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

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ABSTRACT

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 *E. 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 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 a *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), which is produced by specific diadenyl 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). While 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, Streptococcus agalactiae rely only on this DAC for c-di-AMP synthesis (21). Since c-di-AMP is essential for growth of these bacteria (14,25,26), the DAC CdaA is an interesting target for novel antibiotics.

C-da-AMP is also intracellularly degraded by specific phosphodiesterases (PDEs), which can be assigned to three different groups (23,27). The GdpP- and PpgH-type PDEs consist of domains that are involved in signalling 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). Since the DACs and the PDEs determine the cellular c-di-AMP levels 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 cystathione-beta-synthase domain-containing (CBS) 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). While 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 CbpB-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 Listeria 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 (5,55). 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 (41,56,58). S. aureus also contains the low-affinity potassium transport systems KtrCB and KtrCD sharing the accessory protein KtrC (56). A BLASTp sequence analysis revealed that the L. monocytogenes genome codes for the KdpABCD (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 (57). 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\textsuperscript{Bsu}, KimA\textsuperscript{Lmo} (Lmo2130) and KimA\textsuperscript{Sau} (Sacol2443), respectively. KimA\textsuperscript{Lmo} and KimA\textsuperscript{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 (59). Like KimA\textsuperscript{Bsu} also KimA\textsuperscript{Lmo} 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 (45). We also cloned a truncated kimA\textsuperscript{Lmo} gene encoding the ΔC-KimA\textsuperscript{Lmo} variant lacking 152 amino acids of the C-terminal cytosolic domain. Furthermore, we cloned the kimA\textsuperscript{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 (43). 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, collected 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 harbouring the plasmid for kimA\textsuperscript{Sau} expression, the bacteria could not grow in the absence of IPTG. The weak growth of the cells containing the kimA\textsuperscript{Sau} gene could be due to a leaky promoter and due to the high affinity of the encoded KimA\textsuperscript{Sau} transporter for potassium (see below). By contrast, the strains
carrying the \textit{ktrAB}^{Bsu}, \textit{kimA}^{Lmo}, \textit{ktrCD}^{Lmo}, could grow with low amounts of K⁺ when these genes were induced with IPTG (Fig. 2). Moreover, the \textit{kimA}^{Lmo} variant lacking the C-terminal domain (\textit{kimA}^{Lmo \Delta C}) also supported growth of the, albeit less good than the full-length protein. These results indicate that the N-terminal extracellular domain and the 11 transmembrane helices of \textit{KimA}^{Lmo} from \textit{L. monocytogenes} are sufficient for mediating potassium import in \textit{E. coli} (Fig. 2). Expression of \textit{ktrAB}^{Bsu} from \textit{B. subtilis} restores growth on low potassium concentrations agreeing with previous reports that \textit{KtrAB}^{Bsu} is a high-affinity potassium transporter (5,55). Expression of \textit{KimASau} from \textit{S. aureus} in the \textit{E. coli} strain LB650 resulted in much better growth than those strains expressing \textit{KimA}^{Lmo} and KtrCD indicating that \textit{KimA}^{Sau} is likely a high-affinity potassium transporter (Fig. 2). Thus, \textit{KimA}^{Lmo} and \textit{KtrCD}^{Lmo} from \textit{L. monocytogenes} as well as \textit{KimA}^{Sau} from \textit{S. aureus} are indeed potassium transporters. The putative potassium transporter \textit{KdpABC}^{Lmo} did not support growth of the \textit{E. coli} strain LB650 irrespective of whether the \textit{kdpABC}^{Lmo} genes were expressed from the IPTG- and arabinose-dependent plasmids pWH844 and pBAD24 (data not shown; see Experimental procedures). Therefore, the \textit{KdpABC}^{Lmo} system was not further analysed in regards to its affinity to potassium ions and \textit{in vivo} inhibition by c-di-AMP.

\textbf{Apparent affinities of KimA and KtrCD for potassium} - To determine the apparent affinities of \textit{KimA}^{Lmo}, the \textit{KimA}^{Lmo \Delta C}-terminus variant (\textit{ΔC-KimA}^{Lmo}) and KtrCD from \textit{L. monocytogenes} and \textit{KimA}^{Sau} from \textit{S. aureus}, we determined the growth rates of the \textit{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 \textit{B. subtilis} \textit{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 \textit{V}_{\text{max}} values and the apparent affinities are summarized in Table 6. As shown in Fig. 3 and 4, each \textit{E. coli} strain required a different concentration of external potassium to reach half-maximal growth: the strains synthesizing the transporters \textit{KtrCD}^{Lmo}, \textit{KimA}^{Lmo}, \textit{ΔC-KimA}^{Lmo}, \textit{KimA}^{Sau} and \textit{KtrAB}^{Bsu} required 6.30 ± 2.06 mM, 0.35 ± 0.12 mM, 2.99 ± 0.65 mM, 0.14 ± 0.02 mM and 0.03 ±0.01 mM, respectively. These results demonstrate that \textit{KtrCD}^{Lmo} and \textit{KimA}^{Lmo} from \textit{L. monocytogenes} are transporters with low and moderately high affinities for potassium, respectively. Moreover, the C-terminal intracellular domain of \textit{KimA}^{Lmo} is important for full activity of the transporter (Fig. 1, B; Fig. 4). In contrast to \textit{KtrCD}^{Lmo} and \textit{KimA}^{Lmo}, \textit{KimA}^{Sau} from \textit{S. aureus} is a high-affinity potassium transporter, which is inline with the observation that the \textit{E. coli} strain LB650 synthesizing \textit{KimA}^{Sau} and \textit{KtrAB}^{Bsu} grew comparatively well with low amounts of potassium (Fig. 2).

\textbf{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 \textit{B. subtilis}, \textit{L. lactis}, \textit{L. monocytogenes}, \textit{S. agalactiae} and \textit{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 \textit{ktrAB}^{Bsu} and \textit{kimA}^{Lmo} genes encoding high-affinity potassium transporters \textit{KtrAB}^{Bsu} and \textit{KimA}^{Lmo}, respectively, in \textit{E. coli} during growth in M9 minimal medium caused a strong increase of the cellular volume. Moreover, the growth of \textit{E. coli} synthesizing the higher affinity \textit{KtrAB}^{Bsu} transporter was in addition significantly reduced as illustrated by the decline of the optical density (Fig. 5; upper right corners). By contrast, in the absence of the inducer IPTG the growth and the volume of the cells containing the \textit{ktrAB}^{Bsu} and \textit{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 \textit{S. aureus} as well as the \textit{KimA}^{Bsu} transporter from \textit{B. subtilis} are inhibited
by c-di-AMP (7,39). Like KimA\textsubscript{Bu} from \textit{B. subtilis}
the KimA\textsubscript{Lmo} homolog from \textit{L. monocytogenes}
belong to a novel class of high-affinity potassium
transporters (see above; 5). However, whether c-
di-AMP directly binds to KimA\textsubscript{Lmo} and KtrCD\textsubscript{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\textsubscript{Lmo} and KtrCD\textsubscript{Lmo}, we established a
co-expression system using the \textit{E. coli} strain
LB2003 that carries unmarked mutations in the
kdp, kup and trk genes and enable the use of
multiple plasmids encoding chloramphenicol and
ampicillin resistance genes (43). Like the \textit{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, \textit{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 the activity of KimA\textsubscript{Lmo}
and KtrCD\textsubscript{Lmo}. The plasmids pBP384 (kimA\textsubscript{Lmo}), pBP396
(ΔC-kimA\textsubscript{Lmo}) and pBP371 (ktrCD\textsubscript{Lmo}) were used for
the IPTG-dependent expression of ΔC-KimA\textsubscript{Lmo},
KimA\textsubscript{Lmo} and KtrCD\textsubscript{Lmo}, respectively. The empty
plasmid pWH844 served as a negative control. The
\textit{L. monocytogenes} DAC CdaA and the inactive
CdaA\textsuperscript{*} variant D171N (47) 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 mM, 0.35
mM, 3 mM 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\textsuperscript{*} variants, respectively, was not reduced.
Thus, neither the DAC proteins nor c-di-AMP
affect growth of the \textit{E. coli} strain. By contrast,
growth of the bacteria synthesizing KimA\textsubscript{Lmo}
and KtrCD\textsubscript{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
terminated ΔC-KimA\textsubscript{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\textsubscript{Bu} from \textit{S. aureus} (data
not shown). To conclude, the potassium
transporters KimA\textsubscript{Lmo} and KtrCD\textsubscript{Lmo} from \textit{L.
monocytogenes} are both inhibited by c-di-AMP.

\textbf{Effect of potassium on growth of a c-di-
AMP-free \textit{L. monocytogenes} strain---} Previously, it has been
shown that c-di-AMP is essential in \textit{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
\textit{L. monocytogenes}, we constructed a marker-less
deletion of the cdaA gene, encoding the sole c-di-
AMP synthesizing enzyme. As previously
described, we confirmed that the cdaA mutant
is not viable on complex, but on chemically defined
growth medium (10,11). We prepared LSM
defined growth medium (11) without potassium
(LSM-K\textsuperscript{−}) and observed that potassium
concentrations below 1 mM impairs the growth of the
\textit{L. monocytogenes} wild type strain (Fig. 7). The
growth behaviour of the wild type strain was not
affected at potassium chloride concentrations
higher than 1 mM. The \textit{L. monocytogenes} cdaA
mutant shows a slightly slower growth than the
wild type at potassium concentrations above 1
mM. However, high potassium concentrations did
not fully inhibit growth of the \textit{L. monocytogenes}
cdaA mutant strain as it has been shown for a

\textbf{c-di-AMP interaction with the KimA
homologs and KtrCD\textsubscript{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\textsubscript{Lmo}, ΔC-KimA\textsubscript{Lmo},
KimA\textsubscript{Bu}, KtrC\textsubscript{Lmo}, KdpABC\textsubscript{Lmo} and KdpD\textsubscript{Lmo}
(see \textit{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\textsubscript{Lmo} and KimA\textsubscript{Bu}, respectively. This domain
could be involved in the c-di-AMP-dependent
control of KimA potassium transport activity. The
lysate of the \textit{E. coli} strain DH5α containing the
empty plasmids pWH844 or pGP172 served as a negative controls. While the majority of the proteins showed no specific interaction with c-di-AMP in the DReCALA assay, KtrC\textsuperscript{Lmo}, the cytosolic protein of the KtrCD potassium transporter and KdpD\textsuperscript{Lmo}, the sensor kinase of the KdpDE two-component system, gave positive results (Fig. 8). To conclude, the potassium transport activity of KtrCD\textsuperscript{Lmo} from \textit{L. monocytogenes} is inhibited by c-di-AMP \textit{in vivo} and the nucleotide binds to the KtrC subunit of the KtrCD\textsuperscript{Lmo} transporter and to KtrD of the KdpDE\textsuperscript{Lmo} two-component system \textit{in vitro}. Due to toxicity we were unable to purify the full-length KimA\textsuperscript{Lmo} protein. The failure of purification of the C-terminal part of KimA also precludes further \textit{in vitro} characterization.

**Discussion**

Here, we have identified and characterized the potassium transporters KtrCD\textsuperscript{Lmo} and KimA\textsuperscript{Lmo} from \textit{L. monocytogenes}. As stated above, the KtrCD homologs from \textit{L. monocytogenes} and \textit{B. subtilis} show 64\% overall sequence identity and have similar affinities for potassium (see Table 4) (55). We also demonstrate that \textit{S. aureus} possesses a homolog of KimA (Sacol2443). The KimA homologs from \textit{S. aureus} and \textit{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\textsuperscript{Lmo} and KimA\textsuperscript{Lmo} from \textit{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\textsuperscript{Lmo} \textit{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 \textit{B. subtilis} and controls the uptake of potassium by the transporter \textit{in vivo} (39). Unfortunately, we could not show the binding of c-di-AMP to the full-length KimA\textsuperscript{Lmo} protein and to the C-terminal domain of KimA\textsuperscript{Lmo}. However, we speculate that binding of c-di-AMP to the cytosolic domain is required for regulation of KimA\textsuperscript{Lmo} \textit{in vivo} (Fig. 6). Therefore, it might be worth to study the role of the C-terminal domain in controlling the activity of the high-affinity potassium transporter KimA. Surprisingly, the KimA\textsuperscript{Lmo} transport from \textit{L. monocytogenes} has a much lower affinity for potassium than the homolog from \textit{B. subtilis} (39,55). As the external concentrations of potassium are rather low, it is tempting to speculate that \textit{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 \textit{B. subtilis} and \textit{S. aureus} contain two high-affinity potassium transport systems that are active during growth at low potassium concentrations. \textit{B. subtilis} employs the high-affinity potassium transporters KtrAB and KimA under potassium limiting growth conditions (5,39,55). Previously, it has been shown that \textit{S. aureus} relies on the high-affinity transporter KdpFABC, whose synthesis and activity is regulated by the two-component system KdpDE (38,56,58). KimA\textsuperscript{Sac} could also be important for growth of \textit{S. aureus} when the extracellular potassium concentrations are low. In contrast to \textit{S. aureus}, the KdpABC homolog of \textit{L. monocytogenes} does not seem to contribute to potassium uptake. It has been previously shown that the small membrane protein KdpF is required for proper function of the \textit{E. coli} Kdp potassium transport system (57). As described above, no KdpF homolog is present in \textit{L. monocytogenes}. Therefore, the lack of KdpF in \textit{L. monocytogenes} could be the reason why the KdpABC system is not active 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 \textit{kdpABC} genes in \textit{L. monocytogenes} (Fig. 8). However, our comparative RNA-sequencing experiments using the wild type strain and a c-di-AMP-free \textit{cdaA} mutant strain in chemically defined medium revealed that the lack c-di-AMP does not alter the expression of genes involved in potassium uptake (data not shown). In \textit{B. subtilis} it has been shown that the 5'-UTRs of the \textit{kimA} and \textit{ktrAB} genes contain \textit{ydaO} riboswitches preventing synthesis of the transporters in the presence of c-di-AMP (5,62). However, c-di-AMP-dependent riboswitches that could be involved in controlling the expression of potassium transporter genes are absent in \textit{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 either be achieved 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 (60). 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 transporters 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 (61). 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<sup>Lmo</sup> and KimA<sup>Lmo</sup> 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<sup>usu</sup> and KimA<sup>Lmo</sup> 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 (41,58). 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 (5,62). 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 - irrespectively 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 seem 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 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 1. The *E. coli* strains XL1-Blue (Stratagene), Rosetta (DE3) (Novagen) and T7 Express I® (NEB) were used for cloning and protein overproduction. *E. coli* was grown in LB medium and transformants were selected on LB plates (15 g l⁻¹ Bacto agar (Difco)) containing kanamycin (50 µg ml⁻¹), ampicillin, carbenicillin (100 µg ml⁻¹) or chloramphenicol (30 µg ml⁻¹). The *L. monocytogenes* wild type strain EGD-e (laboratory strain collection) was cultivated in LB medium (Oxoid), 0.004 % (w/v) L-proline and 3 µM Thiamine. For the experiments with defined potassium concentrations, the KH₂PO₄ salt was replaced by NaH₂PO₄ and KCl was added as indicated. If not specified different, IPTG was used at a concentration of 50 µM and L-arabinose at 0.005 % (w/v).

**DNA manipulation** – Transformation of *E. coli* was performed using standard procedures (44). 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 a kindly provided by Dr. Jan Pané-Farré (University of Greifswald, Germany). Oligonucleotides were purchased from Sigma-Aldrich (Germany).

**Plasmid construction** – The genes encoding putative potassium transporters were introduced into the vector pWH844 allowing IPTG-dependent expression in *E. coli* (45). The *kimA<sub>Lmo</sub>* and *kimA<sub>Sal</sub>* genes were amplified using the oligonucleotide pairs JH95/JH96 and JH97/JH98, respectively (Table 2). The PCR products were *EcoRI/BamHI*-digested and ligated to pWH844. The resulting plasmids were designated as pBP384 and pBP385 (Table 3). To study the role of the C-terminal domain of KimA<sub>Lmo</sub>, the plasmid pBP396 was constructed. The truncated *kimA<sub>Lmo</sub>* 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/JH62, respectively and fused by Splicing by Overhang Extension (SOE) PCR using primer pair JH59/JH62 (46). The resulting PCR product was digested with EcoRI and *Bam*HI 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 *Bam*HI/PstI and *Kpn*I/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 *Bam*HI/PstI-digested PCR product was ligated to the plasmid pWH844. The plasmids pBP370 and pBP373 were constructed for producing the wild type CdaA enzyme and the inactive D171N variant (47). The *cdaA* gene was amplified using the oligonucleotide pair JH51/JH52 and introduced into the *Xba*I/PstI sites of pBAD33 (48). 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 (49). The pBAD33 and pWH844 expression vectors have compatible selection markers and origin of replications allowing the co-expression of potassium transporter genes (from pWH844) *cdaA* variants (from pBAD33). The plasmids pBP345, pBP346 and pBP347 were constructed to study the binding of c-di-AMP to KtrC, the cytosolic domains of *KimA*<sup>Lmo</sup> (aa 452-607) and *KimA*<sup>Sau</sup> (aa 452-609). The respective genes were amplified using the oligonucleotide pairs GH5/GH6, GH7/GH8 and GH9/GH10, digested with *Bam*HI/*Sal*I and ligated to pWH844 cut with the same enzymes. The genes encoding the full-length *KimA*<sup>Lmo</sup> and *KimA*<sup>Sau</sup> proteins as well as the C-terminally truncated *KimA*<sup>Lmo</sup> variant (aa 1-455) were amplified using oligonucleotide pairs JH142/JH96, JH143/JH98 and JH142/JH120, respectively. The PCR products were digested with SacI/*Bam*HI and ligated to pGP172 (50). The resulting plasmids were designated as pBP265 (*kimA*<sup>Lmo</sup>), pBP267 (*kimA*<sup>Sau</sup>) and pBP266 (*kimA*<sup>Lmo</sup> 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 3). 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 2). The resulting PCR products were fused by SOE PCR using oligonucleotides JH05 and JH08, digested with *Eco*RI and *Bam*HI and ligated to pMAD (46,51), 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-∆*cdaA*) was introduced into the wild type strain EGD-e by electroporation and the cells were plated on LSM medium 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, temperature was shifted to 42°C for 6 h, after which serial dilutions were plated on LSM medium with X-Gal and incubated at 37°C for up to 72 h. Erythromycin-sensitive, X-Gal negative bacteria that did grow on LSM medium but not on BHI were subjected to colony PCR as described previously (52). The *cdaA* deletion and the absence of ectopic suppressor mutations was confirmed by whole genome sequencing (WGS) and Sanger sequencing and the strain designated BPL77 (Table 1).

**Growth of *L. monocytogenes* in LSM medium** — Single colonies of the *L. monocytogenes* wild type and the *cdaA* mutant strains were grown over night in LSM-K<sup>+</sup> with 1 mM KCl. Overnight cultures were harvested by centrifugation at 4000 g for 5 minutes at RT and resuspended in LSM-K<sup>+</sup>. These cell suspensions were used to inoculate 10 ml of LSM-K<sup>+</sup> to an OD<sub>600</sub> of 0.1 and grown for about 4 hours. Cells were washed again as described in LSM-K<sup>+</sup>, the OD<sub>600</sub> adjusted to 0.2 and 100 µl used to inoculate wells of a 96 well plate
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(Microtest Plate 96 Well, F, Sarstedt), containing 100 µL LSM-K + with the 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 measured at an optical density (OD 600) in 15-minute intervals.

Drop dilution assay – Single colonies of the E. coli strain LB650 harbouring the plasmids pWH844, pBP371, pBP372, pBP384, pBP385 or pBP396 were taken from LB-K plates and used to inoculate 4 ml LB-K medium supplemented with kanamycin, ampicillin and chloramphenicol. The cultures were incubated at 37 °C and 220 rpm. The pre-cultures were used to inoculate 4 ml 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. 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 media plates, which were incubated for 24 h at 37°C. M9 plates were prepared by mixing 2 X M9 medium (pre-warmed to 37°C) and 2 X Bacto agar (pre-warmed to 70°C before mixing). The final 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, harvested by centrifugation at 4000 g for 10 min. The pellet was re-suspended in 10 ml M9 medium with glucose, ampicillin, 50 µM IPTG and 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 $V_{\text{max}}$ ($\mu$ [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 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, KCl and with or without L-arabinose. Final concentrations of KCl were equal to the determined $K_M$ values (see Table 1) and 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 analysed using the E. coli strain Rosetta (DE3) for pGP172 and derivatives or strain NEB T7 Express ™ for pWH844 and derivatives. Single colonies were used to inoculate 10 ml LB-K medium containing carbenicillin and chloramphenicol. After incubation overnight at 30°C, the pre-cultures 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 Tris-NaCl buffer (10 mM Tris, pH 8.0, 100 mM NaCl). Cells are lysed by 3 freeze thaw cycles of -80°C and room temperatures. DRAcALA is performed by mixing 1 µL of 32P-c-di-AMP with 20 µL of cell lysates. After a 1 minute incubation, 2 µL of the mixture is spotted on dry nitrocellulose, dried, exposed to phosphorimagener screen and imaged using FLA-7000 phosphorimagener. The fraction bound is calculated using the inner and total areas and intensities as described previously (53).

Microscopic analysis – Derivatives of the LB650 strain harbouring the plasmids pWH844 (empty plasmid), pBP372, or pBP384 were in 4 ml LB-K medium containing ampicillin, kanamycin and chloramphenicol at 37°C. Next day, the cultures were washed twice and used to inoculate 10 ml M9 medium (containing 22.05 mM KH$_2$PO$_4$) 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 1,000-fold primary magnification and the AxioVision Rel 4.8.2 software (Carl Zeiss). Images were later on equally processed using the ImageJ 1.48 software (54).

Statistical analysis – All data are presented as means with $n =$ 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, California, USA).

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Conflict of interest
The authors declare that they have no conflicts of interest with the contents of this article.
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**FOOTNOTES**

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Figure 1. Alignment of KimA homologs and domain organization of the KimA<sub>Lmo</sub> protein. A, MUSCLE alignment of the KimA<sub>bus</sub>, KimA<sub>Lmo</sub> and KimA<sub>aue</sub> homologs from <i>B. subtilis</i>, <i>L. monocytogenes</i> (Lmo2130) and <i>S. aureus</i> (Sacol2443), respectively, generated with the Geneious software package (63). Amino acids in black, grey and white have an amino acid similarity of over 80%, 60-80%, or less than 60%, respectively. B, predicted membrane topology of KimA<sub>Lmo</sub> overlayed with a MUSCLE alignment between KimA<sub>bus</sub> and KimA<sub>Lmo</sub>. The dashed line indicates the position at which the KimA<sub>Lmo</sub> protein was truncated. Amino acids in black are identical; amino acids in grey are similar and amino acids in white are non-similar.
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 (*KimSau^) 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, serial ten-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.
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<sub>Lmo</sub>*)). B, *E. coli* LB650 strain harboring the plasmid pBP384 (*kimA<sub>Lmo</sub>*). C, *E. coli* LB650 strain harboring the plasmids pBP372 (*ktrABB<sub>Sau</sub>*) and pBP385 (*kimS<sub>Au</sub>*). The strains were grown to an OD<sub>600</sub> 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. Multi-well 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 indicate the data means and the dashed lines the fitted curves. Significant differences between the fitted curves are shown (*p* < 0.001 (***) *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 \( \text{KimA}^{\text{lmo}} \) protein. *E. coli* LB650 strains harboring plasmids pWH844 (empty plasmid), pBP384 (\( \text{kimA}^{\text{lmo}} \)) and pBP396 (\( \text{kimA}^{\text{lmo}} \Delta \text{C-terminus} \)) 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. Multi-well 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 indicate the data means and the dashed lines the 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).
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 (ktrABBsu) and pBP384 (kimALmo) 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 right upper corner of the microscopic pictures, was measured in hourly intervals. Scale bar, 2 µm.
Figure 6. Inhibition of potassium transporters by c-di-AMP. The *E. coli* strain LB2003 harboring the plasmids pWH844 (empty plasmid), pBP371 (*ktrCD*<sup>Lmo</sup>), pBP384 (*kimA*<sup>Lmo</sup>), pBP396 (*kimA*<sub>Lmo</sub>ΔC-terminus) and either pBP370 (*cdaA*; filled symbols) or pBP373 (*cdaA* D171N; unfilled symbols) were grown to an OD<sub>600</sub> of 0.3-0.5 in M9 medium and washed for one hour 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<sub>M</sub> values of the transporters. Data are means (n = 3). No significant differences between the strains harbouring the empty plasmid (F<sub>(3,260)</sub> = 0.6496; p = 0.5838). The same is valid for the strains harbouring the plasmid for the expression of the *kimA*<sub>Lmo</sub>ΔC-terminus variant (F<sub>(3,260)</sub> = 0.5085; p = 0.6768). Significant differences between the strains harbouring the plasmid for the expression of the *ktrCD* (F<sub>(3,260)</sub> = 6.588; p = 0.0003) or *kimA* genes (F<sub>(3,260)</sub> = 12.43; p < 0.0001). p < 0.05 (**), p < 0.001 (***) 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* wild type (wt) strain EGD-e (filled symbols) and the cdaA mutant strain (non-filled symbols) were grown over night 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, 100 mM = squares). Bacteria were grown at 37°C. Data are means (*n* = 3). Significant differences between the wild type or the ΔcdaA strains (0 mM KCl) to the strains grown with KCl and between wild type and the ΔcdaA 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).
Figure 8. Interaction between c-di-AMP and potassium transporters determined by DRaCALA. A, Autoradiographs showing the interaction between radiolabeled c-di-AMP (32P-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 plasmids pGP172 (empty plasmid), or the derivatives pBP265 (kimA\textsuperscript{lm0}), pBP266 (kimA\textsuperscript{lm0} ΔC-terminus), pBP267 (kimA\textsuperscript{sou}), or NEB T7 Express I\textsuperscript{q} carrying the plasmids pWH844 (empty plasmid), or the derivatives pBP346 (kimA\textsuperscript{lm0} C-terminal domain), pBP347 (kimA\textsuperscript{sou} C-terminal domain), pBP345 (ktrC\textsuperscript{lm0}), pBP559 (kdpABC\textsuperscript{lm0}) and pBP560 (kdpDL\textsuperscript{lm0}). Both empty vectors showed similar non-binding (data not shown). B, Fraction bound of 32P-c-di-AMP is shown for lysates from E. coli induced overnight for the expression of the indicated gene. Bars indicated data means (n > 3). \( p < 0.01 (**), p < 0.001 (***)\, p < 0.0001 (****)\) compared with empty plasmid alone (F\textsubscript{(8,20)} = 275.9, ANOVA with Dunnett’s post hoc test).
### Table 1. Strains

| Name          | Genotype                                                                 | Description                        | Reference |
|---------------|---------------------------------------------------------------------------|------------------------------------|-----------|
| **E. coli**   |                                                                           |                                    |           |
| XL1-Blue      | recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F’ proAB lacI’ ZΔM15 Tn10 (Tet’)] | Cloning                            | Stratagene|
|               | F’ thi lacZ gal rha kup1 (trkD1) ΔkdpABC5 ΔtrkH (Cm’1) ΔtrkG (Kan’1)            |                                    |           |
| LB650         |                                                                             | Potassium uptake studies           | (44)      |
| LB2003        | F’ aroE rpsL metE thi gal rha kup1 (trkD1) ΔkdpABC5 ΔtrkA aroE’               | Potassium uptake studies           | (44)      |
| Rosetta (DE3) | F’ ompT hsdS6(rS mS) gal dcm (DE3) pRARE (Cm’1)                               | Protein expression                 | Novagen   |
| NEB T7        | MiniF lacf(Cam’8)/fhuA2 lacZ::T7 gene1 [lon] ompT gal sulA11 R(mcr-73::miniTn10–Tet2)2 [dcm] R(2gb-210::Tn10–Tet3) endA1 Δ(mcrC-mrr)114::IS10 | Protein expression and DRaCALA     | New England Biolabs |
| Express 18    |                                                                           |                                    |           |
| B. subtilis   |                                                                           |                                    |           |
| 168           | trpC2                                                                     | Wild type                          | Laboratory collection |
| L. monocytogenes |                                                                                   |                                    |           |
| EGD-e         | Wild type                                                                 | Serotype 1/2a strain               | Laboratory collection |
| BPL77         | ΔcdaA                                                                     | L. monocytogenes strain without a DAC | This work |

### Table 2. Oligonucleotides

| Name | Restriction sites are underlined, complementary regions are in bold | Purpose |
|------|---------------------------------------------------------------------|---------|
| GH5  | 5’-AAAGGATCCATGAAAGAAGGATTTCAGTCATCGGTCTTG                           | pBP345  |
| GH6  | 5’-TTTGTCGACTTTATGGAAATTATTCCATTGTAGTGGTTCAATGTCATTCGCC              | pBP345  |
| GH7  | 5’-AAAGGATCCCATTACGGAAAGAGTTGGACCAACACTTAG                           | pBP346  |
| GH8  | 5’-TTTGTCGACTTATCTTTTTAATGATAAGGATATGTGGAAACTACTACATC                | pBP346  |
| GH9  | 5’-AAAGGATCCATTATCGAGATATCGCAGAACAATTACGTCTG                         | pBP347  |
| ID    | Sequence                                                                 | Reference(s)     |
|-------|--------------------------------------------------------------------------|------------------|
| GH10  | 5'-TTTGTCGACCTATTTTTTAAGTTTAAATGGGAATTGTACATACGTTAACA TTCTTTTGG          | pBP347           |
| JH05  | 5'-AAAGAATTCAAGATTGCGTTCCACGGGATACATTAAAC                                | pBP352           |
| JH06  | 5'-CTCTTTCGGTGACGTGCTCTTTGAAAACCATTATTAAATCAC                             | pBP352           |
| JH07  | 5'-AAGAGGCACGTGACGAAGAGGAGGCAAAAGCGAATGATG                                | pBP352           |
| JH08  | 5'-TTTGATCCCACCTTTCCGCGTCCTTTTG                                         | pBP352           |
| JH51  | 5'-AAATCTAGACCGGAGGTAAGTGGATGGATGTTTTCCAATATGTCGATATTGGCAT               | pBP370/ pBP373   |
| JH52  | 5'-TTCTCAGTCATCATTGCGCTTTTGGCTCTTCTCCA                                   | pBP370/ pBP373   |
| JH59  | 5'-AAAGAATTCAAGAGGAGTGAACATACATGAAAGAGG                                  | pBP371           |
| JH60  | 5'-AATCTTCTGCTAAGTGACCGCTTTTTATTGAAATTTTTCTTGTAGTCTTTGAATACATG          | pBP371           |
| JH61  | 5'-CAATAAAAAAGGCCGTACTTTAGACAGAAGATTAAAGCTTTGTGTTTTGGCACG               | pBP371           |
| JH62  | 5'-TTTGATCTTAAACCAGATAAATAATTTCCTTTTGTAACAGAATC                          | pBP371           |
| JH95  | 5'-AAAGAATTCAAAGGGATAGGAATAACATTGGCCTGGC                               | pBP384/ pBP396   |
| JH96  | 5'-TTTGATCCCCTTTGGATTTCCTTTAAAAGATGAAGGATATGTTGGGAAAC                  | pBP384/ pBP265   |
| JH97  | 5'-AAAGAATTCAAGAGGAGTGATTATGGCTATCAATTTAAAGAGACTTATAGCAG               | pBP385           |
| JH98  | 5'-TTTGATCCGAGTATCTTTTTTAAGTTTAAATGGGAATTTGCACATACGTTAAC               | pBP385/ pBP267   |
| JH120 | 5'-TTTGATCCTTATTTCCGGTAATAGTGCTTGGACATTGAAAGAC                        | pBP396/ pBP266   |
| JH142 | 5'-AAAGAGCTCGATGGCTTCCGGCGCTTAATAAGACTATTAATCG                          | pBP265/ pBP266   |
| JH143 | 5'-AAAGAGCTCGATGTTCAATCTTATAAGACTATTATAGGGCAACC                         | pBP267           |
| JR18  | 5'-P-GAATAACCCGCTTCTATAATGGGACAGTTATATATTAA                             | pBP373           |
| MI1   | 5'-AAAGGATCCTAATAAAGTTTAGGGGTAGGAGGTATTTATGAAGTA TATTGTGATG             | pBP559           |
| MI2   | 5'-TTCTGCAATTACATTATTTCCAATCTATCAATGGCCAATTACCTTTGTAGAAG                | pBP559           |
| MI11  | 5'-AAAGGATCCATGGAAACGAAATCGTCCAAGTCCG                                  | pBP560           |
| MI12  | 5'-TTCTGCAATTTCATTTCCATCTCTCCCGTCTAGT                                  | pBP560           |
**Table 3. Plasmids**

| Name   | Insert/Features                                                                 | Reference  |
|--------|---------------------------------------------------------------------------------|------------|
| pBAD24 | $P_{BAD}$, ampicillin resistance gene                                           | (48)       |
| pBAD33 | $P_{BAD}$, chloramphenicol resistance gene                                       | (48)       |
| pMAD   | $bgaB$, pBR322 ori and ampicillin resistance gene for (*E. coli*); $Pe194^i$ ori and erythromycin resistance gene (*L. monocytogenes*) | (51)       |
| pWH844 | $P_{TS}$, ampicillin resistance gene                                            | (45)       |
| pGP172 | $P_{T7}$, ampicillin resistance gene                                            | (50)       |
| 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$_c$-$ktrC^{Lmo}$                                                    | This work  |
| pBP346 | pWH844, His$_c$-$kimA^{Sau}$ C-terminal domain                                  | This work  |
| pBP347 | pWH844, His$_c$-$kimA^{Sau}$ C-terminal domain                                  | This work  |
| pBP352 | pMAD-$ΔcdaA$ (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  |
| Plasmid   | Description                                      | Source        |
|-----------|--------------------------------------------------|---------------|
| 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     |
**Table 4.** Michaelis-Menten constants of the potassium transporters

|            | Empty vector | KtrAB<sup>Bsu</sup> | KmA<sub>Lmo</sub> | KmA<sub>Lmo</sub> ΔC-terminus | KtrCD<sub>Lmo</sub> | KmA<sub>Sau</sub> |
|------------|--------------|----------------------|-------------------|-------------------------------|----------------------|------------------|
| **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<sup>-1</sup>)]** | 0.82 ± 0.13   | 0.73 ± 0.05          | 0.52 ± 0.04 (***) | 0.40 ± 0.01 (****)            | 0.59 ± 0.12 (**)    | 0.62 ± 0.05 (***) |

Mean values of the $K_M$ and S.E. are shown ($n = 4$). $p$ 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).
c-di-AMP assists osmoadaptation by regulating the Listeria monocytogenes potassium transporters KimA and KtrCD
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