c-di-AMP assists osmoadaptation by regulating the *Listeria monocytogenes* potassium transporters KimA and KtrCD

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Many bacteria and some archaea produce the second messenger cyclic diadenosine monophosphate (c-di-AMP). c-di-AMP controls the uptake of osmolytes in Firmicutes, including the human pathogen *Listeria monocytogenes*, making it essential for growth. c-di-AMP is known to directly regulate several potassium channels involved in osmolyte transport in species such as *Bacillus subtilis* and *Streptococcus pneumoniae*, but whether this same mechanism is involved in *L. monocytogenes*, or even whether similar ion channels were present, was not known. Here, we have identified and characterized the putative *L. monocytogenes'* potassium transporters KimA, KtrCD, and KdpABC. We demonstrate that *Escherichia coli* expressing KimA and KtrCD, but not KdpABC, transport potassium into the cell, and both KimA and KtrCD are inhibited by c-di-AMP *in vivo*. For KimA, c-di-AMP–dependent regulation requires the C-terminal domain. *In vitro* assays demonstrated that the dinucleotide binds to the cytoplasmic regulatory subunit KtrC and to the KdpD sensor kinase of the KdpDE two-component system, which in *Staphylococcus aureus* regulates the corresponding KdpABC transporter. Finally, we also show that *S. aureus* contains a homolog of KimA, which mediates potassium transport. Thus, the c-di-AMP–dependent control of systems involved in potassium homeostasis seems to be conserved in phylogenetically related bacteria. Surprisingly, the growth of an *L. monocytogenes* mutant lacking the c-di-AMP–synthesizing enzyme *cdaA* is only weakly inhibited by potassium. Thus, the physiological impact of the c-di-AMP–dependent control of potassium uptake seems to be less pronounced in *L. monocytogenes* than in other Firmicutes.

Bacteria use complex signal transduction systems to adjust the cellular turgor to the environmental osmolarity (1–3). Under hyperosmotic growth conditions, potassium ions are imported to prevent water efflux from the cytosol and to increase the cellular turgor (4). The potassium ions are thereupon often replaced by compatible solutes such as glycine betaine and ectoine, osmolytes that do not disturb essential cellular processes (4). Depending on the external osmolarity, the import and export of osmolytes have to be tightly controlled to prevent osmotic swelling and shrinking of the cell, respectively (1, 5, 6). Although osmoregulation has been intensively studied, it is still rather unclear how a cell senses the environmental osmolarity to adjust the turgor accordingly. The second messenger cyclic diadenosine monophosphate (c-di-AMP),[2] which is produced by specific diadenylate cyclases (DACs), plays a key role in regulating the turgor in Firmicute bacteria because it controls the uptake and export of osmolytes, including potassium (see below) (5, 7–15). c-di-AMP was discovered during the structural characterization of DNA integrity scanning protein DisA, which is involved in DNA damage response and in controlling sporulation initiation in the Gram-positive bacterium *Bacillus subtilis* (16–20). DisA is present in spore-forming Firmicutes, in actinobacteria (21), and in hyperthermophilic bacteria (17). Whereas DisA is the only c-di-AMP-producing enzyme in actinobacteria, bacteria like *B. subtilis* also contain the DACs CdaA and CdaS, of which the latter is required for efficient spore germination (22, 23). CdaA is attached to the membrane, and DisA and CdaS are soluble proteins (17, 22, 24). CdaA is the most abundant DAC, and many prominent apathogenic and pathogenic Gram-positive bacteria like *Lactococcus lactis*, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Streptococcus agalactiae* rely only on this DAC for c-di-AMP synthesis (21). Because c-di-AMP is essential for growth of these bacteria (14, 25, 26), the DAC CdaA is an interesting target for novel antibiotics.

c-di-AMP is also intracellularly degraded by specific phosphodiesterases (PDEs), which can be assigned to three different groups (23, 27). The GdpP- and PgpH-type PDEs consist of domains that are involved in signaling and enzyme catalysis. Both PDEs are attached to the membrane, suggesting that the enzymes may sense and respond to extracellular cues. The DhhP-type PDEs, which are located in the cytosol, form the third group of c-di-AMP–degrading enzymes (27). Because the DACs and the PDEs determine the cellular c-di-AMP levels

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[2] The abbreviations used are: c-di-AMP, cyclic diadenosine monophosphate; DAC, diadenylate cyclase; DRaCALA, differential radial capillary action of ligand assay; SOE, splicing by overhang extension; PDE, phosphodiesterase; IPTG, isopropyl 1-thio-β-D-galactopyranoside; LB, lysogeny broth; aa, amino acids; LSM, *Listeria* synthetic medium; OD, optical density; ANOVA, analysis of variance.

that are required for optimal growth in environments with changing osmolarities (5, 15), the activities of the enzymes have to be tightly regulated. Recently, it has been observed that the phosphoglucosamine mutase GlmM inhibits the DAC CdaA in *L. lactis* and *S. aureus*, suggesting a link between c-di-AMP metabolism and cell wall biosynthesis (28, 29). However, the molecular mechanisms by which the DACs, the PDEs, and GlmM sense the environmental osmolarity are unknown. Moreover, the sensing mechanisms may vary among the enzymes due to the different domain composition and cellular localization.

Several c-di-AMP targets have been identified. c-di-AMP activates the DNA-binding activity of the transcription factor DarR in *Mycobacterium smegmatis* (30). In *L. monocytogenes*, c-di-AMP inhibits the pyruvate carboxylase PycA (31, 32). Moreover, c-di-AMP binds to the CBS (cystathione-beta-synthase domain–containing) proteins CbpA and CbpB and the PII-like signal transduction DarA in this organism (31, 33). The DarA homologs from *B. subtilis* and *S. aureus* have been structurally and biochemically characterized (34–36). Whereas the biological functions of CbpA, CbpB, and DarA remain to be elucidated, several c-di-AMP targets are involved in the transport of osmolytes, such as potassium, glycine betaine, and carnitine (7, 8, 11–15, 26). c-di-AMP inhibits the KimA, KupA/KupB, KtrCD/KtrCB, and CabP-TrkH potassium uptake systems in *B. subtilis*, *L. lactis*, *S. aureus*, and *S. pneumoniae*, respectively (7, 8, 37–39). Moreover, c-di-AMP stimulates the *S. aureus* potassium and sodium transporter CpaA (9). In *Bacillus thuringiensis* and *S. aureus*, the synthesis of the KdpFABC potassium transporter is also inhibited by binding of c-di-AMP to the sensor kinase KdpD of the KdpDE two-component system (40, 41). In *B. subtilis*, the expression of the *ktrAB* and *kimA* genes, encoding the potassium transporters KtrAB and KimA, respectively, is negatively regulated by c-di-AMP (5). Thus, c-di-AMP plays a central role in osmolyte homeostasis in a variety of bacteria.

We are interested in the c-di-AMP–dependent control of osmolyte homeostasis in the food-borne pathogen *L. monocytogenes* (42). The ability of *L. monocytogenes* to thrive under adverse conditions including high osmolarity depends on the c-di-AMP–dependent control of osmolyte transport, such as carnitine (11). However, the involvement of c-di-AMP in potassium uptake or homeostasis in *L. monocytogenes* has remained elusive. Here we show that the *L. monocytogenes* KimA (Lmo2130) and KtrCD (Lmo1023 and Lmo0993) proteins are high- and low-affinity potassium transporters, respectively. We also show that the transporters are inhibited by c-di-AMP and that unregulated activity leads to rapid osmotic swelling of *Escherichia coli* cells synthesizing KimA from *L. monocytogenes*. The interaction between c-di-AMP and KtrC, as well as between c-di-AMP and KdpD, was also confirmed *in vitro*. Moreover, the C-terminal domain of KimA is important for the c-di-AMP–dependent regulation of potassium uptake. Finally, we show that the control of potassium uptake is not an essential function of c-di-AMP in *L. monocytogenes*.

Results

In silico identification of potassium transporters from L. monocytogenes

Both *B. subtilis* and *S. aureus* contain well-described potassium uptake systems. *B. subtilis* uses the high-affinity transporters KtrAB and KimA and the low-affinity transporter KtrCD (5, 43). By contrast, *S. aureus* relies on the high-affinity transporter KdpFABC, whose synthesis and activity is controlled by the two-component system KdpDE and c-di-AMP, respectively (41, 44, 45). *S. aureus* also contains the low-affinity potassium transport systems KtrCB and KtrCD sharing the accessory protein KtrC (44). A BLASTp sequence analysis revealed that the *L. monocytogenes* genome codes for the KdpABCDE (Lmo2682-Lmo2678) and KtrCD (Lmo1023, Lmo0993) proteins, which show about 31–56% and 51–64% overall amino acid identity with the homologs from *S. aureus* and *B. subtilis*, respectively. The *kdpF* gene that has been shown to be important for proper function of the Kdp system in *E. coli* does not exist in the *L. monocytogenes* genome (46). A homolog of the high-affinity potassium transporter KimA from *B. subtilis* is also present in *L. monocytogenes* and *S. aureus* (5). The KimA homologs from *B. subtilis*, *L. monocytogenes*, and *S. aureus* are from now on designated as $KimA^{Bsu}$, $KimA^{Lmo}$ (Lmo2130), and $KimA^{Sau}$ (Sacol2443), respectively. $KimA^{Lmo}$ and $KimA^{Sau}$ show about 59 and 57% overall amino acid identity, respectively, with the *B. subtilis* homolog (Fig. 1*A*). The membrane topology was illustrated using the web-based tool Protter (47). Like $KimA^{Bsu}$, $KimA^{Lmo}$ also contains an N-terminal extracellular domain, 11 transmembrane helices, and a C-terminal intracellular domain, which might be important for activity control of the transporter (Fig. 1*B*). To conclude, although *B. subtilis*, *L. monocytogenes*, and *S. aureus* are phylogenetically related, each species uses a different set of transporters for potassium uptake.

In vivo activities of the L. monocytogenes potassium transporters

To assess whether KdpABC, KimA, and KtrCD from *L. monocytogenes* are active in potassium transport, we cloned the *kdpABC*, *kimA*, and *ktrCD* genes using the plasmid pWH844, which allows IPTG-dependent expression of heterologous genes in *E. coli* (48). We also cloned a truncated $kimA^{Lmo}$ gene encoding the ΔC-$KimA^{Lmo}$ variant lacking 152 amino acids of the C-terminal cytosolic domain. Furthermore, we cloned the $kimA^{Sau}$ gene from *S. aureus*, to evaluate whether KimA homologs from other Firmicutes are involved in potassium uptake. The resulting plasmids were used to transform the *E. coli* strain LB650 that is unable to take up potassium via the native uptake systems Kup, KdpABC, TrkG, and TrkH (49). The strain is suitable to study potassium transporters because it is only viable in minimal medium supplemented with potassium concentrations above 15 mM KCl (see Fig. 4). The empty plasmid and a plasmid encoding the *B. subtilis ktrAB* genes, which were previously shown to mediate potassium transport in *E. coli* LB650 (5), served as negative and positive controls, respectively. The cells were grown during the day in M9 medium with 50 mM KCl and without IPTG induction, col-

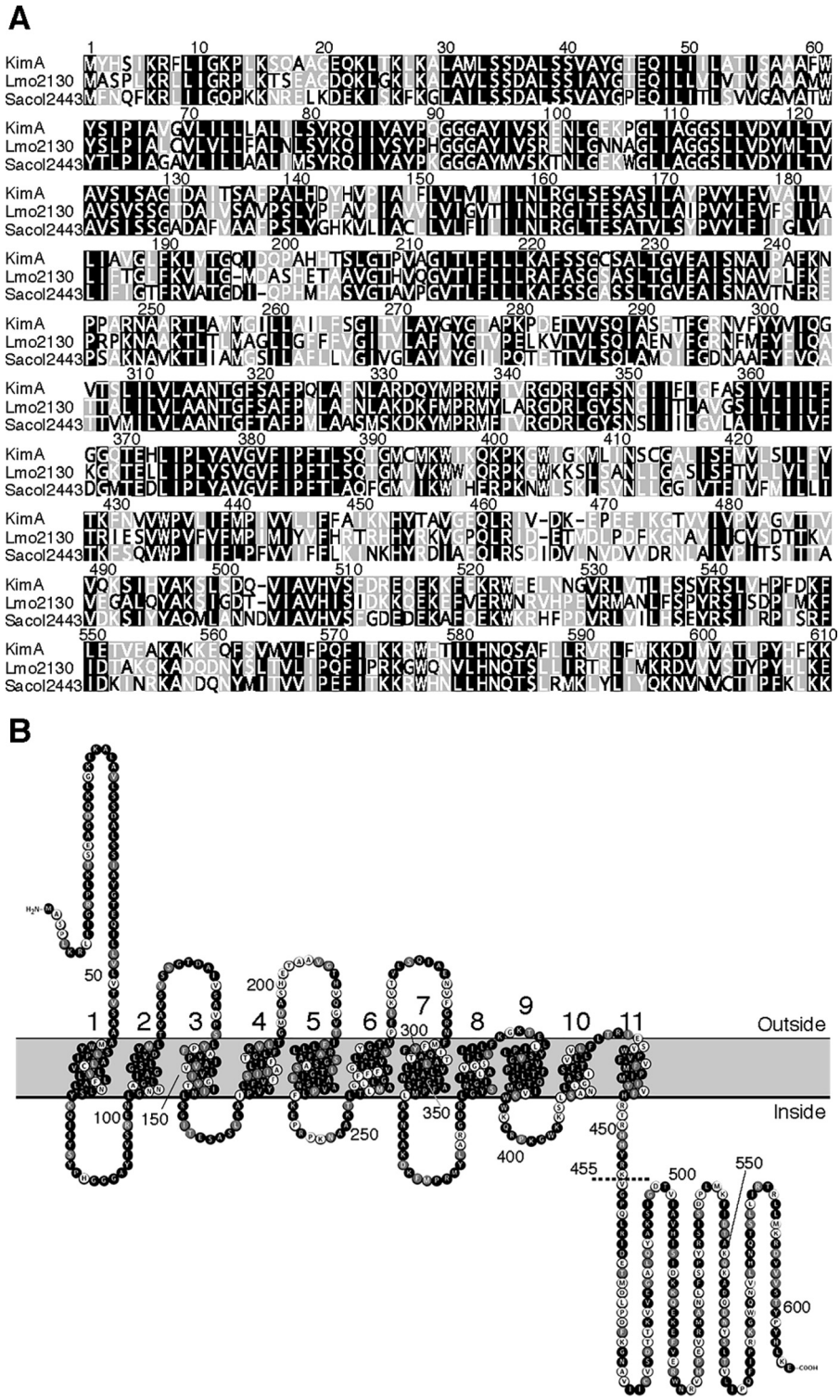

Figure 1. Alignment of KimA homologs and domain organization of the KimA^{Lmo} protein. *A*, MUSCLE alignment of the KimA^{Bsu}, KimA^{Lmo}, and KimA^{Sau} homologs from *B. subtilis*, *L. monocytogenes* (Lmo2130), and *S. aureus* (Sacol2443), respectively, generated with the Geneious software package (63). Amino acids in *black*, *gray*, and *white* have an amino acid similarity of >80, 60–80, or <60%, respectively. *B*, predicted membrane topology of KimA^{Lmo} overlaid with a MUSCLE alignment between KimA^{Bsu} and KimA^{Lmo}. The *dashed line* indicates the position at which the KimA^{Lmo} protein was truncated. Amino acids in *black* are identical; amino acids in *gray* are similar, and amino acids in *white* are nonsimilar.

lected by centrifugation, washed in potassium-free medium, and propagated on M9 minimal medium plates without and with 10 mM IPTG. As shown in Fig. 2, with the exception of the strain harboring the plasmid for *kimA^{Sau}* expression, the bacteria could not grow in the absence of IPTG. The weak growth of the cells containing the *kimA^{Sau}* gene could be due to a leaky promoter and due to the high affinity of the encoded KimA^{Sau} transporter for potassium (see below). By contrast, the strains carrying the *ktrAB^{Bsu}*, *kimA^{Lmo}*, and *ktrCD^{Lmo}*, could grow with low amounts of K^+ when these genes were induced with IPTG

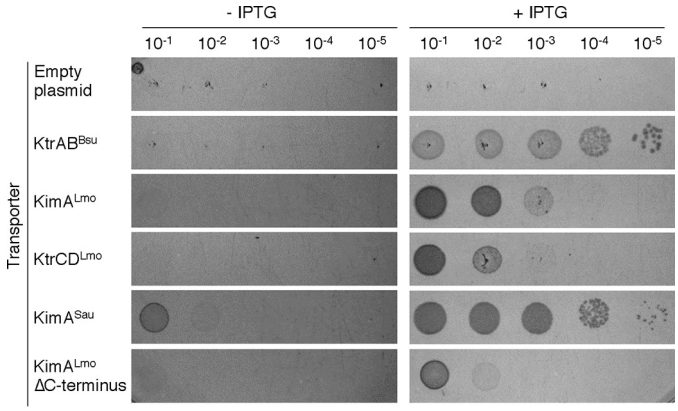

Figure 2. Drop dilution assay to assess the activities of putative potassium transporters. *E. coli* LB650 strains harboring plasmids pWH844 (empty plasmid), pBP372 (*ktrAB*^{Bsu}, positive control), pBP384 (*kimA*^{Lmo}), pBP396 (*kimA*^{Lmo} ΔC terminus), pBP385 (*Kim*^{Sau}), and pBP371 (*ktrCD*^{Lmo}) were grown to an OD_{600} of 0.3–0.5 in M9 minimal medium supplemented with 50 mM KCl. The cells were washed for 1 h in potassium-free M9 medium, the OD_{600} was adjusted to 0.1, the suspension was serially 10-fold diluted, and 5 μl of the diluted cell suspensions were plated on M9 plates containing 10 mM KCl. IPTG was added to a final concentration of 50 μM to induce the expression of the transporter genes. The plates were incubated for 24 h at 37 °C.

(Fig. 2). Moreover, the *kimA*^{Lmo} variant lacking the C-terminal domain (*kimA*^{Lmo} ΔC) also supported growth of the strains, albeit less well than the full-length protein. These results indicate that the N-terminal extracellular domain and the 11 transmembrane helices of KimA^{Lmo} from *L. monocytogenes* are sufficient for mediating potassium import in *E. coli* (Fig. 2). Expression of *ktrAB*^{Bsu} from *B. subtilis* restores growth on low potassium concentrations, agreeing with previous reports that KtrAB^{Bsu} is a high-affinity potassium transporter (5, 43). Expression of KimA^{Sau} from *S. aureus* in the *E. coli* strain LB650 resulted in much better growth than those strains expressing KimA^{Lmo} and KtrCD, indicating that KimA^{Sau} is likely a high-affinity potassium transporter (Fig. 2). Thus, KimA^{Lmo} and KtrCD^{Lmo} from *L. monocytogenes* as well as KimA^{Sau} from *S. aureus* are indeed potassium transporters. The putative potassium transporter KdpABC^{Lmo} did not support growth of the *E. coli* strain LB650 irrespective of whether the *kdpABC*^{Lmo} genes were expressed from the IPTG- and arabinose-dependent plasmids pWH844 and pBAD24 (data not shown; see "Experimental procedures"). Therefore, the KdpABC^{Lmo} system was not further analyzed in regard to its affinity to potassium ions and *in vivo* inhibition by c-di-AMP.

Apparent affinities of KimA and KtrCD for potassium

To determine the apparent affinities of KimA^{Lmo}, the KimA^{Lmo} ΔC terminus variant (ΔC-KimA^{Lmo}) and KtrCD from *L. monocytogenes* and KimA^{Sau} from *S. aureus*, we determined the growth rates of the *E. coli* strain LB650 synthesizing the potassium transporters in M9 minimal medium supplemented with different amounts of potassium. The strains carrying the empty plasmid and expressing the *B. subtilis ktrAB* genes served as negative and positive controls, respectively. The growth rates were plotted against the potassium concentrations and fitted to the Michaelis–Menten equation (5). The V_{max} values and the apparent affinities are summarized in Table 1. As shown in Figs. 3 and 4, each *E. coli* strain required a different concentration of external potassium to reach half-maximal growth; the strains synthesizing the transporters KtrCD^{Lmo}, KimA^{Lmo}, ΔC-KimA^{Lmo}, KimA^{Sau}, and KtrAB^{Bsu} required 6.30 ± 2.06, 0.35 ± 0.12, 2.99 ± 0.65, 0.14 ± 0.02, and 0.03 ± 0.01 mM, respectively. These results demonstrate that KtrCD^{Lmo} and KimA^{Lmo} from *L. monocytogenes* are transporters with low and moderately high affinities for potassium, respectively. Moreover, the C-terminal intracellular domain of KimA^{Lmo} is important for full activity of the transporter (Figs. 1*B* and 4). In contrast to KtrCD^{Lmo} and KimA^{Lmo}, KimA^{Sau} from *S. aureus* is a high-affinity potassium transporter, which is in line with the observation that the *E. coli* strain LB650 synthesizing KimA^{Sau} and KtrAB^{Bsu} grew comparatively well with low amounts of potassium (Fig. 2).

Inhibition of KimA and KtrCD potassium transport activity by c-di-AMP

Several recent studies indicate that c-di-AMP is essential for viability of Gram-positive bacteria like *B. subtilis*, *L. lactis*, *L. monocytogenes*, *S. agalactiae*, and *S. aureus* because the nucleotide controls influx of osmolytes like potassium whose accumulation leads to cell lysis due to water uptake (5, 14, 15, 25, 26). Thus, either synthesis of the potassium transporters or their activity or both need to be tightly regulated. As shown in Fig. 5, the IPTG-dependent overexpression of the *ktrAB*^{Bsu} and *kimA*^{Lmo} genes encoding high-affinity potassium transporters KtrAB^{Bsu} and KimA^{Lmo}, respectively, in *E. coli* during growth in M9 minimal medium caused a strong increase of the cellular volume. Moreover, the growth of *E. coli* synthesizing the higher-affinity KtrAB^{Bsu} transporter was in addition significantly reduced, as illustrated by the decline of the optical density (Fig. 5, *top right corners*). By contrast, in the absence of the inducer IPTG, the growth and the volume of the cells containing the *ktrAB*^{Bsu} and *kimA*^{Lmo} genes were indistinguishable from that of the cells carrying the empty vector. Thus, once sufficient potassium has been taken up by the bacteria to cope with the osmolarity of the environment, the activities of osmolyte transporters have to be reduced to prevent further ion uptake and cell lysis. It has indeed been demonstrated that the activity of the cytoplasmic gating component of the transporters KtrCB and KtrCD from *S. aureus* as well as the KimA^{Bsu} transporter from *B. subtilis* are inhibited by c-di-AMP (7, 39). Like KimA^{Bsu} from *B. subtilis*, the KimA^{Lmo} homolog from *L. monocytogenes* belongs to a novel class of high-affinity potassium transporters (see above) (5). However, whether c-di-AMP directly binds to KimA^{Lmo} and KtrCD^{Lmo} to inhibit the transport activity of the proteins has not been tested so far.

To assess whether c-di-AMP affects the activity of KimA^{Lmo} and KtrCD^{Lmo}, we established a co-expression system using the *E. coli* strain LB2003, which carried unmarked mutations in the *kdp*, *kup*, and *trk* genes, and enable the use of multiple plasmids encoding chloramphenicol and ampicillin resistance genes (49). Like the *E. coli* strain LB650, LB2003 is deficient in the Kdp, Kup, and Trk potassium uptake systems and is therefore only able to grow at low potassium concentrations when synthesizing a potassium transporter. Moreover, *E. coli* lacks c-di-AMP–producing and c-di-AMP–degrading enzymes, which is a prerequisite to assess the phenotypic effect of c-di-AMP on

Table 1
Michaelis–Menten constants of the potassium transporters

Mean values of the K_m and S.E. are shown ($n = 4$). p values were always <0.0001 (****) compared with empty plasmid alone ($F_{(8,18)} = 54.69$, ANOVA with Dunnett's post hoc test). Mean values of the V_{max} and S.E. are shown ($n = 4$). $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****) compared with empty plasmid alone ($F_{(5,18)} = 14.5$, ANOVA with Dunnett's post hoc test).

	Empty vector	KtrAB^{Bsu}	KimA^{Lmo}	KimA^{Lmo} ΔC terminus	KtrCD^{Lmo}	KimA^{Sau}
Apparent K_m (mM KCl)	56.28 ± 14.62	0.03 ± 0.01	0.35 ± 0.12	2.99 ± 0.65	6.30 ± 2.06	0.14 ± 0.02
V_{max} (μ (h^{-1}))	0.82 ± 0.13	0.73 ± 0.05	0.52 ± 0.04 (***)	0.40 ± 0.01 (****)	0.59 ± 0.12 (**)	0.62 ± 0.05 (**)

the activity of KimA^{Lmo} and KtrCD^{Lmo}. The plasmids pBP384 (*kimA^{Lmo}*), pBP396 (Δ*C-kimA^{Lmo}*), and pBP371 (*ktrCD^{Lmo}*) were used for the IPTG-dependent expression of ΔC-KimA^{Lmo}, KimA^{Lmo}, and KtrCD^{Lmo}, respectively. The empty plasmid pWH844 served as a negative control. The *L. monocytogenes* DAC CdaA and the inactive CdaA* variant D171N (50) are encoded by the arabinose-inducible plasmids pBP370 and pBP373, respectively. The strains carrying pWH844, pBP384, pBP396, and pBP371 as well as either of the two DAC-encoding plasmids were grown in M9 minimal medium supplemented with 30, 0.35, 3, and 7 mM KCl, respectively, conditions that allow half-maximal growth of the bacteria. As shown in Fig. 6, growth of the strains carrying the empty plasmid pWH844, and synthesizing the active and the catalytically inactive CdaA and CdaA* variants, respectively, was not reduced. Thus, neither the DAC proteins nor c-di-AMP affect growth of the *E. coli* strain. By contrast, growth of the bacteria synthesizing KimA^{Lmo} and KtrCD^{Lmo} was reduced when the active DAC CdaA was co-produced, indicating that c-di-AMP inhibits the transporter with a moderately high affinity for potassium and to a lesser extent also the low-affinity transporter (Fig. 6). Growth was not affected in the absence of a functional DAC and, thus, of c-di-AMP production. Moreover, c-di-AMP did not affect the activity of the C-terminally truncated ΔC-KimA^{Lmo} variant, indicating that the C-terminal intracellular domain of the transporter contributes to c-di-AMP-dependent regulation (see "Discussion"). Surprisingly, c-di-AMP did not inhibit the activity of KimA^{Sau} from *S. aureus* (data not shown). To conclude, the potassium transporters KimA^{Lmo} and KtrCD^{Lmo} from *L. monocytogenes* are both inhibited by c-di-AMP.

Effect of potassium on growth of a c-di-AMP-free L. monocytogenes strain

Previously, it has been shown that c-di-AMP is essential in *B. subtilis* to control the uptake of potassium to toxic levels (5). To investigate whether c-di-AMP is also essential for the control of potassium uptake in *L. monocytogenes*, we constructed a markerless deletion of the *cdaA* gene, encoding the sole c-di-AMP–synthesizing enzyme. As described previously, we confirmed that the *cdaA* mutant is not viable on complex, but on chemically defined growth medium (10, 11). We prepared *Listeria* synthetic medium (LSM) (11) without potassium (LSM-K^+) and observed that potassium concentrations below 1 mM impair the growth of the *L. monocytogenes* WT strain (Fig. 7). The growth behavior of the WT strain was not affected at potassium chloride concentrations higher than 1 mM. The *L. monocytogenes cdaA* mutant shows a slightly slower growth than the WT at potassium concentrations above 1 mM. However, high potassium concentrations did not fully inhibit growth of the *L. monocytogenes cdaA* mutant strain as it has been shown for a c-di-AMP-free strain of *B. subtilis* (see Fig. 7) (5).

c-di-AMP interaction with the KimA homologs and KtrCD^{Lmo}

To assess the interaction between c-di-AMP and the potassium transporters or their regulators, we performed a differential radial capillary action of ligand assay (DRaCALA) with the proteins KimA^{Lmo}, ΔC-KimA^{Lmo}, KimA^{Sau}, KtrC^{Lmo}, KdpABC^{Lmo}, and KdpD^{Lmo} (see "Experimental procedures"). We also tested the interaction between c-di-AMP and the 156- and 158-amino-acid-long C-terminal cytosolic domains of KimA^{Lmo} and KimA^{Sau}, respectively. This domain could be involved in the c-di-AMP– dependent control of KimA potassium transport activity. The lysate of the *E. coli* strain DH5α containing the empty plasmid pWH844 or pGP172 served as a negative controls. Whereas the majority of the proteins showed no specific interaction with c-di-AMP in the DRaCALA assay, KtrC^{Lmo}, the cytosolic protein of the KtrCD potassium transporter, and KdpD^{Lmo}, the sensor kinase of the KdpDE two-component system, gave positive results (Fig. 8). To conclude, the potassium transport activity of KtrCD^{Lmo} from *L. monocytogenes* is inhibited by c-di-AMP *in vivo*, and the nucleotide binds to the KtrC subunit of the KtrCD^{Lmo} transporter and to KtrD of the KdpDE^{Lmo} two-component system *in vitro*. Due to toxicity, we were unable to purify the full-length KimA^{Lmo} protein. The failure of purification of the C-terminal part of KimA also precludes further *in vitro* characterization.

Discussion

Here, we have identified and characterized the potassium transporters KtrCD^{Lmo} and KimA^{Lmo} from *L. monocytogenes*. As stated above, the KtrCD homologs from *L. monocytogenes* and *B. subtilis* show 64% overall sequence identity and have similar affinities for potassium (see Table 1) (43). We also demonstrate that *S. aureus* possesses a homolog of KimA (Sacol2443). The KimA homologs from *S. aureus* and *L. monocytogenes* belong to a novel class of high-affinity potassium transporters that are active at low external potassium concentrations (5). Moreover, the potassium transport activity of KtrCD^{Lmo} and KimA^{Lmo} from *L. monocytogenes* is inhibited by c-di-AMP (Fig. 6). Furthermore, we show that the C-terminal cytosolic domain is important for the c-di-AMP–mediated regulation of KimA^{Lmo} *in vivo* because the C-terminally truncated variant lacking 156 amino acids did not respond to the nucleotide. Recently, it has been shown that c-di-AMP binds to the KimA homolog from *B. subtilis* and controls the uptake of potassium by the transporter *in vivo* (39). Unfortunately, we could not show the binding of c-di-AMP to the full-length

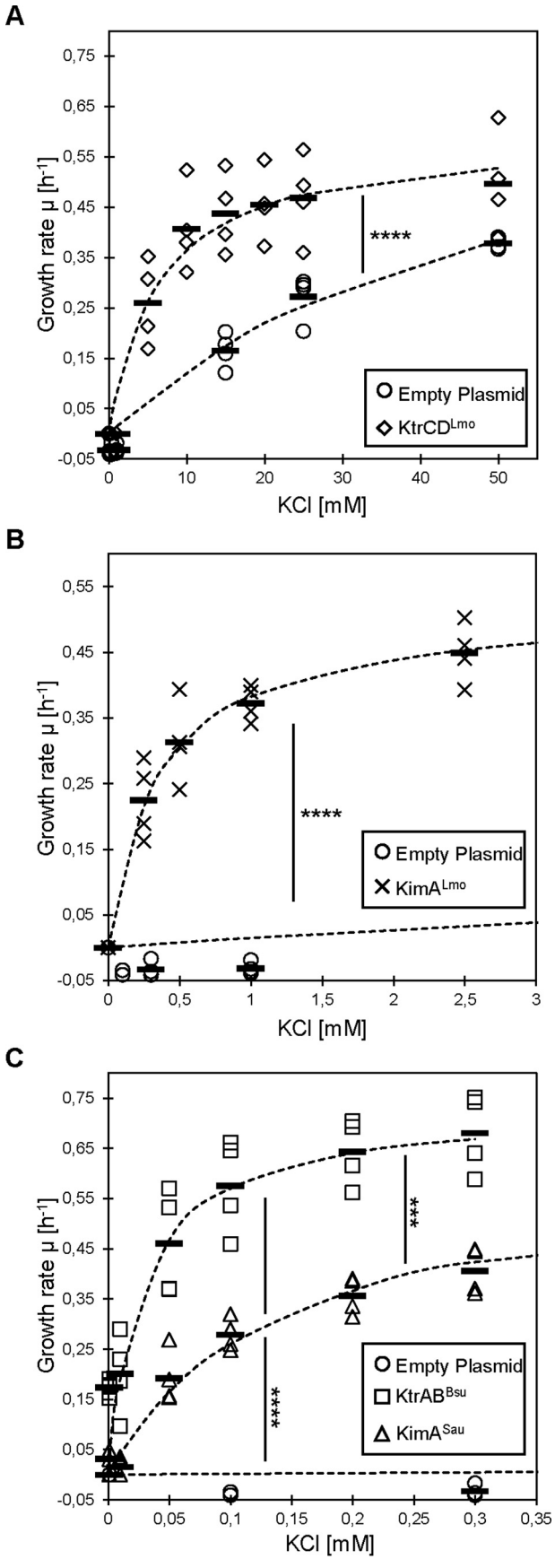

Figure 3. Potassium-dependent growth of *E. coli* synthesizing potassium transporters from *L. monocytogenes* and *S. aureus*. *A*, *E. coli* LB650 strain harboring the plasmids pWH844 (empty plasmid) and pBP371 (*ktrCD*^{Lmo}). *B*, *E. coli* LB650 strain harboring the plasmid pBP384 (*kimA*^{Lmo}). *C*, *E. coli* LB650 strain harboring the plasmids pBP372 (*ktrAB*^{Bsu}) and pBP385 (*kim*^{Sau}). The strains were grown to an OD_{600} of 0.3–0.5 in M9 minimal medium supplemented with 50 mM KCl. The cells were washed for 1 h in potassium-free M9 medium. Multiwell plate reader growth assays with different KCl concentrations were performed ($n = 4$). The growth rates were plotted against the KCl concentrations and fitted to the Michaelis–Menten equation. *Bars*, means; *dashed lines*, fitted curves. Significant differences between the fitted curves are shown ($p < 0.0001$ (****)) ($F_{(2,2997)} = 1480$, one-way ANOVA with Tukey's post hoc test).

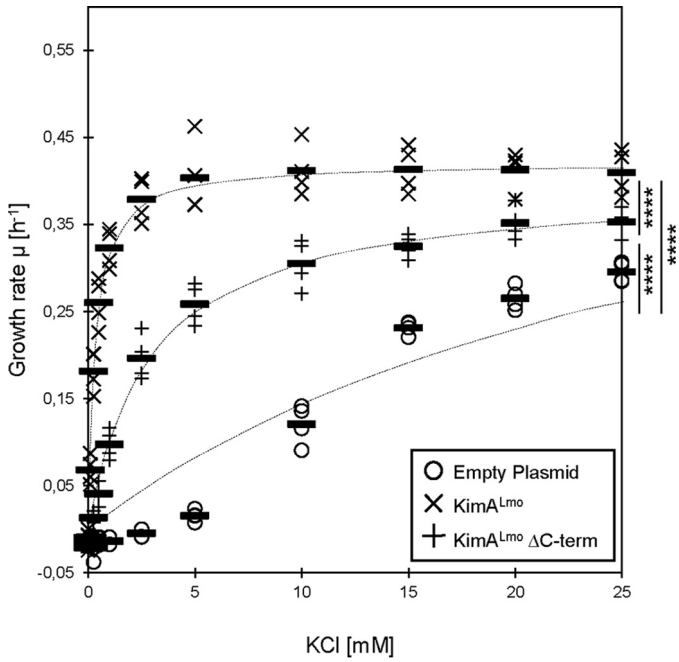

Figure 4. Potassium-dependent growth of *E. coli* synthesizing the full-length and the C-terminally truncated KimA^{Lmo} protein. *E. coli* LB650 strains harboring plasmids pWH844 (empty plasmid), pBP384 (*kimA*^{Lmo}), and pBP396 (*kimA*^{Lmo} ΔC terminus) were grown to an OD_{600} of 0.3–0.5 in M9 minimal medium supplemented with 50 mM KCl. The cells were washed for 1 h in potassium-free M9 medium. Multiwell plate reader growth assays with different KCl concentrations were performed ($n = 4$). The growth rates were plotted against the KCl concentrations and fitted to the Michaelis–Menten equation. *Bars*, means; *dashed lines*, fitted curves. Significant differences between the *fitted curves* are shown ($p < 0.0001$ (****)) ($F_{(4,2307)} = 540.1$, one-way ANOVA with Tukey's post hoc test).

KimA^{Lmo} protein and to the C-terminal domain of KimA^{Lmo}. However, we speculate that binding of c-di-AMP to the cytosolic domain is required of for regulation of KimA^{Lmo} *in vivo* (Fig. 6). Therefore, it might be worthwhile to study the role of the C-terminal domain in controlling the activity of the high-affinity potassium transporter KimA. Surprisingly, the KimA^{Lmo} transporter from *L. monocytogenes* has a much lower affinity for potassium than the homolog from *B. subtilis* (39, 43). As the external concentrations of potassium are rather low, it is tempting to speculate that *L. monocytogenes* possesses an additional high-affinity potassium transporter to be able to compete with other bacteria when the extracellular potassium is scarce. The phylogenetically related bacteria *B. subtilis* and *S. aureus* contain two high-affinity potassium transport systems that are active during growth at low potassium concentrations. *B. subtilis* employs the high-affinity potassium transporters KtrAB and KimA under potassium-limiting growth conditions (5, 39, 43). Previously, it has been shown that *S. aureus* relies on the

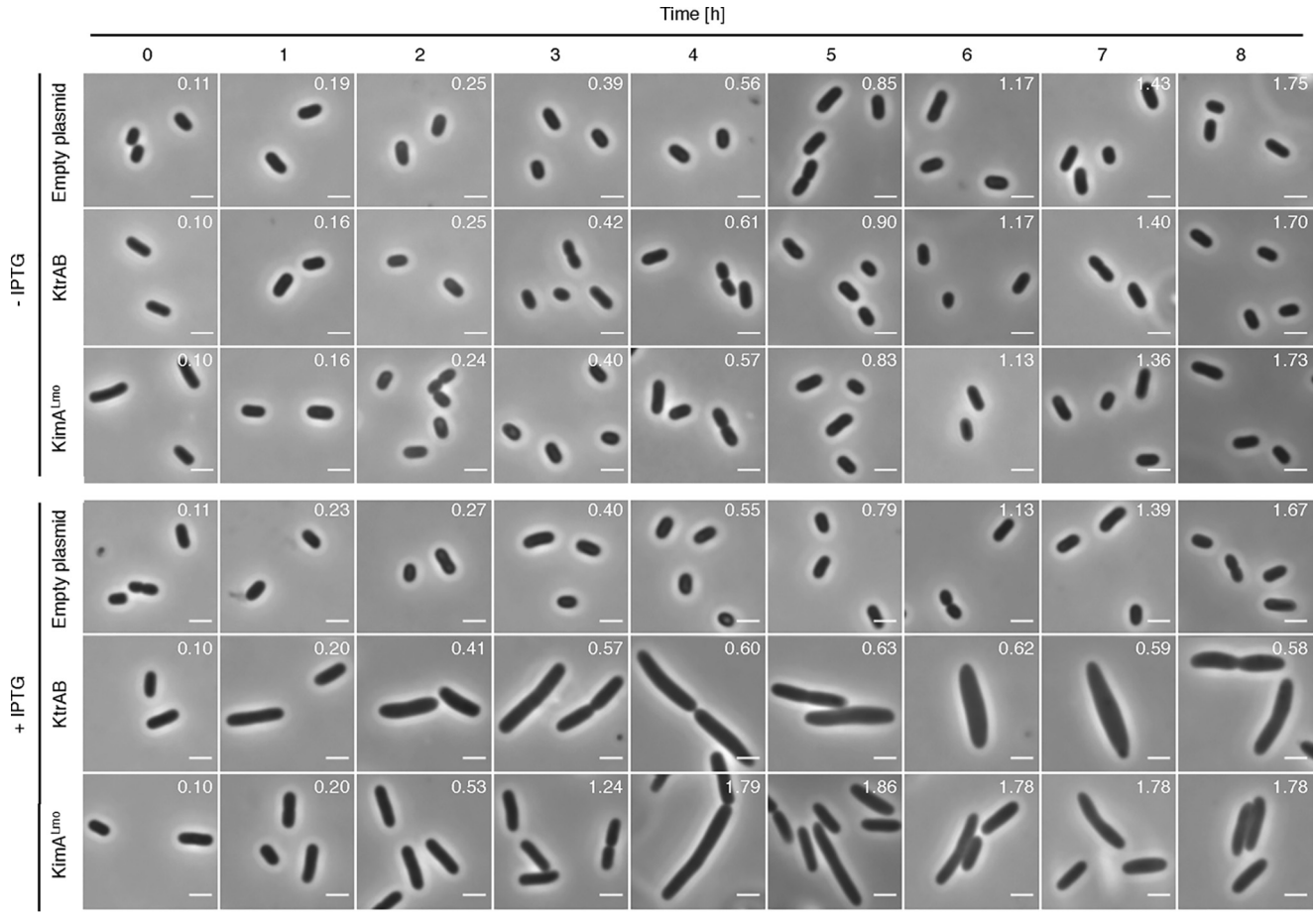

Figure 5. Impact of unregulated potassium import on the cell volume of *E. coli*. Derivatives of the *E. coli* strain LB650 harboring plasmids pWH844 (empty plasmid), pBP372 (*ktrAB*^{Bsu}), and pBP384 (*kimA*^{Lmo}) were grown overnight in LB-K medium. The cells were washed and cultivated in M9 medium without and with 1 mM IPTG for the induction of the transporter genes. The OD_{600}, which is shown in the *top right corners* of the microscopic pictures, was measured in hourly intervals. *Scale bar*, 2 μm.

high-affinity transporter KdpFABC, whose synthesis and activity is regulated by the two-component system KdpDE (38, 44, 45). KimA^{Sau} could also be important for growth of *S. aureus* when the extracellular potassium concentrations are low. In contrast to *S. aureus*, the KdpABC homolog of *L. monocytogenes* does not seem to contribute to potassium uptake. It has been shown previously that the small membrane protein KdpF is required for proper function of the *E. coli* Kdp potassium transport system (46). As described above, no KdpF homolog is present in *L. monocytogenes*. Therefore, the lack of KdpF in *L. monocytogenes* could be the reason why the KdpABC system is not active in potassium transport. Interestingly, we found that c-di-AMP binds to the sensor kinases KdpD of the KdpDE two-component system that might be involved in controlling the expression of the *kdpABC* genes in *L. monocytogenes* (Fig. 8). However, our comparative RNA-Seq experiments using the WT strain and a c-di-AMP–free *cdaA* mutant strain in chemically defined medium revealed that the lack of c-di-AMP does not alter the expression of genes involved in potassium uptake (data not shown). In *B. subtilis*, it has been shown that the 5′-UTRs of the *kimA* and *ktrAB* genes contain *ydaO* riboswitches preventing synthesis of the transporters in the presence of c-di-AMP (5, 51). However, c-di-AMP– dependent riboswitches that could be involved in controlling the expression of potassium transporter genes are absent in *L. monocytogenes.* Therefore, it remains to be elucidated under which conditions the potassium transporter genes are transcribed. Moreover, it has to be investigated whether c-di-AMP controls the expression of the *kimA*, *ktrC, ktrD*, and *kdpABC* genes in *L. monocytogenes* at all.

As stated above, during growth under hyperosmotic conditions many bacteria take up potassium ions to prevent water efflux from the cytosol and to increase the cellular turgor (1–4). Once the cellular turgor has been adjusted to the environmental osmolarity, the transport of potassium ions across the cell membrane has to be reduced to prevent osmotic swelling and cell lysis (1–4). A reduction of the ion uptake might be achieved either by proteolytic degradation or by controlling the activity of the transporters through binding of low-molecular weight ligands. It has indeed been shown that transport systems are rapidly degraded when the respective substrates are not available (52). However, the cellular turgor is a physical variable that changes rapidly and needs to be tightly adjusted (1–4). Thus, it is obvious that the proteolytic degradation of transport systems would be too slow to allow the bacteria to prevent potassium uptake to toxic levels. However, the tight control of the cellular turgor requires the existence of low-molecular weight ligands, which specifically modulate the activity of potassium transport-

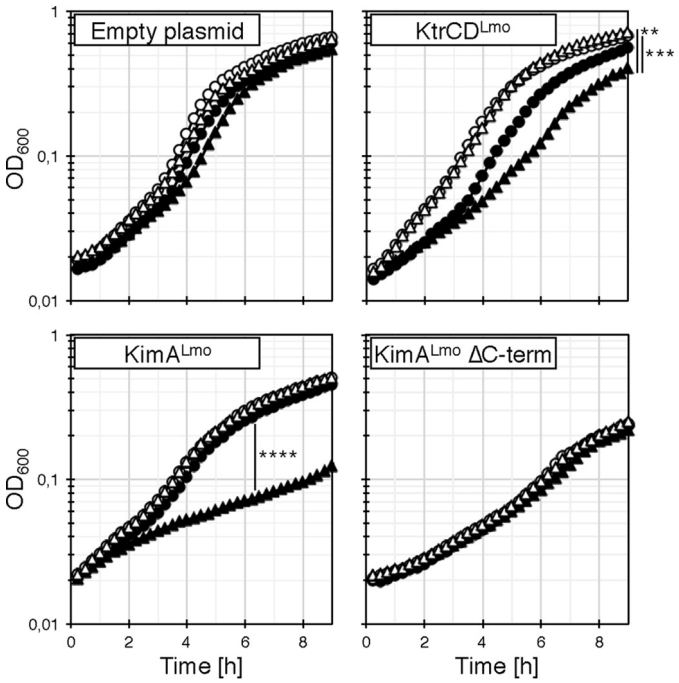

Figure 6. Inhibition of potassium transporters by c-di-AMP. The *E. coli* strain LB2003 harboring the plasmids pWH844 (empty plasmid), pBP371 (*ktrCD*^{Lmo}), pBP384 (*kimA*^{Lmo}), pBP396 (*kimA*^{Lmo} ΔC terminus) and either pBP370 (*cdaA*; *filled symbols*) or pBP373 (*cdaA* D171N; *unfilled symbols*) was grown to an OD_{600} of 0.3–0.5 in M9 medium and washed for 1 h in potassium-free M9 medium. The growth assays were performed with (*triangles*) or without (*circles*) 0.005% (w/v) L-arabinose and at KCl concentrations that are equal to the K_m values of the transporters. Data are means ($n = 3$), and there were no significant differences between the strains harboring the empty plasmid ($F_{(3,260)} = 0.6496$; $p = 0.5838$). The same is valid for the strains harboring the plasmid for the expression of the *kimA*^{Lmo} ΔC terminus variant ($F_{(3,260)} = 0.5085$; $p = 0.6768$). There were significant differences between the strains harboring the plasmid for the expression of the *ktrCD* ($F_{(3,260)} = 6.588$; $p = 0.0003$) or *kimA* genes ($F_{(3,260)} = 12.43$; $p < 0.0001$). $p < 0.05$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****) (one-way ANOVA with Tukey's post hoc test).

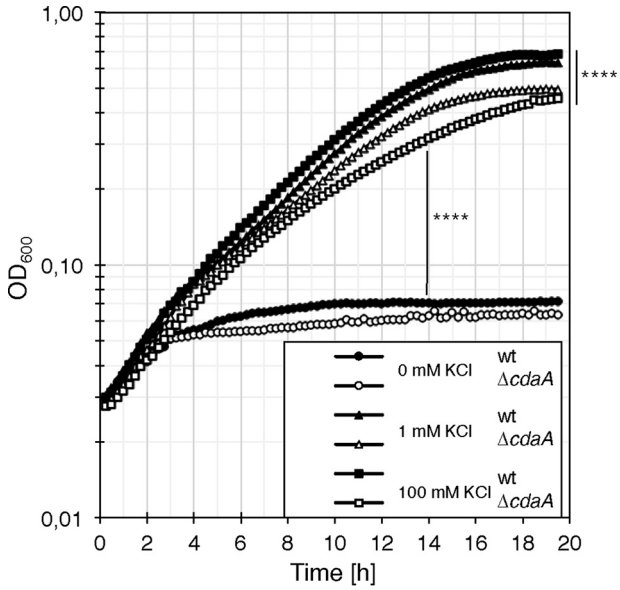

Figure 7. Effect of potassium on growth of the *L. monocytogenes cdaA* mutant. The *L. monocytogenes* WT strain EGD-e (*filled symbols*) and the *cdaA* mutant strain (*nonfilled symbols*) were grown overnight in LSM-K^+ with 1 mM potassium shaking at 37 °C. Cells were washed in LSM-K^+, grown in LSM-K^+, washed again, and subsequently used to inoculate LSM-K^+ medium with the indicated concentrations of KCl (0 mM (*circles*), 1 mM (*triangles*), and 100 mM (*squares*)). Bacteria were grown at 37 °C. Data are means ($n = 3$). Significant differences between the WT or the Δ*cdaA* strains (0 mM KCl) and the strains grown with KCl and between WT and the Δ*cdaA* strains grown with 100 mM KCl are depicted ($p < 0.0001$ (****)) ($F_{(4,462)} = 46.38$, one-way ANOVA with Tukey's post hoc test).

ers and other osmolyte uptake systems. In *S. aureus*, it has been shown that the low-affinity potassium transporters KtrCB and KtrCD are inhibited by the second messenger c-di-AMP that binds to the RCK_C (regulator of conductance of K^+) domain of the KtrC gating component (7). Moreover, c-di-AMP binds to the CabP protein and prevents potassium uptake by the CabP-TrkH protein complex in *S. pneumoniae* (8). The cytoplasmatic regulatory subunit KtrC of the KtrCD potassium transporter is also bound by c-di-AMP in *Mycoplasma pneumoniae* (53). Recently, it has been demonstrated that the potassium importers KupA and KupB of *L. lactis* are inhibited by c-di-AMP (38). Here, we show that the potassium transporters KtrCD^{Lmo} and KimA^{Lmo} from *L. monocytogenes* are inhibited by the second messenger c-di-AMP. This study also revealed that the uncontrolled influx of potassium ions via the KtrAB^{Bsu} and KimA^{Lmo} results in osmotic swelling of *E. coli* (Fig. 5). Recently, it has been shown that c-di-AMP inhibits the potassium transport activity of the KimA homolog from *B. subtilis* (39). In this organism, c-di-AMP is required to reduce potassium uptake to toxic levels. As described above, c-di-AMP also controls the uptake of potassium at the level of transcription. For instance, c-di-AMP inhibits the sensor kinase KdpD of the KdpDE two-component system and thus reduces the expression of the *kdpFABC* operon encoding the high-affinity KdpFABC potassium transport system from *S. aureus* (41, 45). Moreover, c-di-AMP prevents the expression of the *ktrAB* and *kimA* mRNAs in *B. subtilis*, thereby reducing expression of the high-affinity potassium transporters KtrAB and KimA, respectively (5, 51). It should be noted that c-di-AMP also inhibits the uptake of other osmolytes, such as glycine betaine and carnitine (11–13, 15, 26). Thus, c-di-AMP plays a central role in controlling the activities of potassium transporters and other osmolyte uptake systems, and the c-di-AMP–dependent regulation can occur at two different levels in a variety of bacteria.

Recently, it has been demonstrated that the control of potassium uptake is an essential function of c-di-AMP in *B. subtilis* (5). A *B. subtilis* strain lacking all c-di-AMP–producing enzymes was only viable in medium containing low potassium concentrations. c-di-AMP is also essential in bacteria like *L. monocytogenes*, *S. agalactiae*, and *S. aureus* to prevent uptake of osmolytes to toxic levels (10, 15, 26). However, in these bacteria, the control of glycine betaine and amino acid uptake seems to be the essential function of c-di-AMP. This could explain why an increase of external osmolarity, either by sodium or potassium chloride, rescues the growth defect of a *cdaA* mutant strain in complex media, irrespective of the ion (11). We furthermore show that high amounts of potassium only slightly inhibit the growth of the *cdaA* mutant in defined medium (Fig. 7). Thus, the physiological impact of the c-di-AMP–dependent control of the potassium transporters seems to be less pronounced in *L. monocytogenes* than in bacteria like *B. subtilis* (5). In fact, phylogenetically related bacteria have evolved species-specific mechanisms to regulate the cellular turgor using different osmolytes, but they all use c-di-AMP in

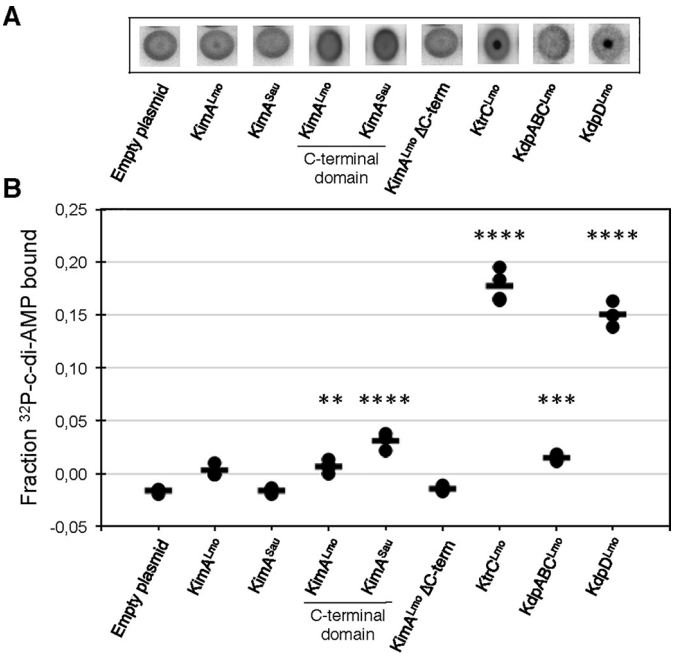

Figure 8. Interaction between c-di-AMP and potassium transporters determined by DRaCALA. *A*, autoradiographs showing the interaction between [^{32}P]c-di-AMP and the potassium transporters as well as the truncated variants that are present in whole-cell lysates of the *E. coli* strains Rosetta (DE3) carrying the plasmid pGP172 (empty plasmid) or the derivatives pBP265 (*kimA*^{Lmo}), pBP266 (*kimA*^{Lmo} ΔC terminus), pBP267 (*kimA*^{Sau}), or NEB T7 Express *I*^{q} carrying the plasmid pWH844 (empty plasmid) or the derivatives pBP346 (*kimA*^{Lmo} C-terminal domain), pBP347 (*kimA*^{Sau} C-terminal domain), pBP345 (*ktrC*^{Lmo}), pBP559 (*kdpABC*^{Lmo}), and pBP560 (*kdpD*^{Lmo}). Both empty vectors showed similar nonbinding (data not shown). *B*, fraction bound of [^{32}P]c-di-AMP is shown for lysates from *E. coli* induced overnight for the expression of the indicated gene. *Bars*, means ($n > 3$). $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****) compared with empty plasmid alone ($F_{(8,20)} = 275.9$, ANOVA with Dunnett's post hoc test).

this essential process (6). It remains to be elucidated how c-di-AMP controls potassium homeostasis in *L. monocytogenes*. Moreover, it will be crucial to identify the osmo-signal–sensing mechanism of the c-di-AMP system, which could be conserved among different bacteria (6, 15).

Experimental procedures

Bacterial strains and growth conditions

The bacterial strains are listed in Table 2. The *E. coli* strains XL1-Blue (Stratagene), Rosetta (DE3) (Novagen), and T7 Express *I*^{q} (New England Biolabs) were used for cloning and protein overproduction. *E. coli* was grown in LB medium, and transformants were selected on LB plates (15 g/liter Bacto agar (Difco)) containing kanamycin (50 μg ml^{-1}), ampicillin, carbenicillin (100 μg ml^{-1}), or chloramphenicol (30 μg ml^{-1}). The *L. monocytogenes* WT strain EGD-e (laboratory strain collection) was cultivated in brain heart infusion medium (Sigma-Aldrich, Darmstadt, Germany). For the deletion of the *cdaA* gene, the LSM was used as described previously (11), with the following minor changes (equimolar substitutions): riboflavin-5′-monophosphate instead of riboflavin; L-isoleucine, L-methionine, and L-valine instead of the DL-enantiomers; and L-cysteine·HCl·H_2O instead of L-cysteine·2HCl. For experiments with defined potassium concentrations, the KH_2PO_4 component of the "phosphate stock solution" was replaced by equimolar concentrated NaH_2PO_4, and for the other 43 components, no chemicals containing potassium salts were used. The LSM without potassium was subsequently adjusted to the depicted concentrations, by the addition of KCl. Erythromycin and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) were used in the deletion process at concentrations of 5 or 100 μg ml^{-1}, respectively. For pouring minimal medium agar plates, 2-fold concentrated medium was prewarmed to 37 °C and mixed with 70 °C prewarmed 2-fold Bacto agar, directly before pouring the plates. The *B. subtilis* WT strain 168 (laboratory strain collection) was cultivated in LB medium. Potassium transporter–deficient *E. coli* strains LB650 and LB2003 were cultivated in LB-K medium (NaCl substituted by 1% KCl (w/v)) (49). M9 medium was used for *E. coli* growth experiments with the following composition: 37.85 mM Na_2HPO_4, 22.05 mM KH_2PO_4, 18.75 mM NH_4Cl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.5 μM $FeCl_3$, 28 mM D-glucose or glycerol as sources of carbon. For the *E. coli* strain LB650, the M9 medium was supplemented with amino acids L-valine, L-isoleucine, L-methionine, L-proline, and L-serine (each 0.02% (w/v)) and 3 μM thiamine. For the *E. coli* strain LB2003, the M9 medium was supplemented with 0.0066% (w/v) casein hydrolysate (acid) (Oxoid), 0.004% (w/v) L-proline, and 3 μM thiamine. For experiments with defined potassium concentrations, the KH_2PO_4 salt was replaced by NaH_2PO_4, and KCl was added as indicated. If not otherwise specified, IPTG was used at a concentration of 50 μM, and L-arabinose was used at 0.005% (w/v).

DNA manipulation

Transformation of *E. coli* was performed using standard procedures (54). Plasmid DNA was extracted using the NucleoSpin Plasmid Kit (Macherey and Nagel). Restriction enzymes, T4 DNA ligase, and DNA polymerases were used as recommended by the manufacturers. DNA fragments were purified using the PCR purification kit (Qiagen). DNA sequences were determined by the Microsynth sequencing laboratories (Göttingen, Germany). Chromosomal DNA of *L. monocytogenes* or *B. subtilis* was isolated using the NucleoSpin Microbial DNA Kit (Macherey and Nagel). Chromosomal DNA of *S. aureus* COL was kindly provided by Dr. Jan Pané-Farré (University of Greifswald, Germany). Oligonucleotides were purchased from Sigma-Aldrich (Darmstadt, Germany).

Plasmid construction

The genes encoding putative potassium transporters were introduced into the vector pWH844, allowing IPTG-dependent expression in *E. coli* (48). The *kimA*^{Lmo} and *kimA*^{Sau} genes were amplified using the oligonucleotide pairs JH95/JH96 and JH97/JH98, respectively (Table 3). The PCR products were EcoRI/BamHI-digested and ligated to pWH844. The resulting plasmids were designated as pBP384 and pBP385 (Table 4). To study the role of the C-terminal domain of KimA^{Lmo}, the plasmid pBP396 was constructed. The truncated *kimA*^{Lmo} gene was amplified with the oligonucleotide pair JH95/JH120, digested with EcoRI/BamHI, and ligated to pWH844. The plasmid pBP371 for the expression of the *L. monocytogenes ktrCD* genes was constructed as follows. The *ktrC* and *ktrD* genes were amplified using the oligonucleotide pairs JH59/JH60 and JH61/

Table 2
Strains

Name	Genotype	Description	Reference
E. coli			
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q} *ZΔM15* Tn*10* (*Tet*^{r})]	Cloning	Stratagene
LB650	F^{-} *thi lacZ gal rha kup1* (*trkD1*) Δ*kdpABC5* Δ*trkH* (Cm^{r}) Δ*trkG* (Kan^{r})	Potassium uptake studies	Ref. 54
LB2003	F^{-} *aroE rpsL metE thi gal rha kup1* (*trkD1*) Δ*kdpABC5* Δ*trkA aroE*$^{+}$	Potassium uptake studies	Ref. 54
Rosetta (DE3)	F^{-} *ompT hsdS*$_{B}$(r_B^- m_B^-) *gal dcm* (DE3) pRARE (Cm^{r})	Protein expression	Novagen
NEB T7 Express *I*^{q}	MiniF *lacI*^{q}(Cam^{R})/*fhuA2 lacZ::T7 gene1 [lon] ompT gal sulA11 R(mcr-73:: miniTn10*–Tet^{S}*)2 [dcm] R(zgb-210::Tn10*–Tet^{S}*) endA1 Δ(mcrC-mrr)114::IS10*	Protein expression and DRaCALA	New England Biolabs
B. subtilis			
168	*trpC2*	WT	Laboratory collection
L. monocytogenes			
EGD-e	WT	Serotype 1/2a strain	Laboratory collection
BPL77	Δ*cdaA*	*L. monocytogenes* strain without a DAC	This work

Table 3
Oligonucleotides
Restriction sites are underlined, and complementary regions are in boldface type.

Name	Sequence	Purpose
GH5	5′-AAAGGATCCATGAAAGAAGGATTTGCAGTCATCGGTCTTG	pBP345
GH6	5′-TTTGTCGACTTATTGAATTTTTTCTTGTAGTCGTTCAATGTCATCATCC	pBP345
GH7	5′-AAAGGATCCCATTACCGGAAAGTTGGACCACAACTTAG	pBP346
GH8	5′-TTTGTCGACTTATTCTTTTAAATGATAAGGATATGTGGAAACTACTACATCC	pBP346
GH9	5′-AAAGGATCCCATTATCGAGATATCGCAGAACAATTACGTTCTG	pBP347
GH10	5′-TTTGTCGACCTATTTTTTAAGTTTAAATGGAATTGTACATACGTTAACATTCTTTTG	pBP347
JH05	5′-AAAGAATTCAGAATTGCGTTCCACGGATACATTAAAAC	pBP352
JH06	5′-**CCTCCTTTCGTCGACGTGCCTCTT**GAAAACCATTTATAATCAC	pBP352
JH07	5′-**AAGAGGCACGTCGACGAAAGGAGG**CAAAAGCGAATGATG	pBP352
JH08	5′-TTTGGATCCCACTTTCCGGCGTGCCTTCTTG	pBP352
JH51	5′-AAATCTAGACACGGAGGTGAAGTGATGGATTTTTCCAATATGTCGATATTGCAT	pBP370/pBP373
JH52	5′-TTTCTGCAGTCATTCGCTTTTGCCTCCTTTCCA	pBP370/pBP373
JH59	5′-AAAGAATTCAAGGAGGTAACGTACACATGAAAGAAGG	pBP371
JH60	5′-**AATCTTCTGCTAAGTACGGCTTTTTATTG**AATTTTTTCTTGTAGTCGTTCAATG	pBP371
JH61	5′-**CAATAAAAAGCCGTACTTAGCAGAAGATT**AAAGCTTGTTTTGGCACG	pBP371
JH62	5′-TTTGGATCCTTAACCAGTAATAATTTTCTCTTTTGGTAAACGAATC	pBP371
JH95	5′-AAAGAATTCAAAGGTAGGGAATACAATGGCTTCGCC	pBP384/pBP396
JH96	5′-TTTGGATCCCTCTTGTTATTCTTTTAAATGATAAGGATATGTGGAAAC	pBP384/pBP265
JH97	5′-AAAGAATTCAAAGGAATAGGAGATTATGTTCAATCAATTTAAAAGAC	pBP385
JH98	5′-TTTGGATCCGAATCTATTTTTTAAGTTTAAATGGAATTGTACATACGTTAAC	pBP385/pBP267
JH120	5′-TTTGGATCCTTATTTCCGGTAATGATGTCTTGTACGATGGAAAAC	pBP396/pBP266
JH142	5′-AAAGAGCTCGATGGCTTCGCCGCTAAAAAGACTATTAATCG	pBP265/pBP266
JH143	5′-AAAGAGCTCGATGTTCAATCAATTTAAAAGACTTATTATAGGGCAACC	pBP267
JR18	5′-P-GAATACACCGCTTCATAATGGAGCAGTTATTATTAA	pBP373
MI1	5′-AAAGGATCCTAATAAGTTTAGAGGTGAGGATTTATGAAGTATATTGTGATG	pBP559
MI2	5′-TTTCTGCAGTTACATTTTCAATCTATCTAATGCCAAATTCACTTGTAAG	pBP559
MI11	5′-AAAGGATCCATGGAAACGAATCGTCCAAGTCCGG	pBP560
MI12	5′-TTTCTGCAGTCATTTTCCATCTCCTCCGTCTAGTG	pBP560
MI21	5′-AAAGGTACCGAAGTATATTGTGATGCAGGATGTG	pBP563

JH62, respectively, and fused by splicing by overhang extension (SOE) PCR using primer pair JH59/JH62 (55). The resulting PCR product was digested with EcoRI and BamHI and ligated to pWH844. The plasmids pBP559 and pBP563 were constructed for the expression of the *L. monocytogenes kdpABC* genes. The *kdpABC* genes were amplified using the oligonucleotide pairs MI1/MI2 and MI21/MI2, respectively. The PCR products were digested with BamHI/PstI and KpnI/PstI and ligated to the plasmids pWH844 and pBAD24, respectively. The plasmid pBP560 served for the expression of the *L. monocytogenes kdpD* gene. The *kdpD* gene was amplified by PCR with the oligonucleotide pair MI11/MI13. The BamHI/PstI-digested PCR product was ligated to the plasmid pWH844. The plasmids pBP370 and pBP373 were constructed for producing the WT CdaA enzyme and the inactive D171N variant (50). The *cdaA* gene was amplified using the oligonucleotide pair JH51/JH52 and introduced into the XbaI/PstI sites of pBAD33 (56). For the construction of plasmid pBP373, we used the oligonucleotide pair JH51/JH52 together with the 5′-phosphorylated oligonucleotide JR18 to introduce the D171N mutation via the combined chain reaction (57). The pBAD33 and pWH844 expression vectors have compatible selection markers and origin of replications, allowing the co-expression of potassium transporter (from pWH844) and *cdaA* genes (from pBAD33). The plasmids pBP345, pBP346, and pBP347 were constructed to study the binding of c-di-AMP to KtrC and the cytosolic domains of KimA^{Lmo} (aa 452–607) and KimA^{Sau} (aa 452–609). The respective genes were amplified using the oligonucleotide pairs GH5/GH6, GH7/GH8, and GH9/GH10, digested with BamHI/SalI, and ligated to pWH844 cut with the same enzymes. The genes encoding the full-length KimA^{Lmo} and KimA^{Sau} proteins as well as the C-terminally truncated KimA^{Lmo} variant (aa 1–455) were amplified using oligonucleotide pairs JH142/JH96, JH143/JH98, and JH142/JH120, respectively. The PCR products were digested with SacI/BamHI and ligated to pGP172 (58). The resulting plasmids were

Table 4
Plasmids

Name	Insert/Features	Reference
pBAD24	P_{BAD}, ampicillin resistance gene	Ref. 56
pBAD33	P_{BAD}, chloramphenicol resistance gene	Ref. 56
pMAD	*bgaB*, pBR322 ori and ampicillin resistance gene for (*E. coli*); pE194^{ts} ori and erythromycin resistance gene (*L. monocytogenes*)	Ref. 59
pWH844	P_{T5}, ampicillin resistance gene	Ref. 48
pGP172	P_{T7}, ampicillin resistance gene	Ref. 58
pBP265	pGP172, Strep-tag II-kimA^{Lmo}	This work
pBP266	pGP172, *Strep*-tag II- *kimA*^{Lmo} ΔC terminus	This work
pBP267	pGP172, *Strep*-tag II-*kimA*^{Sau}	This work
pBP345	pWH844, His_6-*ktrC*^{Lmo}	This work
pBP346	pWH844, His_6-*kimA*^{Sau} C-terminal domain	This work
pBP347	pWH844, His_6-*kimA*^{Sau} C-terminal domain	This work
pBP352	pMAD-Δ*cdaA* (*cdaA* up- and downstream region)	This work
pBP370	pBAD33, *cdaA*	This work
pBP371	pWH844, *ktrC*^{Lmo} and *ktrD*^{Lmo}	This work
pBP372	pWH844, *ktrAB*^{Bsu}	This work
pBP373	pBAD33, *cdaA* (D171N)	This work
pBP384	pWH844, *kimA*^{Lmo}	This work
pBP385	pWH844, *kimA*^{Sau}	This work
pBP396	pWH844, *kimA*^{Lmo} ΔC terminus	This work
pBP559	pWH844, *kdpABC*^{Lmo}	This work
pBP560	pWH844, *kdpD*^{Lmo}	This work
pBP563	pBAD24, *kdpABC*^{Lmo}	This work

designated as pBP265 (*kimA*^{Lmo}), pBP267 (*kimA*^{Sau}), and pBP266 (*kimA*^{Lmo} ΔC terminus). The plasmids are suitable for the IPTG-dependent overproduction of the transporters with an N-terminal *Strep*-tag II in the *E. coli* strain Rosetta (DE3). For the chromosomal deletion of the *cdaA* gene, pBP352 was constructed (Table 4). The up- and downstream regions of *cdaA*, while leaving the *cdaA* ORF out, were amplified using oligonucleotide pairs JH05/JH06 and JH07/JH08, respectively (Table 3). The resulting PCR products were fused by SOE PCR using oligonucleotides JH05 and JH08, digested with EcoRI and BamHI, and ligated to pMAD (55, 59), which was digested using the same enzymes.

Deletion of the cdaA gene

The chromosomal deletion of the *cdaA* gene in strain BPL77 was performed as follows. The plasmid pBP352 (pMAD-Δ*cdaA*) was introduced into the WT strain EGD-e by electroporation, and the cells were plated on LSM with erythromycin and X-Gal at 30 °C for up to 72 h. Single blue colonies were streaked on the same medium and incubated for up to 72 h at 42 °C to facilitate the selection for integrants. Blue colonies were used to inoculate 5 ml of LSM without antibiotics at 30 °C for 4 h, and the temperature was shifted to 42 °C for 6 h, after which serial dilutions were plated on LSM with X-Gal and incubated at 37 °C for up to 72 h. Erythromycin-sensitive, X-Gal–negative bacteria that did grow on LSM but not on brain heart infusion were subjected to colony PCR as described previously (60). The *cdaA* deletion and the absence of ectopic suppressor mutations was confirmed by whole-genome sequencing and Sanger sequencing, and the strain was designated BPL77 (Table 2).

Growth of L. monocytogenes in LSM

Single colonies of the *L. monocytogenes* WT and the *cdaA* mutant strains were grown overnight in LSM-K^+ with 1 mM KCl. Overnight cultures were harvested by centrifugation at 4000 × *g* for 5 min at room temperature and resuspended in LSM-K^+. These cell suspensions were used to inoculate 10 ml of LSM-K^+ to an OD_{600} of 0.1 and grown for about 4 h. Cells were washed again as described in LSM-K^+, the OD_{600} was adjusted to 0.2, and 100 μl were used to inoculate wells of a 96-well plate (Microtest Plate 96 Well, F, Sarstedt), containing 100 μl of LSM-K^+ with a 2-fold concentration of the indicated potassium concentrations. The 96-well plate was incubated at 37 °C with medium orbital shaking at 237 cpm (4 mm) in an Epoch 2 microplate spectrophotometer (BioTek Instruments), and growth was measured at an optical density (OD_{600}) in 15-min intervals.

Drop dilution assay

Single colonies of the *E. coli* strain LB650 harboring the plasmid pWH844, pBP371, pBP372, pBP384, pBP385, or pBP396 were taken from LB-K plates and used to inoculate 4 ml of LB-K medium supplemented with kanamycin, ampicillin, and chloramphenicol. The cultures were incubated at 37 °C and 220 rpm. The precultures were used to inoculate 4 ml of M9 medium supplemented with glucose, antibiotics, and 50 mM KCl to an OD_{600} of 0.001. The cultures were incubated for about 16 h at 37 °C. The next day, the cultures were used to inoculate 10 ml of the same medium to an OD_{600} of 0.1. At an OD_{600} between 0.3 and 0.5, the cells were harvested by centrifugation at 4000 × *g* for 10 min at room temperature. The cell pellets were washed twice in 10 ml of M9 medium lacking KCl. The cell suspension was adjusted to an OD_{600} of 0.1, and 5 μl of the diluted cells were spotted onto M9 minimal medium plates, which were incubated for 24 h at 37 °C. M9 plates were prepared by mixing 2× M9 medium (prewarmed to 37 °C) and 2× Bacto agar (prewarmed to 70 °C before mixing). The finial medium contained glucose as a carbon source, 10 mM KCl, and 50 μM IPTG when required.

Determination of kinetic parameters of the potassium transporters

To determine the growth characteristics of the *E. coli* strain LB650 synthesizing potassium transporters from *L. monocytogenes* and *S. aureus*, the bacteria were grown until the early exponential phase and harvested by centrifugation at 4000 × *g* for 10 min. The pellet was resuspended in 10 ml of M9 medium with glucose, ampicillin, and 50 μM IPTG without KCl. The cells were incubated for 1 h at 37 °C, harvested by centrifugation, and washed twice. The cultures were adjusted to an OD_{600} of 0.2, and 50 μl were used to inoculate a 96-well plate (Microtest Plate 96 Well, F, Sarstedt) containing 50 μl of M9 medium with glucose, ampicillin, 50 μM IPTG, and KCl concentrations ranging from 0 to 100 mM. The 96-well plate was incubated at 37 °C with medium orbital shaking at 237 cpm (4 mm) in an Epoch 2 microplate spectrophotometer (BioTek Instruments). The growth rates were calculated ($\mu = (2.303 \cdot (\log(OD_2) - \log(OD_1)))/(t_2 - t_1)$), plotted against the KCl concentrations, and fitted to the Michaelis–Menten equation using the solver tool of Excel 2013 (Microsoft) to calculate V_{max} (μ (h^{-1})) and the apparent K_m (mM KCl).

c-di-AMP in vivo inhibition assay

The potassium transporter– deficient *E. coli* strain LB2003 was co-transformed with the plasmid pWH844 or derivatives

(pBP371, pBP384, or pBP396) and the pBAD33 derivatives (pBP370 or pBP373) on LB-K plates containing 0.5% (w/v) glucose, ampicillin, and chloramphenicol. Single colonies were used to inoculate 4 ml of LB-K medium containing 0.2% (w/v) glucose, ampicillin, and chloramphenicol, and the exponentially growing cultures were used to inoculate M9 medium containing 0.2% (w/v) glycerol and 0.02% (w/v) glucose to an OD_{600} of 0.001. The cultures were incubated overnight at 37 °C and used to re-inoculate the same medium (without glucose) to an OD_{600} of 0.1. After reaching early exponential phase (OD_{600} = 0.3–0.5), the cells were washed, and 50 μl of the suspensions were used to inoculate a 96-well plate. The M9 medium was supplemented with glycerol, 50 μM IPTG, ampicillin, chloramphenicol, and KCl with or without L-arabinose. Final concentrations of KCl were equal to the determined K_m values (see Table 2), and either no or 0.005% (w/v) L-arabinose was present, as indicated. Growth was monitored in an Epoch 2 microplate spectrophotometer (BioTek Instruments).

Protein expression and DRaCALA

The binding of c-di-AMP to the potassium transporters was analyzed using the *E. coli* strain Rosetta (DE3) for pGP172 and derivatives or strain NEB T7 Express I^q for pWH844 and derivatives. Single colonies were used to inoculate 10 ml of LB-K medium containing carbenicillin and chloramphenicol. After incubation overnight at 30 °C, the precultures were used to inoculate 1.5 ml of LB-K medium to an OD_{600} of 0.1. 1 mM IPTG was added at an OD_{600} of 1.0–1.5 to induce gene expression. After incubation for 4 h, the cultures were harvested by centrifugation (4000 × *g*, 10 min, 4 °C), the cell pellets were resuspended in 150 μl of Tris-NaCl buffer (10 mM Tris, pH 8.0, 100 mM NaCl). Cells were lysed by three freeze/thaw cycles of −80 °C and room temperature. DRaCALA was performed by mixing 1 μl of [^{32}P]c-di-AMP with 20 μl of cell lysates. After a 1-min incubation, 2 μl of the mixture was spotted on dry nitrocellulose, dried, exposed to a PhosphorImager screen, and imaged using an FLA-7000 PhosphorImager. The fraction bound was calculated using the inner and total areas and intensities, as described previously (61).

Microscopic analysis

Derivatives of the LB650 strain harboring the plasmids pWH844 (empty plasmid), pBP372, or pBP384 were in 4 ml of LB-K medium containing ampicillin, kanamycin, and chloramphenicol at 37 °C. The next day, the cultures were washed twice and used to inoculate 10 ml of M9 medium (containing 22.05 mM KH_2PO_4) with or without 1 mM IPTG to an OD_{600} of 0.1. Cells were transferred to standard microscope slides (Carl Roth) and examined using an Axioskop 40 FL fluorescence microscope, equipped with an Axio-Cam MRm digital camera, objectives of the Neofluar series at 1000-fold primary magnification, and the AxioVision Rel 4.8.2 software (Carl Zeiss). Images were later equally processed using ImageJ 1.48 software (62).

Statistical analysis

All data are presented as means with *n* representing the number of independent experiments. Data were statistically evaluated by analysis of variance (ANOVA) tests with post hoc Dunnett's or Tukey tests using the GraphPad Prism version 8.2.1 software (GraphPad Software, La Jolla, CA).

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