MICROBIOLOGY

Control of potassium homeostasis is an essential function of the second messenger cyclic di-AMP in *Bacillus subtilis*

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The second messenger cyclic di–adenosine monophosphate (c-di-AMP) is essential in the Gram-positive model organism *Bacillus subtilis* and in related pathogenic bacteria. It controls the activity of the conserved *ydaO* riboswitch and of several proteins involved in potassium (K^+) uptake. We found that the YdaO protein was conserved among several different bacteria and provide evidence that YdaO functions as a K^+ transporter. Thus, we renamed the gene and protein KimA (K^+ importer A). Reporter activity assays indicated that expression beyond the c-di-AMP–responsive riboswitch of the *kimA* upstream regulatory region occurred only in bacteria grown in medium containing low K^+ concentrations. Furthermore, mass spectrometry analysis indicated that c-di-AMP accumulated in bacteria grown in the presence of high K^+ concentrations but not in low concentrations. A bacterial strain lacking all genes encoding c-di-AMP–synthesizing enzymes was viable when grown in medium containing low K^+ concentrations, but not at higher K^+ concentrations unless it acquired suppressor mutations in the gene encoding the cation exporter NhaK. Thus, our results indicated that the control of potassium homeostasis is an essential function of c-di-AMP.

INTRODUCTION

Gram-positive bacteria synthesize the signaling nucleotide cyclic di–adenosine monophosphate (c-di-AMP). In the Gram-positive model organism *Bacillus subtilis* and in many other firmicutes, c-di-AMP is both essential (bacteria lacking the ability to synthesize this messenger die) and toxic if degradation is blocked (*1*–*3*). As a second messenger, c-di-AMP controls K^+ transport by binding to transport and regulatory proteins and to a regulatory RNA called a riboswitch (*2*). In firmicutes, c-di-AMP binds to the peripheral membrane proteins KtrA and KtrC, which are subunits of two distinct K^+ transporters, and inhibits their activity and, thus, K^+ uptake (*4*, *5*). In *Staphylococcus aureus*, c-di-AMP inhibits the sensor kinase KdpD and, thus, the expression of the genes encoding the high-affinity K^+ transporter KdpABC (*4*, *6*). In *B. subtilis*, c-di-AMP binds to a regulatory RNA molecule, the *ydaO* riboswitch (*7*, *8*). Binding of c-di-AMP prevents transcription elongation of the target genes beyond the riboswitch (*8*). The *B. subtilis* genome encodes two copies of the riboswitch, one upstream of *ydaO*, which encodes a putative membrane protein of unknown function, and a second copy in front of the *ktrAB* operon, which encodes the high-affinity K^+ transporter KtrAB. The physiological cues to which this riboswitch—and thus the signals that promote the synthesis and accumulation of c-di-AMP—responds have not yet been elucidated.

We studied the impact of c-di-AMP on K^+ uptake in *B. subtilis*. Two K^+ uptake systems, KtrAB and KtrCD, have been identified in *B. subtilis* (*9*). Our results revealed that expression of the product of *ydaO* is regulated by c-di-AMP and encodes a high-affinity K^+ transporter, which we renamed KimA (K^+ importer A). Furthermore, we identified K^+ as the physiological trigger that controls c-di-AMP–dependent regulation at the *kimA* riboswitch and found that the intracellular c-di-AMP amounts reflect K^+ availability. At low K^+ concentrations, c-di-AMP was nonessential, but strains lacking c-di-AMP tolerated high K^+ concentrations only if they acquired suppressor mutations in the *nhaK* gene, which encodes a transporter that facilitates K^+ export. Thus, the results indicated that the essential function of c-di-AMP in *B. subtilis* is control of K^+ homeostasis.

RESULTS

KimA is a potassium transporter

B. subtilis (strain JH642) has two paralogous potassium transporters with a ~10-fold difference in affinity: KtrAB (the high-affinity transporter) and KtrCD (the low-affinity transporter) (*9*). The strain JH642 carries an 18-kb deletion encompassing the *ydaO* (*kimA*) gene, which is controlled by the c-di-AMP–responsive riboswitch (*8*, *10*). To test the relative importance of KtrAB and KtrCD in *B. subtilis* (strain 168; for a list of the bacterial strains used in this study, see table S1), we deleted the corresponding genes (*ktrAB*, *ktrC*, and *ktrD*) in various combinations and assayed growth of the resulting mutant strains at low (0.5 mM) or high (50 mM) K^+ concentrations. As previously shown and in contrast to *B. subtilis* GHB15 (based on JH642) (Fig. 1A), the *B. subtilis* 168 mutant strain lacking both Ktr systems (strain GP2169) grew at the low K^+ concentration (Fig. 1A). This result indicated the existence of an additional potassium transporter in *B. subtilis* 168.

This phenotypic analysis of the K^+-sensitive growth of *ktr* mutants in the two *B. subtilis* genetic backgrounds, as well as the regulation of *ydaO* (renamed *kimA* from here on) by the c-di-AMP–controlled riboswitch (*8*), suggested that *kimA* might encode a potassium transporter. To test this hypothesis, we constructed a *B. subtilis* 168 *kimA* deletion mutant and combined this mutation with deletions of the *ktrAB* and *ktrC* or *ktrD* genes (Fig. 1A). Deletion of either *ktrC* or

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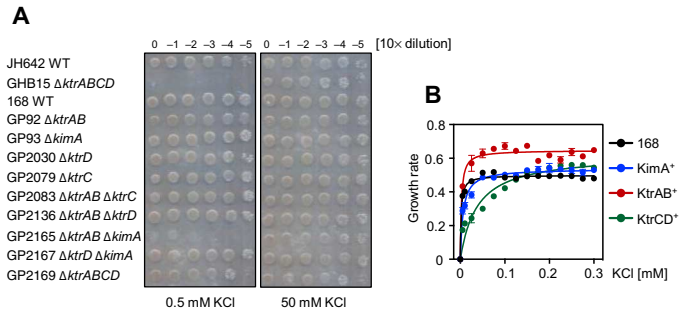

Fig. 1. Growth of various combinations of transporter mutants and the *kimA* mutant in the presence of low and high K^+. (**A**) Growth of wild-type (WT) *B. subtilis* strains JH642 and 168 and the mutants deficient in KtrAB, KtrCD, and KimA (for *B. subtilis* 168) transporter systems. Data are representative of five experiments. (**B**) Dependence of growth rate on external K^+ concentration for *B. subtilis* WT cells (168) and mutant strains containing only KtrAB (GP2169), KtrCD (GP2165), or KimA (GP2169). Saturation curves were fitted according to the Michaelis-Menten equation (n = 3 experiments).

ktrD disrupts function of the transporter KtrCD (*9*), and thus, these individual mutant strains are deficient in KtrCD activity. Mutants that lacked only one of the known [Δ*ktrAB* (GP92), Δ*ktrC* (GP2079), or Δ*ktrD* (GP2030)] or putative [Δ*kimA* (GP93)] potassium transporters grew in the presence of 0.5 mM KCl. The mutant in which only the KtrAB system was active [Δ*ktrD* Δ*kimA* (GP2167)] also grew on low K^+ medium. In contrast, the Δ*ktrAB* Δ*kimA* double mutant (GP2165) expressing only the low-affinity KtrCD transporter exhibited impaired growth on low K^+ medium. All mutants grew on medium containing 50 mM KCl (Fig. 1A).

By growing *B. subtilis* strains expressing only one of the transporters KtrAB or KtrCD or the putative transporter KimA in the presence of various KCl concentrations, we found that each strain required a different concentration of external K^+ to reach half-maximal growth: The KtrAB-only strain (GP2167) required 2.4 ± 0.4 μM, the KtrCD-only strain (GP2165) required 38 ± 4 μM, and the KimA-only strain (GP2169) required 6.0 ± 0.5 μM (Fig. 1B). These differences in growth rate on external K^+ are consistent with the affinities of KtrAB [Michaelis constant (K_M), 1 mM] and KtrCD (K_M, 10 mM) (*9*) and suggested that KimA is a high-affinity K^+ transporter. We confirmed that KimA supported growth on low K^+ medium when introduced into an *Escherichia coli* (LB650) lacking all known K^+ transport systems (fig. S1A). The dependence of growth rate on external KCl concentration was similar for *E. coli* complemented with KtrAB or KimA (fig. S1B). Together, these results demonstrate that KimA can support growth under low K^+ conditions in the absence of other known K^+ transporters, consistent with KimA functioning as a K^+ uptake transporter.

We searched for homologs of KimA in the nonredundant protein database using BLAST (Basic Local Alignment Search Tool) (*11*) and found that KimA is widespread among diverse bacterial taxa, including firmicutes, actinobacteria, cyanobacteria, and proteobacteria (for an alignment, see fig. S2). Sequence analysis showed several features consistent with KimA functioning as a transporter, including the presence of 11 transmembrane helices. Strikingly, residues highly conserved among homologous proteins are mostly found in the transmembrane helices (Fig. 2). Thus, we renamed the *ydaO* gene *kimA* and refer to the encoded potassium transporter protein as KimA.

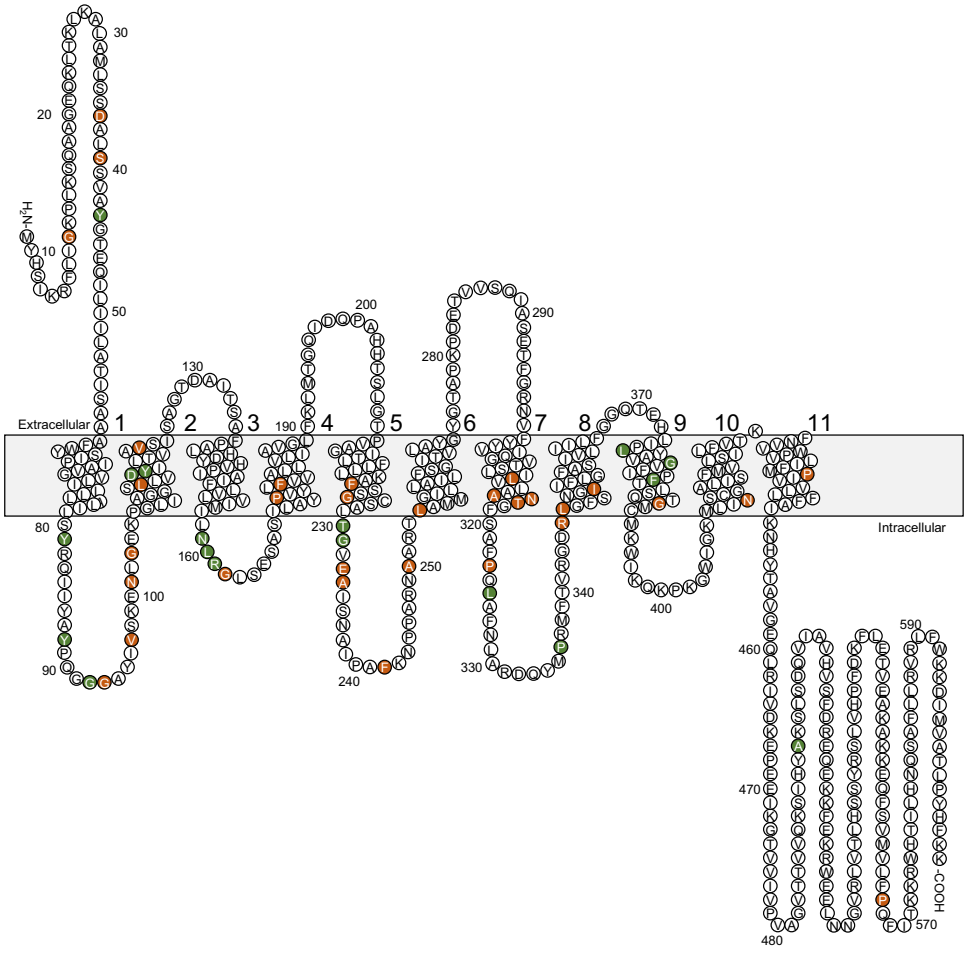

Fig. 2. KimA is the founding member of a widespread family of K^+ transporters. The ProtterBlot shows the organization of the KimA protein. The membrane is shown in light gray. Amino acids colored in orange represent amino acids conserved between *B. subtilis*, *S. aureus*, *S. pneumoniae*, *L. monocytogenes*, *Lactococcus casei*, *Clostridium difficile*, *Streptomyces coelicolor*, *Mycobacterium tuberculosis*, *Chloroflexus aggregans*, *Chlamydia trachomatis*, *Deinococcus gobiensis*, *Gloeobacter kilaueensis*, *Anabaena* sp., *Synechococcus* sp., *Francisella* sp., *Myxococcus xanthus*, and *Rhizobium* sp. Amino acids colored in green are those that are also conserved in the *E. coli* low-affinity putrescine importer PlaP. The protein is shown to scale according to UniProt (accession number P96589) and ProtterBlot (http://wlab.ethz.ch/protter).

kimA expression is controlled by K^+ availability

Because our results indicated that KimA functioned as a high-affinity potassium transporter, we hypothesized that external K^+ may regulate the expression of the encoding gene. We introduced a construct

encoding KimA labeled with a FLAG tag into *B. subtilis* (GP2405) to facilitate immunological detection of the KimA protein. KimA-FLAG was detectable at a low (0.1 mM) but not at a high (5 mM) KCl concentration (Fig. 3A). Dose-response analysis using the *kimA* control region encompassing the promoter and the riboswitch fused to a *lacZ* reporter gene in strain BP144 showed that this regulatory region of *kimA* promoted reporter expression the most at low external KCl concentrations and that expression decreased as the KCl concentration increased (Fig. 3B).

The c-di-AMP–binding riboswitch in the leader region of the *kimA* mRNA refolds upon binding of the second messenger, thus preventing transcription beyond the riboswitch. Accordingly, the *kimA* riboswitch has been classified as a genetic OFF switch (*8*). The K^+-dependent regulation of *kimA* may result from an effect on a transcriptional regulator acting at the promoter, an effect on the riboswitch, or both. To distinguish between these possibilities, we separated the *kimA* promoter sequence from the riboswitch-encoding sequence. We fused the promoter sequence to *lacZ* (GP2182) and placed the riboswitch sequence between a constitutively active artificial promoter (p_a) and the *lacZ* reporter gene (GP2198) (Fig. 3C). This constitutively active promoter fused to the reporter was unaffected by K^+ concentration (Fig. 3D). The *kimA* promoter reporter lacking the riboswitch (GP2182) exhibited high activity at the low K^+ concentration and low activity at the high K^+ concentration (Fig. 3D). The riboswitch reporter strain (GP2198) also exhibited K^+-dependent expression; only at the low external K^+ concentration did transcription proceed past the riboswitch to produce β-galactosidase activity (Fig. 3E).

To assess the role of c-di-AMP in the riboswitch-dependent *kimA* expression, we tested the reporter's activities in bacteria with artificially increased production of c-di-AMP. For this purpose, we introduced the reporter into *B. subtilis* that overexpressed the sporulation-specific diadenylate cyclase CdaS (GP2200) (*12*, *13*). The riboswitch-regulated reporter exhibited K^+-dependent activity in the wild-type strain (GP2198; Fig. 3E). In contrast, in the CdaS-overexpressing strain (GP2200), riboswitch-regulated reporter activity was suppressed at both high and low K^+ concentrations (Fig. 3E).

Because the *kimA* riboswitch is controlled by c-di-AMP (*8*), the physiological regulation of *kimA* expression by K^+ is likely caused by the intracellular concentrations of this second messenger. Therefore, we analyzed the intracellular K^+ and c-di-AMP pools of *B. subtilis* 168 grown at low and high K^+ concentrations (Fig. 4A). The intracellular concentrations of K^+ faithfully reflected the external supply of the ion. However, a 50-fold difference in the external concentrations was buffered to a 5-fold difference for internal K^+ concentrations, indicating that the bacteria can adjust their intracellular K^+ pools to a wide range of external concentrations. Consistent with K^+-dependent regulation of c-di-AMP production, c-di-AMP levels were increased twofold at the high K^+ concentration in the wild-type strain (Fig. 4A). This result suggests that the c-di-AMP concentration may report the availability of potassium. As an initial attempt to obtain insight into the mechanism(s) by which the K^+ availability controls

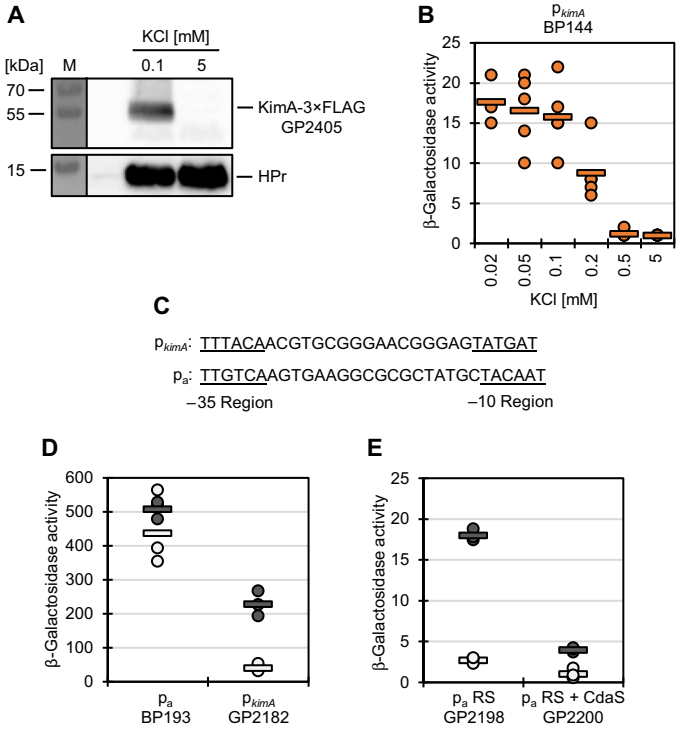

Fig. 3. *KimA* expression and abundance are reduced by high concentrations of external K^+. (**A**) *B. subtilis* expressing FLAG-tagged KimA (GP2405) was cultivated in MSSM medium at low (0.1 mM) and high (5 mM) K^+ concentrations. KimA-FLAG was detected using antibodies against the FLAG tag. The constitutively expressed HPr protein served as a control. (**B**) Control of *kimA* gene expression. *B. subtilis* BP144 harbors the native *kimA* promoter region fused to the reporter gene *lacZ*. Promoter activity was analyzed by quantification of β-galactosidase activity. (**C**) Sequences of the native *kimA* and artificial p_a promoter regions. Conserved regions are underlined. (**D**) Promoter-dependent reporter activity in bacteria grown in MSSM medium with 5 mM KCl (light gray symbols) or 0.1 mM KCl (dark gray symbols). *B. subtilis* BP193 carries the p_a promoter, and *B. subtilis* GP2182 carries the native *kimA* core promoter (without the riboswitch) fused to the reporter gene *lacZ*. (**E**) Riboswitch-dependent reporter activity in WT cells (p_a RS, GP2198) and a mutant strain with high c-di-AMP concentrations (p_a RS + CdaS, GP2200). In both strains, the riboswitch was placed downstream of the constitutively active p_a promoter. Each data point in (A), (B), (D), and (E) represents a biologically independent replicate ($n \geq 3$). The bars indicate the means of the replicates. β-Galactosidase activities are given as units per milligram of protein.

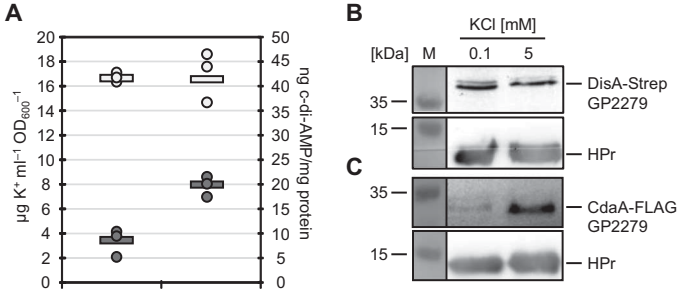

Fig. 4. K^+ concentrations control c-di-AMP synthesis. (**A**) *B. subtilis* WT strain (168) was grown at 5 mM KCl (light gray symbols) or 0.1 mM KCl (dark gray symbols), and K^+ and c-di-AMP were determined by inductively coupled plasma optical emission spectrometry (ICP-OES) and mass spectrometry, respectively. Each data point represents a biologically independent replicate ($n = 3$). The bars indicate the means of the replicates. OD_{600}, optical density at 600 nm. (**B**) *B. subtilis* expressing Strep-tagged DisA (GP2279) was cultivated in MSSM medium at high (5 mM) and low (0.1 mM) KCl concentrations. DisA-Strep was detected using antibodies against the Strep tag. (**C**) *B. subtilis* expressing FLAG-tagged CdaA (GP2279) was cultivated in MSSM medium at high (5 mM) and low (0.1 mM) KCl concentrations. CdaA-FLAG was detected using antibodies against the FLAG tag. The constitutively expressed HPr protein served as a loading control in (B) and (C).

c-di-AMP synthesis, we compared the intracellular concentration of the vegetative diadenylate cyclases DisA and CdaA. For this purpose, we used strain GP2297 that expresses DisA and CdaA with immunogenic Strep and FLAG tags, respectively (Fig. 4B). Whereas the amounts of DisA were similar irrespective of the K^+ concentration, CdaA was poorly expressed at the low extracellular K^+ concentration. In contrast, CdaA abundance was high at 5 mM extracellular K^+. This result suggests that CdaA abundance may be a major determinant of c-di-AMP concentrations in response to potassium availability.

Together, the data suggest that changes in external K^+ availability control the cellular c-di-AMP concentration to regulate gene expression through the *kimA* riboswitch. Collectively, the results are consistent with a model in which high K^+ stimulates an increase in c-di-AMP production, resulting in repression of *kimA* riboswitch-controlled gene expression and a reduction in the abundance of the K^+ importer KimA.

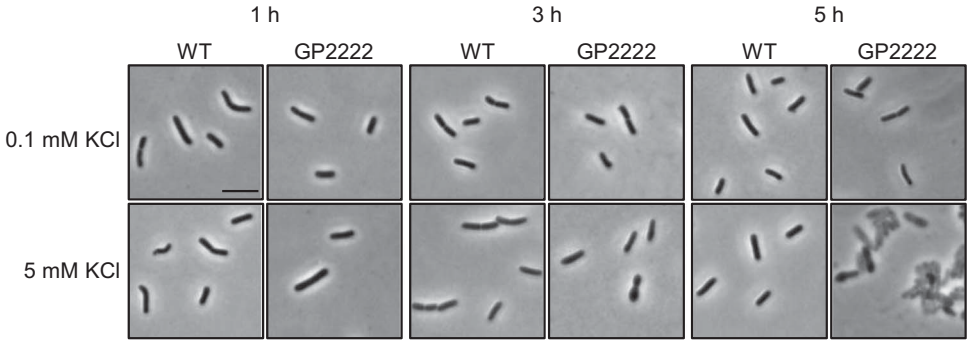

Fig. 5. K^+ is toxic for a strain lacking c-di-AMP. *B. subtilis* WT (168) and the mutant strain lacking c-di-AMP (GP2222) were precultivated at 0.1 mM KCl. At t_0, cells were washed and reinoculated in MSSM medium with the indicated low and high KCl concentrations. Samples were analyzed after 1, 3, and 5 hours of growth. Scale bar, 5 μM.

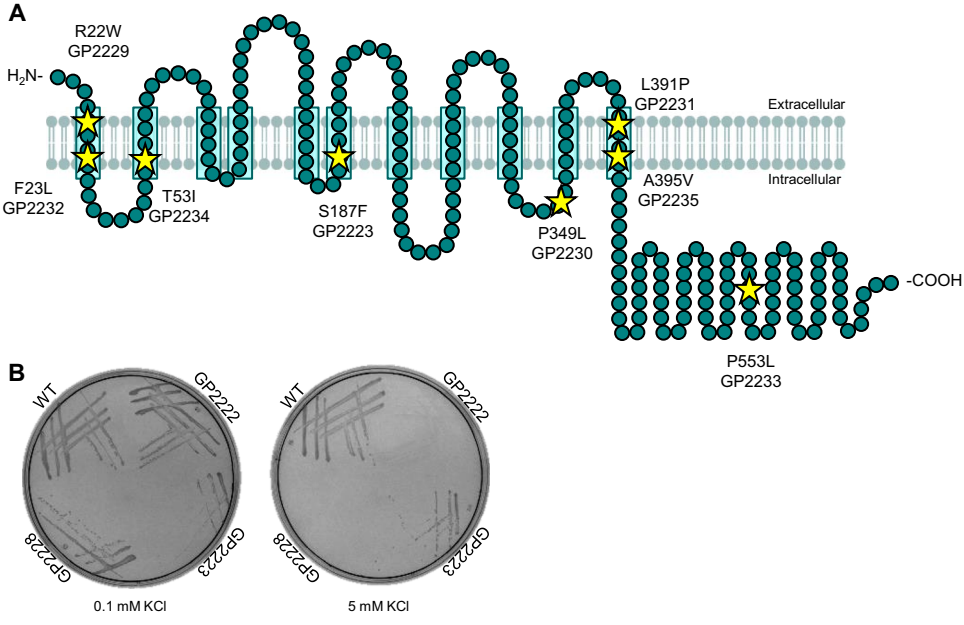

Fig. 6. Mutations in the NhaK cation exporter overcome K^+ toxicity in the absence of c-di-AMP. (**A**) Green dots represent the amino acids of the NhaK protein. Yellow stars indicate amino acid substitutions that individually enable growth on 5 mM KCl–containing medium. The substitutions are indicated along with the names of the suppressor strains. The transmembrane domains are highlighted in turquoise. The protein is not shown to scale. (**B**) Growth of *B. subtilis* WT (168), GP2222 (Δ*cdaA* Δ*cdaS* Δ*disA*), GP2223 (Δ*cdaA* Δ*cdaS* Δ*disA* $nhaK_{S187F}$), and GP2228 (Δ*cdaA* Δ*cdaS* Δ*disA* Δ*nhaK*) when plated on MSSM plates containing 0.1 or 5 mM KCl.

B. subtilis is viable without c-di-AMP at low potassium concentrations

c-di-AMP is essential for the viability of *B. subtilis* and many other firmicutes (*2*). For *Listeria monocytogenes*, c-di-AMP is essential to prevent the toxic accumulation of (p)ppGpp (*14*). Accordingly, deletion of the single diadenylate cyclase gene of *L. monocytogenes* is possible if cells are cultivated on minimal medium under conditions that do not trigger (p)ppGpp synthesis (*14*). However, previous attempts to construct a *B. subtilis* strain devoid of all three diadenylate cyclases have been unsuccessful (*12*, *15*, *16*). Even on standard minimal medium supplemented with glucose (*12*), deletion of the diadenylate cyclase genes has not been possible in *B. subtilis*, suggesting that the functions of c-di-AMP differ between different bacteria. The low c-di-AMP concentration that we observed in *B. subtilis* grown in low K^+ medium suggested that this second messenger might be dispensable under these conditions. *B. subtilis* lacking *cdaA*, *cdaS*, and *disA* (and thus all diadenylate cyclase–encoding genes; GP2222) was viable on minimal medium supplemented with 0.1 mM KCl (Fig. 5). However, within 3 hours on minimal medium containing 5 mM KCl, we observed bacterial swelling; by 5 hours, many of the cells had lysed, indicating that K^+ becomes toxic to *B. subtilis* in the absence of c-di-AMP (Fig. 5). Mass spectrometry analysis of c-di-AMP concentrations of the GP2222 strain confirmed that no c-di-AMP was present.

Cultivation of GP2222 on 5 mM KCl–containing medium resulted in the appearance of suppressor mutants after 5 days of cultivation. Whole-genome analysis of eight of these mutants revealed the presence of single point mutation in the *nhaK* gene, which encodes a widely distributed cation/proton antiporter-I family and functions as both a Na^+ and K^+ efflux transporter (*17*) (Fig. 6A). Toxicity of K^+ and suppression of this toxicity by the *nhaK* mutations suggested that the mutant proteins had enhanced K^+ efflux activity. Unlike GP2222, which lacks the ability to synthesize c-di-AMP yet grows on 0.1 mM KCl and produces suppressor mutants when grown on 5 mM KCl, deletion of *nhaK* in this background (GP2228) did not allow growth at the 5 mM KCl–containing medium and prevented the accumulation of suppressor mutants (Fig. 6B). Thus, our data indicate that maintenance of K^+ homeostasis is the essential function of c-di-AMP in *B. subtilis*.

DISCUSSION

We have established an additional layer in the control of potassium homeostasis in the Gram-positive model organism *B. subtilis*: c-di-AMP acts as a second messenger that responds to changes in extracellular K^+ concentration and controls K^+ homeostasis in this bacterium. K^+ is the most abundant cation in any living cell and is essential for the function of the ribosome and of many enzymes and for the maintenance of intracellular pH (*18*, *19*). On the other hand, excess K^+ inhibits bacterial growth, suggesting that the ion is toxic; however, the reasons for this toxicity are poorly understood (*20*, *21*).

Because K^+ is essential for growth, efficient uptake systems are required to accumulate this ion even at low environmental concentrations. This is achieved by the utilization of different transport systems: Low-affinity transporters are responsible for K^+ uptake at high extracellular concentrations, whereas high-affinity transporters take over at low concentrations (*18*). We have discovered and characterized KimA, a high-affinity potassium transporter that is widely conserved in bacteria. Among the bacteria that are closely related to *B. subtilis*, such as *L. monocytogenes* and *S. aureus*, each species seems to have two high-affinity K^+ uptake systems. These are KimA, which is present in all three species, and either KtrAB (*B. subtilis*) or KdpABC (*L. monocytogenes* and *S. aureus*). In *B. subtilis*, the expression of genes encoding both high-affinity transport systems is negatively controlled by a K^+-responsive, c-di-AMP–binding riboswitch (*8*). In contrast, the expression of the genes in the Kdp system is negatively controlled by binding of c-di-AMP to the two-component sensor kinase KdpD (*6*). Moreover, all three species contain the low-affinity K^+ transporter KtrCD, the activity of which is inhibited upon binding of c-di-AMP (*4*). It will be interesting to study whether KimA and KtrAB are active under the same conditions and how their transport properties may be differentially controlled.

c-di-AMP is the only essential second messenger (*2*). Four lines of evidence indicate that the control of K^+ homeostasis is the function that makes c-di-AMP essential: (i) c-di-AMP binds K^+ transporters not only in *B. subtilis* and close relatives but also in *Corynebacterium glutamicum* and *Mycoplasma pneumoniae* (*2*); (ii) c-di-AMP negatively controls the expression of genes encoding high-affinity potassium transporters by binding to the *kimA* and *ktrAB* riboswitches in *B. subtilis* and to the KdpD sensor kinase in *S. aureus* and *L. monocytogenes* (*4*, *6*, *8*, *22*); (iii) the signaling nucleotide is synthesized in response to K^+ availability (this work); and (iv) the second messenger is dispensable at low K^+ concentrations, whereas mutations in the cation exporter NhaK are required to overcome K^+ toxicity in the absence of c-di-AMP signaling (this work).

The intimate link between c-di-AMP and K^+ homeostasis is evident not only for *B. subtilis* but also for related pathogens such as *L. monocytogenes*, *S. aureus*, and *Streptococcus pneumoniae*. A strain of *L. monocytogenes* has been constructed that is viable in the absence of c-di-AMP only if the formation of the signaling nucleotide (p)ppGpp, which indicates amino acid starvation, is suppressed or if the bacteria are grown on minimal medium (*14*). We were not able to delete all diadenylate cyclases under such conditions in *B. subtilis*, indicating that c-di-AMP–mediated signaling may have distinct functions in the different bacteria, although they are closely related. One important difference between *B. subtilis* and *L. monocytogenes* is the citric acid cycle, which is complete in the former bacterium and incomplete in the latter bacterium (*23*). This difference may be relevant because c-di-AMP also controls the activity of *L. monocytogenes* pyruvate carboxylase, the enzyme important for the generation of the acetyl–coenzyme A acceptor oxaloacetate (*24*). In contrast, this enzyme plays only a minor role in *B. subtilis* with its complete citric acid cycle. The control of pyruvate carboxylase by c-di-AMP is linked to osmoregulation in *L. monocytogenes* (*23*). This link might be the basis for the difference in c-di-AMP signaling between the two bacteria: K^+

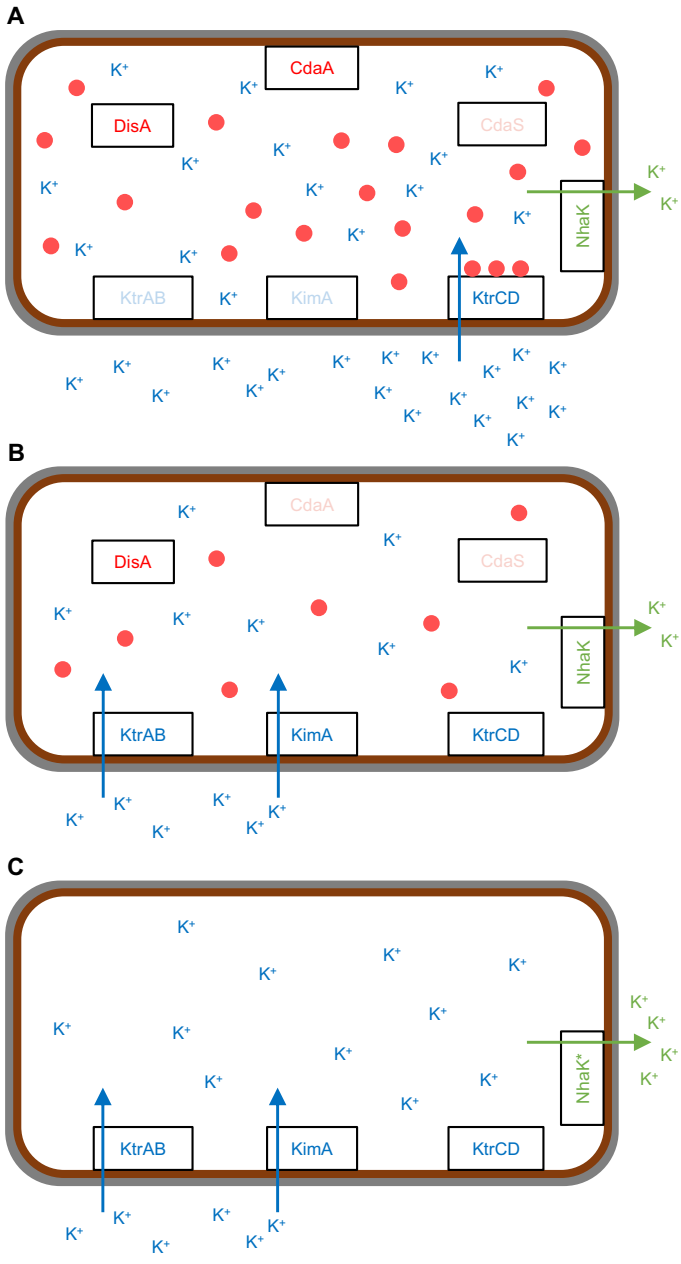

Fig. 7. Schematic overview of c-di-AMP–mediated control of K^+ uptake. (**A**) High external K^+ concentrations trigger c-di-AMP synthesis by DisA and CdaA. c-di-AMP prevents the expression of the genes encoding KtrAB and KimA and binds to the low-affinity transporter KtrCD to limit K^+ uptake. Moderate K^+ efflux is possible through NhaK. (**B**) Low external K^+ concentrations result in decreased CdaA abundance and, thus, in low c-di-AMP concentrations. K^+ is taken up by the high-affinity K^+ transporters KtrAB and KimA. Moderate K^+ efflux is mediated by NhaK. (**C**) In the absence of c-di-AMP, K^+ influx cannot be controlled. To prevent intoxication, excess K^+ is exported by mutated NhaK (NhaK*). The red circles represent c-di-AMP. Faded names indicate proteins that are not present under the condition shown.

accumulation is a rapid response to osmotic stress, followed by the uptake or synthesis of compatible solutes (*25*, *26*). As shown in this work, c-di-AMP is central to the control of K^+ homeostasis in *B. subtilis*. A suppressor mutation in a gene similar to *nhaK* (*lmo2353*) has also been detected in a *L. monocytogenes* strain lacking the single diadenylate cyclase gene (*14*). This observation suggests that c-di-AMP may be involved in the control of K^+ homeostasis even in *L. monocytogenes*. In addition, c-di-AMP controls the uptake of compatible solutes in *L. monocytogenes* and *S. aureus* (*22*, *27*). However, *B. subtilis* synthesizes the compatible solute proline from the citric acid cycle intermediate 2-oxoglutarate to adapt to osmotic stress (*26*). The differences in the citric acid cycle and in osmoadaptation may be responsible for the different impact of c-di-AMP on cellular physiology between the three related bacteria. Together, our work uncovered a physiologically relevant connection between the cellular amount of the second messenger c-di-AMP and the control of K^+ homeostasis (Fig. 7, A to C).

MATERIALS AND METHODS

Strains, media, and growth conditions

E. coli DH5α (*28*) was used for cloning, and *E. coli* LB650 (*29*) was used for potassium transporter complementation assays. All *B. subtilis* strains used in this study are derivatives of the laboratory strains 168 and JH642 (table S1). Bacteria were grown in Luria-Bertani (LB) or in sporulation medium (*28*, *30*). To study potassium requirements in *B. subtilis* and for the quantification of c-di-AMP, we used a modified sodium Spizizen minimal (MSSM) medium. The fivefold concentrated basic potassium-free medium stock solution contained 4.1% $Na_2HPO_4 \times 2\ H_2O$, 1.6% $NaH_2PO_4 \times H_2O$, 0.5% sodium citrate, and 0.1% $MgSO_4 \times 7\ H_2O$ (pH 7.5). The following trace elements were prepared as 100-fold concentrated stock solution (per liter of stock solution): 0.55 g of $CaCl_2$, 0.1 g of $MnCl_2 \times 4\ H_2O$, 0.17 g of $ZnCl_2$, 0.033 g of $CuCl_2 \times 2\ H_2O$, 0.06 g of $CoCl_2 \times 6\ H_2O$, and 0.06 g of $Na_2MoO_4 \times 2\ H_2O$. A 100-fold concentrated stock solution for iron supplementation was prepared as follows (per 100 ml of stock solution): 0.0135 g of $FeCl_3 \times 6\ H_2O$, and 0.1 g of sodium citrate $\times 3\ H_2O$. Glucose was added to a final concentration of 0.5% (w/v), and sodium glutamate was added to a final concentration of 1% (w/v). Tryptophan was added to a final concentration of 0.05 mg/ml. Potassium chloride was added as indicated. To test deletion of all diadenylate cyclases in minimal medium, we used MSSM medium supplemented with 0.2% $(NH_4)_2SO_4$ and 0.1 mM KCl. For *E. coli* complementation experiments, a slightly modified MSSM medium was used (MSSM-EC): Glucose was used at 1% (w/v), and casamino acids (0.66%), proline (0.004%), and thiamine (0.003%) were added. To grow the *E. coli* LB650 derivative strains on complex medium, the sodium chloride in the LB medium was replaced by potassium chloride (LB-K). The media were supplemented with ampicillin (100 μg/ml), kanamycin (10 and 50 μg/ml for *B. subtilis* and *E. coli*, respectively), chloramphenicol (5 and 30 μg/ml for *B. subtilis* and *E. coli*, respectively), tetracyclin (12.5 μg/ml), spectinomycin (150 μg/ml), or erythromycin and lincomycin (2 and 25 μg/ml, respectively) if required.

DNA manipulation

Transformation of *E. coli* and plasmid DNA extraction were performed using standard procedures (*28*). 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 (*30*). *B. subtilis* was transformed with plasmid and genomic DNA according to the two-step protocol (*30*).

Plasmids

Plasmids pAC5 (*31*), pAC6 (*32*), and pAC7 (*33*) were used to construct reporter fusions, respectively, of the *kimA* control regions with the *lacZ* gene. The artificial promoter p_a was obtained by hybridization of the corresponding oligonucleotides (see Fig. 3C). The promoter has an intermediate constitutive activity in *B. subtilis*. The constitutive activity of this promoter under the growth conditions used in this study was verified (strain BP193; Fig. 2D). The details of the construction and plasmid designations are summarized in table S2. The resulting plasmids were linearized with Sca I and used to transform the relevant *B. subtilis* strains (see table S1). To express the DisA protein fused to a Strep tag under the control of its native promoter, we constructed plasmid pBP599. Briefly, the region of the *disA* gene encoding the C-terminal part of the protein was amplified and cloned between the Bam HI and Sal I restriction sites of the vector pGP1389 (*34*). Finally, pBP599 was used to transform *B. subtilis* 168, resulting in strain BP610. For *E. coli* complementation assays, *ktrAB* and *kimA* were cloned into the expression vector pWH844 (*35*), as indicated in table S2.

Phenotypic characterization

In *B. subtilis*, amylase activity was detected after growth on plates containing nutrient broth (7.5 g/liter), 17 g of Bacto agar per liter (Difco), and 5 g of hydrolyzed starch per liter (Connaught). Starch degradation was detected by sublimating iodine onto the plates. Quantitative studies of *lacZ* expression in *B. subtilis* were performed as follows: Cells were grown in glutamate-supplemented MSSM medium as indicated and harvested in the exponential phase. β-Galactosidase–specific activities were determined with cell extracts obtained by lysozyme treatment as described previously (*30*). One unit of β-galactosidase is defined as the amount of enzyme that produces 1 nmol of *o*-nitrophenol per minute at 28°C.

To assay growth of *B. subtilis* mutants at different potassium concentrations, a drop dilution assay was performed. Briefly, precultures in MSSM medium at the indicated potassium concentration were washed three times and resuspended to an OD_{600} of 1.0 in MSSM basal salts solution. Dilution series were then pipetted onto MSSM plates with 0.5 or 50 mM KCl.

For *E. coli* complementation assays, strain LB650 (*29*) was transformed with the empty cloning vector pWH844 (*35*) and with the complementation plasmids pBP372 (*ktrAB*) and pGP2913 (*kimA*). Transformants were selected on LB-K plates. Transformants were cultivated in LB-K, washed in MSSM basal salts solution, and plated on MSSM-EC plates containing isopropyl-β-D-thiogalactopyranoside (IPTG) to induce expression of the potassium transporters and potassium chloride at the indicated concentrations.

Microscopy

For light microscopy, cells were grown at 37°C in low phosphate concentration overnight. The cells were washed three times with potassium-free medium and adjusted to an OD_{600} of 0.1. Cells were reinoculated in medium containing low or high potassium concentration. Phase-contrast images were obtained with the Axioskop 40 microscope, equipped with digital camera AxioCam MRm and AxioVision Rel 4.8 software for image processing (Carl Zeiss).

Growth assays

Precultures of the *E. coli* strains LB650/pWH844 (cloning vector), LB650/pBP372 (*ktrAB*$^+$), and LB650/pGP2913 (*kimA*$^+$) were grown in MSSM-EC containing 50 mM KCl. Expression of the potassium transporter genes *ktrAB* and *kimA* was induced by the addition of 1 mM IPTG (for LB650/pBP372) or 0.1 mM IPTG (LB650/pGP2913). After the cultures reached midexponential growth phase (OD_{578} of about 1.5 to 2.0), cells were harvested by centrifugation, washed twice with potassium-free MSSM-EC medium, and used for the inoculation of the main cultures (0.5 ml) to an OD_{578} of 0.05. This medium was supplemented with potassium concentrations ranging from 0 to 300 μM. Bacteria were grown at 37°C in 48-well microtiter plates in a combined microplate incubator and reader (Epoch 2, BioTek). To assess the growth characteristics of *B. subtilis* strains expressing only one of the three potassium transporters, precultures of the strains were grown in glutamate-supplemented MSSM medium containing 25 mM KCl. These cultures were then washed twice in potassium-free MSSM medium and used to inoculate main cultures containing various concentrations of potassium to an OD_{578} of 0.1. The cultures were grown in 48-well microtiter plates in the Epoch 2 microplate incubator/reader at 37°C. Growth rates were determined from the growth curves by fitting them according to the exponential growth equation. These data were then plotted against the potassium concentration of the corresponding cultures, and the resulting curve was fitted using the Michaelis-Menten equation. Potassium concentrations corresponding to the half-maximal growth rate were calculated. Fitting and data calculations were conducted using the GraphPad Prism sofware package—version 6 for Mac.

Construction of deletion strains

Deletion of the *ktrC* and *kimA* genes was achieved by transformation with polymerase chain reaction (PCR) products constructed using oligonucleotides (see table S2) to amplify DNA fragments flanking the target genes and intervening antibiotic resistance cassettes, as described previously (*36*, *37*).

To construct a strain devoid of all three diadenylate cyclases, strain GP991 (Δ*cdaS* Δ*disA*) was transformed with the chromosomal DNA of GP997 (Δ*cdaA*). For transformation, potassium salts were replaced by the corresponding sodium salts in all solutions. In the outgrowth medium, the yeast extract was omitted. Transformants were selected on MSSM medium containing 0.1 mM KCl and ammonium as the nitrogen source. Deletion of the three genes was verified by PCR with primer pairs internal to *cdaA*, *cdaS*, and *disA*. Primer pairs for the *cdaR* and *dgcW* genes served as positive controls. Moreover, the genome sequence of GP2222 excluded the presence of secondary mutations.

Mapping suppressors by whole-genome sequencing and PCR analysis

Chromosomal DNA from *B. subtilis* was isolated using the peqGOLD Bacterial DNA Kit (Peqlab). To identify the mutations in the mutant strains GP2222 and GP2223, the genomic DNA was subjected to whole-genome sequencing. The reads were mapped on the reference genome of *B. subtilis* 168 (GenBank accession number: NC_000964) (*38*). Mapping of the reads was performed using the Geneious software package (Biomatters Ltd.) (*39*). Single-nucleotide polymorphisms 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.

Western blot analysis

To facilitate the analysis of KimA production in *B. subtilis* by Western blot analysis, a triple FLAG tag was fused to the C terminus of the protein. For this purpose, plasmid pGP2789 and strain *B. subtilis* GP2405 were constructed using the vector pGP1331 (*40*), as outlined in table S1. For Western blot 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. Proteins were detected using specific antibodies raised against *B. subtilis* HPr (*41*) or antibodies recognizing the FLAG tag (Sigma), respectively. The primary antibodies were visualized by using anti-rabbit IgG (immunoglobulin G)–AP (alkaline phosphatase) secondary antibodies (Promega) and the CDP* detection system (Roche Diagnostics) (*42*).

Analysis of the cyclic dinucleotide pools

The concentration of c-di-AMP in *B. subtilis* cells was determined by a liquid chromatography–tandem mass spectrometry method, as described previously (*3*). Briefly, *B. subtilis* cells (20 ml) were grown in MSSM medium supplemented with the desired nitrogen source and at the indicated potassium concentration to an OD_{600} of 1.0. The samples (10 ml) were centrifuged (0°C, 20,800*g*), shock-frozen in liquid nitrogen, and stored at −80°C. This sample was used for c-di-AMP extraction (*3*). The chromatographic separation was performed on a Series 200 HPLC (high-performance liquid chromatography) system (PerkinElmer Life Sciences) or an LC-10AD HPLC system (Shimadzu), as described previously (*12*). Detection of c-di-AMP was performed on an API 3000 or API 4000 triple quadrupole mass spectrometer equipped with an electrospray ionization source (AB Sciex) using selected reaction monitoring (SRM) analysis in positive ionization mode. The SRM transitions labeled as "quantifier" were used to quantify the compound of interest, whereas "identifier" SRM transitions were monitored as confirmatory signals. The quantifier SRM transitions were most intense and were therefore used for quantification.

Determination of intracellular potassium pools

For the determination of intracellular K^+ pools, *B. subtilis* cells (100 ml) were grown as described for c-di-AMP extraction. Cell pellets were resuspended in 800 μl of MSSM basal salts solution and transferred onto ash-free filter discs (pore size, 0.45 μm; diameter, 47 mm). The cells were dried overnight at room temperature, followed by 3 hours at 70°C. The dried filter discs containing the bacteria cells were cut into small pieces and reduced into a fluid state through pressure and 2 ml of 65% HNO_3 for 7 hours at 185°C in 25-ml Teflon beakers (PDS-6 Pressure Digestion System, Loftfield). After the digestion process, the fluid content in the beakers was transferred into an Erlenmeyer flask and diluted with demineralized water to a volume of 50 ml. The total K^+ content of the bacterial cells in this solution was determined by ICP-OES analysis (Optima 5300 DV, PerkinElmer). This common type of emission spectroscopy technique uses the ICP to produce element atoms and ions that emit electromagnetic radiation at wavelengths of specific characteristics of a particular chemical element. The intensity of light emission at 766.49 nm is indicative of the K^+ concentration in the sample. The plasma is built by argon gas ionized in an intense electromagnetic field at a temperature of about 7000° to

10,000°C, generated as the result of the collisions between the neutral argon atoms and the charged particles (*43*).

SUPPLEMENTARY MATERIALS

www.sciencesignaling.org/cgi/content/full/10/475/eaal3011/DC1
Fig. S1. Activity of *B. subtilis* K^+ transporters in *E. coli.*
Fig. S2. Alignment of KimA homologs.
Table S1. Bacterial strains used in this study.
Table S2. Plasmids and oligonucleotides used in this study.

REFERENCES AND NOTES

1. R. M. Corrigan, A. Gründling, Cyclic di-AMP: Another second messenger enters the fray. *Nat. Rev. Microbiol.* **11**, 513–524 (2013).
2. F. M. Commichau, A. Dickmanns, J. Gundlach, R. Ficner, J. Stülke, A jack of all trades: The multiple roles of the unique essential second messenger cyclic di-AMP. *Mol. Microbiol.* **97**, 189–204 (2015).
3. J. Gundlach, F. M. P. Mehne, C. Herzberg, J. Kampf, O. Valerius, V. Kaever, J. Stülke, An essential poison: Synthesis and degradation of cyclic di-AMP in *Bacillus subtilis*. *J. Bacteriol.* **197**, 3265–3274 (2015).
4. R. M. Corrigan, I. Campeotto, T. Jeganathan, K. G. Roelofs, V. T. Lee, A. Gründling, Systematic identification of conserved bacterial c-di-AMP receptor proteins. *Proc. Natl. Acad. Sci. U.S.A.* **110**, 9084–9089 (2013).
5. Y. Bai, J. Yang, T. M. Zarella, Y. Zhang, D. W. Metzger, G. Bai, Cyclic di-AMP impairs potassium uptake mediated by a cyclic di-AMP binding protein in *Streptococcus pneumoniae*. *J. Bacteriol.* **196**, 614–623 (2014).
6. J. A. Moscoso, H. Schramke, Y. Zhang, T. Tosi, A. Dehbi, K. Jung, A. Gründling, Binding of cyclic di-AMP to the *Staphylococcus aureus* sensor kinase KdpD occurs via the universal stress protein domain and downregulates the expression of the Kdp potassium transporter. *J. Bacteriol.* **198**, 98–110 (2016).
7. K. F. Block, M. C. Hammond, R. R. Breaker, Evidence for widespread gene control function by the *ydaO* riboswitch candidate. *J. Bacteriol.* **192**, 3983–3989 (2010).
8. J. W. Nelson, N. Sudarsan, K. Furukawa, Z. Weinberg, J. X. Wang, R. R. Breaker, Riboswitches in eubacteria sense the second messenger c-di-AMP. *Nat. Chem. Biol.* **9**, 834–839 (2013).
9. G. Holtmann, E. P. Bakker, N. Uozumi, E. Bremer, KtrAB and KtrCD: Two K^+ uptake systems in *Bacillus subtilis* and their role in adaptation to hypertonicity. *J. Bacteriol.* **185**, 1289–1298 (2003).
10. A. Srivatsan, Y. Han, J. Peng, A. K. Tehranchi, R. Gibbs , J. D. Wang, R. Chen, High-precision, whole genome sequencing of laboratory strains facilitates genetic studies. *PLOS Genet.* **4**, e1000139 (2008).
11. S. F. Altschul, W. Gish, W. Miller, E. W. Myers, D. J. Lipman, Basic local alignment search tool. *J. Mol. Biol.* **215**, 403–410 (1990).
12. F. M. P. Mehne, K. Gunka, H. Eilers, C. Herzberg, V. Kaever, J. Stülke, Cyclic-di-AMP homeostasis in *Bacillus subtilis*: Both lack and high-level accumulation of the nucleotide are detrimental for cell growth. *J. Biol. Chem.* **288**, 2004–2017 (2013).
13. F. M. P. Mehne, K. Schröder-Tittmann, R. T. Eijlander, C. Herzberg, L. Hewitt, V. Kaever, R. J. Lewis, O. P. Kuipers, K. Tittmann, J. Stülke, Control of the diadenylate cyclase CdaS in *Bacillus subtilis*: An autoinhibitory domain limits cyclic di-AMP production. *J. Biol. Chem.* **289**, 21098–21107 (2014).
14. A. T. Whiteley, A. J. Pollock, D. Portnoy, The PAMP c-di-AMP is essential for *Listeria monocytogenes* growth in rich but not minimal media due to a toxic increase in (p)ppGpp. *Cell Host Microbe* **17**, 788–798 (2015).
15. J. Gundlach, A. Dickmanns, K. Schröder-Tittmann, P. Neumann, J. Kaesler, J. Kampf, C. Herzberg, E. Hammer, F. Schwede, V. Kaever, K. Tittmann, J. Stülke, R. Ficner, Identification, characterization and structure analysis of the cyclic di-AMP-binding P_{II}-like signal transduction protein DarA. *J. Biol. Chem.* **290**, 3069–3080 (2015).
16. Y. Luo, J. D. Helmann, Analysis of the role of *Bacillus subtilis* σ^M in β-resistance reveals an essential role for c-di-AMP in peptidoglycan homeostasis. *Mol. Microbiol.* **83**, 623–639 (2012).
17. M. Fujisawa, A. Kusumoto, Y. Wada, T. Tsuchiya, M. Ito, NhaK, a novel monovalent cation/H+ antiporter of *Bacillus subtilis*. *Arch. Microbiol.* **183**, 411–420 (2005).
18. W. Epstein, The roles and regulation of potassium in bacteria. *Prog. Nucleic Acid Res. Mol. Biol.* **75**, 293–320 (2003).
19. P. Nissen, J. Hansen, N. Ban, P. B. Moore, T. A. Steitz, The structural basis of ribosome activity in peptide bond synthesis. *Science* **289**, 920–930 (2000).
20. M. V. Radchenko, R. Waditee, S. Oshimi, M. Fukuhara, T. Takabe, T. Nakamura, Cloning, functional expression and primary characterization of *Vibrio parahaemolyticus* K^+/H^+ antiporter genes in *Escherichia coli*. *Mol. Microbiol.* **59**, 651–663 (2006).
21. M. V. Radchenko, K. Tanaka, R. Waditee, S. Oshimi, Y. Matsuzaki, M. Fukuhara, H. Kobayashi, T. Takabe, T. Nakamura, Potassium/proton antiport system of *Escherichia coli*. *J. Biol. Chem.* **281**, 19822–19829 (2006).
22. T. N. Huynh, P. H. Choi, K. Sureka, H. E. Ledvina, J. Campillo, L. Tong, J. J. Woodward, Cyclic di-AMP targets the cystathionine beta-synthase domain of the osmolyte transporter OpuC. *Mol. Microbiol.* **102**, 233–243 (2016).
23. A. T. Whiteley, N. E. Garelis, B. N. Peterson, P. H. Choi, L. Tong, J. J. Woodward, D. A. Portnoy, c-di-AMP modulates *Listeria monocytogenes* central metabolism to regulate growth, antibiotic resistance, and osmoregulation. *Mol. Microbiol.* **104**, 212–233 (2017).
24. K. Sureka, P. H. Choi, M. Precit, M. Delince, D. A. Pensinger, T. N. Huynh, A. R. Jurado, Y. A. Goo, M. Sadilek, A. T. Iavarone, J.-D. Sauer, L. Tong, J. J. Woodward, The cyclic dinucleotide c-di-AMP is an allosteric regulator of metabolic enzyme function. *Cell* **158**, 1389–1401 (2014).
25. J. M. Wood, Osmosensing by bacteria. *Sci. STKE* **357**, pe43 (2006).
26. B. Kempf, E. Bremer, Uptake and synthesis of compatible solutes as microbial stress responses to high-osmolality environments. *Arch. Microbiol.* **170**, 319–330 (1998).
27. C. F. Schuster, L. E. Bellows, T. Tosi, I. Campeotto, R. M. Corrigan, P. Freemont, A. Gründling, The second messenger c-di-AMP inhibits the osmolyte uptake system OpuC in *Staphylococcus aureus*. *Sci. Signal.* **9**, ra81 (2016).
28. J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, ed. 2, 1989).
29. S. Stumpe, E. P. Bakker, Requirement of a large K^+ uptake capacity and of extracytoplasmic protease activity for protamine resistance of *Escherichia coli*. *Arch. Microbiol.* **167**, 126–136 (1997).
30. F. Kunst, G. Rapoport, Salt stress is an environmental signal affecting degradative enzyme synthesis in *Bacillus subtilis*. *J. Bacteriol.* **177**, 2403–2407 (1995).
31. I. Martin-Verstraete, M. Débarbouillé, A. Klier, G. Rapoport, Mutagenesis of the *Bacillus subtilis* "–2, –24" promoter of the levanase operon and evidence for the existence of an upstream activating sequence. *J. Mol. Biol.* **226**, 85–99 (1992).
32. J. Stülke, I. Martin-Verstraete, M. Zagorec, M. Rose, A. Klier, G. Rapoport, Induction of the *Bacillus subtilis ptsGHI* operon by glucose is controlled by a novel antiterminator, GlcT. *Mol. Microbiol.* **25**, 65–78 (1997).
33. Y. Weinrauch, T. Msadek, F. Kunst, D. Dubnau, Sequence and properties of *comQ*, a new competence regulatory gene of *Bacillus subtilis*. *J. Bacteriol.* **173**, 5685–5693 (1991).
34. M. Lehnik-Habrink, J. Newman, F. M. Rothe, A. s. Solovyova, C. Rodrigues, C. Herzberg, F. M. Commichau, R. J. Lewis, J. Stülke, RNase Y in *Bacillus subtilis*: A natively disordered protein that is the functional equivalent of RNase E from *Escherichia coli*. *J. Bacteriol.* **193**, 5431–5441 (2011).
35. F. Schirmer, S. Ehrt, W. Hillen, Expression, inducer spectrum, domain structure, and function of MopR, the regulator of phenol degradation in *Acinetobacter calcoaceticus* NCIB8250. *J. Bacteriol.* **179**, 1329–1336 (1997).
36. A. Wach, PCR-synthesis of marker cassettes with long flanking homology regions for gene disruptions in *S. cerevisiae*. *Yeast* **12**, 259–265 (1996).
37. A. M. Guérout-Fleury, K. Shazand, N. Frandsen, P. Stragier, Antibiotic resistance cassettes for *Bacillus subtilis*. *Gene* **167**, 335–336 (1995).
38. V. Barbe, S. Cruveiller, F. Kunst, P. Lenoble, G. Meurice, A. Sekowska, D. Vallenet, T. Wang, I. Moszer, C. Médigue, A. Danchin, From a consortium sequence to a unified sequence: The *Bacillus subtilis* 168 reference genome a decade later. *Microbiology* **155**, 1758–1775 (2009).
39. M. Kearse, R. Moah, A. Wilson, S. Stones-Havas, M. Cheung, S. Sturrock, S. Buxton, A. Cooper, S. Markowitz, C. Duran, T. Thierer, B. Ashton, P. Meintjes, A. Drummond, Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* **28**, 1647–1649 (2012).
40. M. Lehnik-Habrink, H. Pförtner, L. Rempeters, N. Pietack, C. Herzberg, J. Stülke, The RNA degradosome in *Bacillus subtilis*: Identification of CshA as the major RNA helicase in the multi-protein complex. *Mol. Microbiol.* **77**, 958–971 (2010).
41. V. Monedero, S. Poncet, I. Mijakovic, S. Fieulaine, V. Dossonet, I. Martin-Verstraete, S. Nessler, J. Deutscher, Mutations lowering the phosphatase activity of HPr kinase/phosphatase switch off carbon metabolism. *EMBO J.* **20**, 3928–3937 (2001).
42. M. Schmalisch, I. Langbein, J. Stülke, The general stress protein Ctc of *Bacillus subtilis* is a ribosomal protein. *J. Mol. Microbiol. Biotechnol.* **4**, 495–501 (2002).
43. M. Aceto, O. Abollino, M. C. Bruzzoniti, E. Mentasti, C. Sarzanini, M. Malandrino, Determination of metals in wine with atomic spectroscopy (flame-AAS, GF-AAS and ICP-AES); a review. *Food Addit. Contam.* **19**, 126–133 (2002).

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Control of potassium homeostasis is an essential function of the second messenger cyclic di-AMP in *Bacillus subtilis*

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