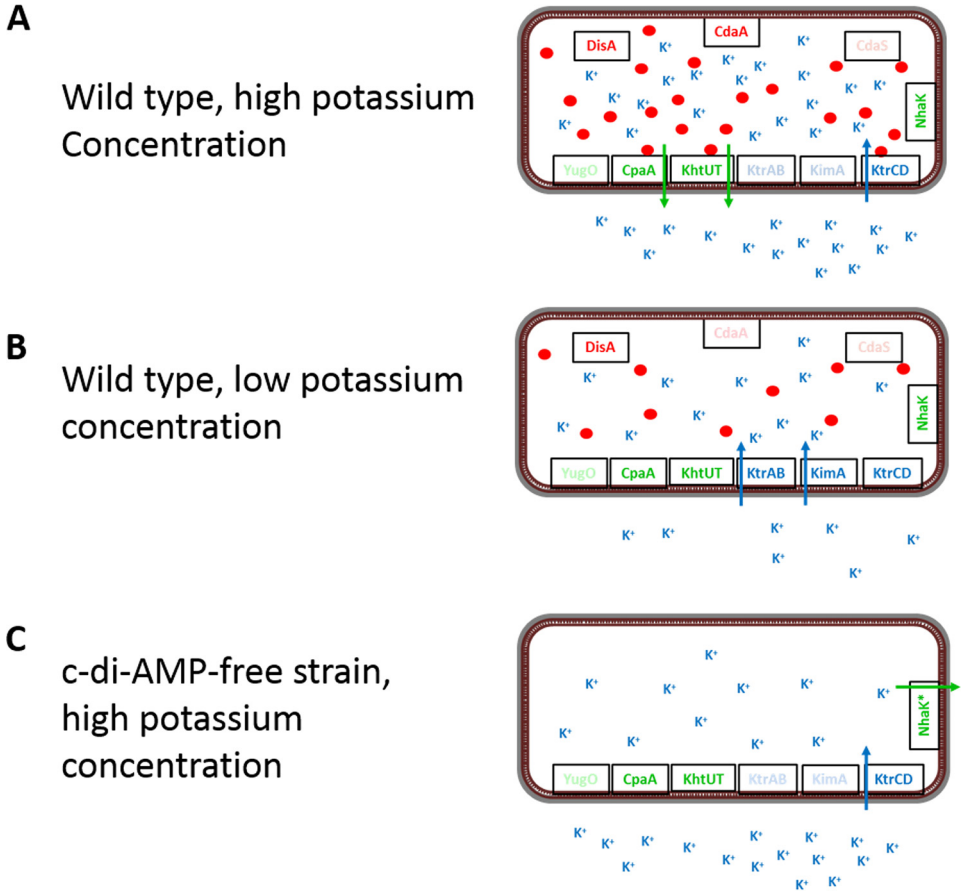

Figure 5. Control of potassium uptake and export in *B. subtilis* by c-di-AMP. *A*, in the WT strain, the potassium exporters CpaA and KhtUT, as well as the low-affinity transporter KtrCD, are expressed at high potassium concentrations. Potassium is taken up by KtrCD and triggers the production of c-di-AMP by CdaA and DisA. Upon accumulation of potassium and the second messenger, c-di-AMP binds to KtrC to prevent further potassium uptake. Simultaneous binding of c-di-AMP to CpaA and KhtUT triggers potassium export. Thus, the cell prevents potassium intoxication. *B*, at low potassium concentrations, all three uptake systems as well as the two exporters are expressed. Potassium is transported by the high-affinity transporters KtrAB and KimA. The low intracellular potassium concentration results only in a very limited synthesis of c-di-AMP that is not sufficient to inhibit or activate potassium uptake or export, respectively. *C*, a strain lacking c-di-AMP is unable to grow at high potassium concentrations because of the unlimited influx of the ion via KtrCD. The potassium exporters are inactive in the absence of the second messenger. Therefore, growth of this strain in the presence of potassium requires a novel active potassium exporter. Under these conditions, the bacteria acquire mutations affecting the NhaK cation/proton antiporter that result in increased potassium export (4).

DNA manipulation and genome sequencing

Transformation of *E. coli* and plasmid DNA extraction were performed using standard procedures (45). All commercially available plasmids, restriction enzymes, T4 DNA ligase, and DNA polymerases were used as recommended by the manufacturers. Chromosomal DNA of *B. subtilis* was isolated as described (46). *B. subtilis* was transformed with plasmid and genomic DNA according to the two-step protocol (46).

To identify the mutations in the suppressor mutant strains GP2737 and GP2738, the genomic DNA was subjected to whole-genome sequencing (47). Briefly, the reads were mapped on the reference genome of *B. subtilis* 168 (GenBank™ accession number NC_000964) (48). Mapping of the reads was performed using the Geneious software package (Biomatters Ltd.) (49). SNPs were considered as significant when the total coverage depth exceeded 25 reads with a variant frequency of ≥90%. All identified mutations were verified by PCR amplification and Sanger sequencing.

Plasmid constructions

The selected genes were amplified using chromosomal DNA of *B. subtilis* 168 as the template and appropriate nucleotides that attached BamHI (or BglII) and SalI restriction sites to the fragments and cloned between the BamHI and SalI sites of the expression vector pWH844 (31) (Table 1). The mutant *kimA* allele encoding KimA (W520G) was amplified using the oligonucleotides JN465 and JN466 (4) and cloned between the BamHI and PstI sites of pWH844. The resulting plasmid was pGP2993. The construct of KimA used in the co-expression of KimA and diadenylate cyclases was cloned by restriction-free cloning into a modified pBAD24 vector that bears a His tag in the C terminus, resulting in the plasmid pB24C3H-KimA.

Identification of c-di-AMP–binding proteins by DRaCALA

Expression of the genes upon induction using 1 mM of IPTG was verified by analyzing the protein patterns of the expression strains by SDS-PAGE. ^{32}P-Labeled c-di-AMP synthesis was performed using purified diadenylate cyclase DisA (8). The analysis of protein–ligand interaction was performed using

E. coli whole cell lysates that were grown to an A_{600} of 0.5–1.0 and induced for 4 h by 1 mM IPTG as described (37, 50) or grown overnight in the presence of 50 μg/ml carbenicillin and 1 mM IPTG. All binding reactions were performed in 1× binding buffer (10 mM Tris, pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$) containing ~10 pM [^{32}P]c-di-AMP. Protein–ligand mixtures were spotted on nitrocellulose membrane (Amersham Biosciences Hybond-ECL; GE Healthcare) and allowed to dry. The areas and intensities of spots were quantified by exposing phosphorus imaging screens and scanning by FUJI FLA-7000 phosphorus imager. The competition assays were performed with indicated concentrations of unlabeled ATP and c-di-AMP (Axxora).

Preparation of membrane fractions

Cultures of *B. subtilis* were harvested by centrifugation (4,400 × *g*, 10 min, 4 °C). The following steps were done as described previously (51). Briefly, the cells were lysed by sonication, the cellular debris was removed, and the fractions of the cell extract were separated by ultracentrifugation. The membrane pellet was washed for three times and finally resuspended in phosphate buffer (50 mM Na_2HPO_4, 50 mM NaH_2PO_4, pH 6.8). To assess the quality of the preparations, the fractions were analyzed for the presence of CggR and RNase Y using polyclonal rabbit antibodies raised against these proteins (35, 52).

Western blotting analysis

The DarA protein was purified using *E. coli* DH5α carrying the expression vector pGP2601 (21) as described previously (21). Purified His_6-DarA was used to generate rabbit polyclonal antibodies. For Western blotting analysis, *B. subtilis* cell extracts were separated on 12.5% SDS-polyacrylamide gels. After electrophoresis, the proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad) by electroblotting. The primary antibodies were visualized by using anti-rabbit IgG-AP secondary antibodies (Promega) and the CDP* detection system (Roche Diagnostics) as described previously (53).

Determination of cellular potassium pools

The cellular potassium pools were determined as described previously (25). Briefly, *B. subtilis* cells were cultivated in MSSM medium supplemented with 0.1 mM KCl. Cells were pelleted, transferred onto ash-free filter discs, and dried. The dried filter disks were cut into small pieces and reduced into a fluid state through pressure and 2 ml of 65% HNO_3 for 7 h at 185 °C in 25-ml Teflon beakers (PDS-6 Pressure Digestion System; Loftfield, Göttingen, Germany). After digestion, the fluid content was transferred into an Erlenmeyer flask and diluted with demineralized water to a volume of 50 ml. The total potassium content of the cells in this solution was determined by inductively coupled plasma optical emission spectrometry analysis (Optima 5300 DV; PerkinElmer Life Sciences). Light emission at 766.49 nm that is indicative of the potassium concentration in the sample was recorded.

Determination of specific growth parameters

The growth characteristics of *E. coli* LB2003 complemented with plasmid-based KimA, KimA-W520G or empty vector were determined as follows. Potassium was used as the growth-limiting factor. The bacteria were inoculated in LB medium containing 50 mM KCl and precultured in MSSM medium, supplemented with thiamin (1 mg/ml) and 50 mM KCl. The cultures were grown until exponential phase and harvested, and the cells were incubated for 1 h in potassium-free medium and washed three times in 1× MSSM buffer. Afterward, the cells were adjusted to A_{600} 1.0 and used to inoculate a 96-well plate (Microtest plate 96-well) containing MSSM medium adjusted to the required potassium concentrations and 50 μM IPTG to induce expression of *kimA*. Growth was tracked in an Epoch 2 microplate spectrophotometer (BioTek Instruments) at 37 °C with linear shaking at 237 cpm (4 mm) for 20 h, and optical density at 600 nm was measured in 10-min intervals. The exponential growth phase was used to determine the growth rate μ (h^{-1}). The growth rates were then plotted against the potassium concentrations. This allowed fitting to the Michaelis–Menten equation and calculation of v_{max} (h^{-1}) and the apparent K_m (mM KCl) using the solver tool of Excel 2012 (Microsoft). The experiments were repeated with three biological replicates.

Co-expression of KimA and diadenylate cyclases under potassium limitation

E. coli LB2003 was transformed with plasmids pB24C3H-KimA, pBP370, and pBP373 that encode KimA, CdaA, and the inactive mutant of CdaA D171N, alone and in combination. The cells were cultivated in K medium (34 mM Na_2HPO_4, 17 mM NaH_2PO_4, 1 mM trisodium-citrate, 7.6 mM $(NH_4)_2SO_4$) supplemented with 20 μg/ml L-methionine, 6 μM Fe(II)SO_4, 1 μg/ml thiamine, 0.4 μM Mg_2SO_4, 0.2% glycerol, 30 mM potassium (K30, 12 mM K_2HPO_4, 6 mM KH_2PO_4,), and the corresponding antibiotics. An overnight culture was harvested and washed two times in K medium (46 mM Na_2HPO_4, 23 mM NaH_2PO_4, 1 mM trisodium-citrate, 7.6 mM $(NH_4)_2SO_4$) supplemented with 10 mM KCl. These samples were then used to inoculate fresh K medium with the same supplements plus 0.002% L-arabinose to induce protein expression and different concentrations of KCl varying from 0.1 to 30 mM. The cultures were incubated at 37 °C with continuous orbital shaking, and A_{600} measurements were taken every hour.

Author contributions—J. G., R. D., I. H., V. T. L., and J. S. supervision; J. G., I. H., and V. T. L. validation; J. G., L. K., C. H., A. T., A. P., I. T., M. W., and D. H. investigation; L. K., C. H., A. T., A. P., I. T., M. W., and D. H. methodology; A. T., I. H., and V. T. L. visualization; I. H., V. T. L., and J. S. conceptualization; I. H. and J. S. writing-original draft; V. T. L. and J. S. funding acquisition; J. S. project administration.

Acknowledgments—We thank Suzanna Grubek for the help with the cloning experiments. We are grateful to Johannes Gibhardt and Fabian Commichau for helpful discussions.

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