Cyclic di-AMP Oversight of Counter-Ion Osmolyte Pools Impacts Intrinsic Cefuroxime Resistance in *Lactococcus lactis*

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ABSTRACT  The broadly conserved cyclic di-AMP (c-di-AMP) is a conditionally essential bacterial second messenger. The pool of c-di-AMP is fine-tuned through diadenylate cyclase and phosphodiesterase activities, and direct binding of c-di-AMP to proteins and riboswitches allows the regulation of a broad spectrum of cellular processes. c-di-AMP has a significant impact on intrinsic β-lactam antibiotic resistance in Gram-positive bacteria; however, the reason for this is currently unclear. In this work, genetic studies revealed that suppressor mutations that decrease the activity of the potassium (K⁺) importer KupB or the glutamine importer GlnPQ restore cefuroxime (CEF) resistance in diaadenylate cyclase (cdaA) mutants of *Lactococcus lactis*. Metabolite analyses showed that glutamine is imported by GlnPQ and then rapidly converted to glutamate, and GlnPQ mutations or c-di-AMP negatively affects the pools of the most abundant free amino acids (glutamate and aspartate) during growth. In a high-c-di-AMP mutant, GlnPQ activity could be increased by raising the internal K⁺ level through the overexpression of a c-di-AMP-insensitive KupB variant. These results demonstrate that c-di-AMP reduces GlnPQ activity and, therefore, the level of the major free anions in *L. lactis* through its inhibition of K⁺ import. Excessive ion accumulation in cdaA mutants results in greater spontaneous cell lysis under hypotonic conditions, while CEF-resistant suppressors exhibit reduced cell lysis and lower osmoresistance. This work demonstrates that the overaccumulation of major counter-ion osmolyte pools in c-di-AMP-defective mutants of *L. lactis* causes cefuroxime sensitivity.

IMPORTANCE  The bacterial second messenger cyclic di-AMP (c-di-AMP) is a global regulator of potassium homeostasis and compatible solute uptake in many Gram-positive bacteria, making it essential for osmoregulation. The role that c-di-AMP plays in β-lactam resistance, however, is unclear despite being first identified a decade ago. Here, we demonstrate that the overaccumulation of potassium or free amino acids leads to cefuroxime sensitivity in *Lactococcus lactis* mutants partially defective in c-di-AMP synthesis. It was shown that c-di-AMP negatively affects the levels of the most abundant free amino acids (glutamate and aspartate) in *L. lactis*. Regulation of these major free anions was found to occur via the glutamine transporter GlnPQ, whose activity increased in response to intracellular potassium levels, which are under c-di-AMP control. Evidence is also presented showing that they are major osmolytes that enhance osmoresistance and cell lysis. The regulatory reach of c-di-AMP can be extended to include the main free anions in bacteria.
Cyclic di-AMP (c-di-AMP) is a class of diffusible nucleotide second messengers that transmit signals based on their intracellular concentration (1). Once a c-di-AMP concentration threshold is reached, it is able to bind to protein and riboswitch receptors to alter their activity (2). Most Firmicutes contain one diadenylate cyclase (DAC) (named either CdaA or DacA) and one or two phosphodiesterases (PDEs) (GdpP or PgpH), which fine-tune the intracellular c-di-AMP level (3). Despite a significant number of different c-di-AMP receptors being identified, most play a role in the regulation of K+ or compatible solute (e.g., carnitine and glycine-betaine) accumulation (2). In several bacterial genera, a common role for c-di-AMP in adaptation to environmental osmolarity has been identified and aligns well with the receptors to which c-di-AMP binds (4). PDE mutants with elevated c-di-AMP grow poorly in high-osmolarity media (5). In contrast, a lack of c-di-AMP can restrict bacterial growth and result in lysis of cells in rich media due to the overaccumulation of a range of osmolytes, including peptides, K+, and glycine-betaine (6–10). Growth of cdaA mutants can be rescued in high-osmolarity growth media (6, 11) or through the omission of major osmolytes that are imported by bacteria present in growth media (6–8), suggesting that the internal osmotic pressure is high in cdaA mutants.

Another common phenotype observed in bacterial mutants with altered c-di-AMP levels is varying resistance to cell wall-acting antibiotics of the β-lactam family. β-Lactam antibiotics inhibit the transpeptidase activity of penicillin-binding proteins needed to cross-link the glycan strands in peptidoglycan. β-Lactam treatment results in decreased peptidoglycan cross-linking and cell lysis (12). In different species, gdpP mutants have shown elevated β-lactam resistance (13–15), and cdaA (dacA) mutants exhibited β-lactam sensitivity (6, 16, 17). Clinical isolates of Staphylococcus aureus that have developed intrinsic β-lactam resistance have also been found to contain destructive mutations in the gdpP gene (18–21). The mechanism by which c-di-AMP regulates β-lactam resistance is, however, unclear despite the first evidence of a connection being reported a decade ago. Two hypotheses have been proposed to explain how c-di-AMP might regulate β-lactam resistance. The first is that c-di-AMP regulates cell wall peptidoglycan synthesis. This could be possible in part via the phosphoglucosamine mutase enzyme GlmM, which is involved in peptidoglycan precursor biosynthesis. The glmM gene is colocated in an operon with the most common c-di-AMP synthesis gene, and GlmM regulates c-di-AMP synthesis through direct binding to CdaA/DacA (22–24). The second hypothesis is that a lack of c-di-AMP results in the overaccumulation of intracellular osmolytes that increase internal osmotic pressure to such an extent that the β-lactam-weakened cell wall is unable to prevent lysis (5, 6). This is supported by results showing that β-lactam resistance in c-di-AMP-depleted mutants can be rescued in high-osmolarity media containing sucrose or NaCl (6, 14).

In this study, we identified and characterized genes that contribute to sensitivity to the β-lactam antibiotic cefuroxime (CEF) in c-di-AMP synthesis mutants of the model lactic acid bacterium Lactococcus lactis. CEF was chosen since the depletion of c-di-AMP in Bacillus subtilis and Listeria monocytogenes results in strong CEF hypersensitivity (6, 14, 16). We found that the overaccumulation of K+ and the major anionic amino acids (Glu and aspartate [Asp]) contributes to CEF sensitivity in L. lactis cdaA mutants. CEF-resistant suppressor mutants became sensitive to osmotic stress and lysed significantly less in a hypotonic environment, suggesting that mutants with reduced osmotic pressure have greater stability. We also identified the mechanism by which c-di-AMP regulates the dominant anionic amino acid levels in L. lactis.

RESULTS

CEF-resistant suppressors of cdaA mutants commonly contain mutations in a K+ or amino acid uptake system. Previously, we identified a large number of independent mutations in cdaA in a suppressor screen of different high-c-di-AMP gdpP
mutants under elevated NaCl concentrations (22, 25). Most of the cdaA mutants, which were all more osmoresistant than their gdpP mutant parent, contained single amino acid changes in CdaA, while some possessed frameshift mutations or mutations affecting the cdaA ribosome-binding site (Fig. 1A). Here, we tested a range of different cdaA

![Image of experiment results](image_url)

**FIG 1** Isolation of cefuroxime-resistant suppressors from mutants defective in cdaA. (A) Osmoresistance and CEF resistance of the wild type, a gdpP mutant (gdpP-1), and a variety of osmoresistant cdaA suppressor mutants (labeled with green writing) obtained from parent gdpP-1 and gdpP-2 strains. For this and all other dilution spot plates below, spots are 10 μl of 10-fold serial dilutions of mid-log-phase cultures starting from a 10⁻¹ dilution at the top. (B) CEF resistance of strains using a disk diffusion assay with 0.15 μg CEF per disk. (C) Confirmation of CEF-resistant suppressors (labeled with blue writing) obtained from the cdaA-1 and cdaA-2 strains.

mutants under elevated NaCl concentrations (22, 25). Most of the cdaA mutants, which were all more osmoresistant than their gdpP mutant parent, contained single amino acid changes in CdaA, while some possessed frameshift mutations or mutations affecting the cdaA ribosome-binding site (Fig. 1A). Here, we tested a range of different cdaA
suppressor mutants for their CEF resistance. Out of 11 cdaA mutants, 6 were CEF sensitive (Fig. 1A). We found that in a disk diffusion assay with CEF, strongly growing colonies frequently formed within the inhibition zone of the cdaA-1 (cdaA mutant 1) strain but not its gdpP-1 parent strain (Fig. 1B). Purification of these colonies and retesting using the disk diffusion assay showed that they underwent one or more mutations to become resistant to CEF (see an example of the glnP-3 strain in Fig. 1B). To better understand why L. lactis cdaA mutant strains are sensitive to CEF, we characterized suppressor mutations that restored CEF resistance.

CEF-sensitive cdaA-1 and cdaA-2 strains were chosen for further study. They both have lower c-di-AMP levels than a gdpP mutant strain (see Fig. S1 in the supplemental material). They were plated with inhibitory levels of CEF, and 12 suppressor mutants were obtained and confirmed (Fig. 1C). Whole-genome sequencing (WGS) of these suppressors revealed that distinct mutations occurred in suppressors from the two different parent strains cdaA-1 and cdaA-2 (Table S1).

For the cdaA-2 strain, 4 out of 5 CEF-resistant suppressors possessed mutations in kupB (Table S1). Additional isolation of CEF-resistant suppressors and sequencing of kupB revealed three more independent mutations in this gene (G285, P526L, and F550L) (Fig. 2A). KupB is a K⁺ importer and has been previously identified as a c-di-AMP receptor protein in L. lactis (26), and gain-of-function mutations in kupB restored osmoresistance in a high-c-di-AMP gdpP mutant (25). Interestingly, one suppressor strain (kupB-4) also contained a transposon insertion upstream of busAA (Table S1). Analysis of this IS905 insertion revealed that it is not oriented in a direction that would provide activation of downstream genes, like that described previously (27, 28). Therefore, the insertion likely disrupts transcription initiated from the native busAA promoter located 135 bp upstream.

Interestingly, the KupB amino acid changes identified in our CEF-resistant suppressor screen are mostly located in transmembrane helices within the proximity of residues that interact with K⁺ or protons in KimA from B. subtilis (29) (Fig. S2). To determine if the mutations in kupB caused a gain or loss of function, we examined the effect of the mutation 27 bp upstream of kupB found in the kupB-2 strain on gene expression using a lacZ reporter. It was found that the G→T mutation reduced expression from the kupB promoter by 30% (Fig. 2B). Inspection of the upstream region did not reveal any obvious changes in −10 or −35 sigma factor recognition boxes, so the reason for this downregulation is unclear at this stage. Next, we introduced a wild-type (WT) copy

![FIG 2 Loss-of-function mutations in kupB restore CEF resistance in the cdaA-2 strain. (A) Location of mutations in KupB in CEF-resistant suppressors of the cdaA-2 strain. (B) Promoter activity of wild-type and kupB-2 mutant promoters in the WT strain background. Means ± standard deviations (SD) from three independent biological replicate experiments are shown. *** P < 0.001 using an unpaired t test. (C) CEF resistance of strains, including the kupB-2 strain containing a WT copy of kupB introduced on the pGh9 plasmid.](http://mbio.asm.org/content/12/2/e00324-21/F2)

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of kupB into the kupB-2 suppressor, which restored CEF sensitivity (Fig. 2C). Taken together, these results demonstrate that reduced K$^+$ uptake in the kupB suppressor mutants increases CEF resistance.

For the cdaA-1 strain, 6 out of 7 CEF-resistant suppressors contained mutations in the amino acid ATP-binding cassette (ABC) transport system GlnPQ (Table S1). Additional isolation of CEF-resistant suppressors and sequencing of glnPQ revealed three more independent mutations in these genes (G569W and S621N in GlnP and H199Q in GlnQ) (Fig. 3A). GlnP is composed of a fusion of two substrate-binding domains (SBDs) to the transmembrane permease domain, and GlnQ is an ATPase (30–33). The primary substrate of GlnPQ is glutamine (Gln); however, other amino acids can be imported through this transporter with various affinities. To determine if the mutations in glnPQ caused a gain or loss of function, we introduced the wild-type glnP gene in the glnP-1 strain, which lowered CEF resistance (Fig. 3B). Curing of the glnP expression plasmid from this strain resulted in the restoration of CEF resistance (Fig. 3B). Next, we compared the resistances of strains to the toxic Gln analog L-5-hydroxyglutamine. It was found that 2 of the 3 glnPQ suppressor mutants (glnP-1 and glnQ-1) grew well in the presence of the toxic analog (Fig. 3C). It is likely that the glnP-1 and glnQ-1 strains contain more destructive glnPQ mutations than the glnP-3 strain, which grew poorly at this concentration of analog tested. Next, we compared the growths of strains in chemically defined medium (CDM) with various Gln levels. The glnQ-1 strain grew poorly compared with its cdaA-1 parent strain in low-Gln medium (Fig. 3D). Interestingly, the cdaA-1 and gdpP-1 strains grew better and worse, respectively, than the WT at low Gln concentrations (Fig. 3D), suggesting that c-di-AMP may negatively influence the Gln uptake ability.

To obtain a c-di-AMP synthesis-defective strain completely defective in GlnPQ activity, we plated the cdaA-1 strain onto agar containing an inhibitory concentration of the toxic analog L-5-N-hydroxyglutamine. Two analog-resistant suppressors were obtained (Fig. S3), and analysis of glnP revealed single nucleotide changes that introduced a TAA stop codon at codon 44 (glnP-7) or codon 442 (glnP-6). Both the glnP-6 and glnP-7 strains were more CEF resistant than their parent cdaA-1 strain (Fig. 3E). They were also unable to fully grow in CDM unless very high levels of Gln were provided (Fig. 3F), showing that the Gln acquisition ability of the glnP-6 and glnP-7 strains is severely impaired. Taken together, these results demonstrate that destructive glnPQ mutations lead to reduced uptake of Gln (and possibly other amino acids), which results in CEF resistance in a c-di-AMP synthase-defective strain.

CEF-resistant suppressors possess an osmosensitive phenotype. We hypothesized that the CEF-resistant suppressors have lower concentrations of intracellular osmolytes (K$^+$ or free amino acid pool), which either directly or indirectly results in reduced internal osmotic pressure leading to greater cell stability during CEF-induced cell wall weakening. A lower level of intracellular osmolytes would also be expected to reduce osmoreistance. The CEF-resistant suppressors tested were all found to be more sensitive to osmotic stress than their parent strains (cdaA-1 or cdaA-2) (Fig. S4A). Some CEF-resistant strains (kupB-1, kupB-4, glnP-1, and glnQ-1) were found to be highly NaCl sensitive, suggesting that their mutations are more severe. The expression of glnP rescued NaCl resistance in the glnP-1 strain (Fig. 3B), and the L-5-N-hydroxyglutamine-selected suppressors glnP-6 and glnP-7 were highly NaCl sensitive (Fig. 3E), confirming that in cdaA mutants, GlnPQ activity is required for growth under high osmolarity. We next determined if higher growth medium osmolarity could rescue the CEF resistance of the cdaA-1 and cdaA-2 strains. It was found that the addition of increased NaCl enhanced the growth of these strains on CEF-containing agar (Fig. S4B). Together, these data show that mutations that allow for CEF resistance lower the osmotic pressure within the cell, and CEF-sensitive cdaA mutant cells can be stabilized by elevated external osmolarity.

CEF-resistant suppressors exhibit reduced cell lysis. In c-di-AMP-depleted mutants of B. subtilis and L. monocytogenes, elevated cell lysis occurs during growth in rich media (14, 16). We examined if cdaA mutants of L. lactis would exhibit greater lysis...
FIG 3 Loss-of-function mutations in GlnPQ restore CEF resistance in the cdaA-1 strain. (A) Location of GlnPQ mutations in CEF-resistant suppressors obtained from the cdaA-1 strain. Membrane-spanning regions were predicted using TOPCONS. Note that GlnPQ functions as a homodimer but is shown here as a monomer. (B) CEF and NaCl resistance of strains, including the glnP-1 strain containing a WT copy of glnP introduced on the pGh9 plasmid. A strain in which the plasmid was cured from the glnP-1-pGh9-glnP strain was also included. (C) Growth of strains with the toxic glutamine analog L-5-N-hydroxyglutamine. (D) Growth (after 24 h) of strains in chemically defined medium with various glutamine concentrations. (E) CEF and NaCl resistance of strains, including the L-5-N-hydroxyglutamine-resistant suppressors obtained from the cdaA-1 strain (glnP-6 and glnP-7). (F) Growth (after 24 h) of strains in chemically defined medium with various glutamine concentrations. In panels C, D, and F, means ± SD from three independent biological replicate experiments are shown.
during growth with CEF. Culture supernatants were examined for the presence of DNA and RNA by gel electrophoresis as an indicator of cell lysis. Both the cdaA-1 and cdaA-2 strains were found to lyse significantly when cultured with CEF, while CEF-resistant suppressors, the WT, and gdpP mutants showed no or minimal lysis (Fig. 4A). L. lactis cdaA-1 was also found to undergo some lysis during growth in medium without CEF (Fig. 4A). To explore the role of osmotic pressure in the stability of strains, we compared the amounts of spontaneous lysis of washed cells grown to mid-log-phase, which were suspended in pure water (a hypotonic solution). L. lactis cdaA-1 cells grown in GM17 and heart infusion (HI) media were relatively stable when suspended in water; however, cells grown in glucose-yeast (GY) broth were much more prone to lysis. It was found that both the cdaA-1 and cdaA-2 strains lysed more in water than CEF resistance suppressors, the WT, and gdpP mutants (Fig. 4B). To lower the internal osmotic pressure of cells, cdaA mutants were pregrown in GY medium with increasing concentrations of NaCl. This resulted in greater stability of cells following their resuspension in water, most likely due to a lowering of cell turgor pressure (Fig. 4C). These results suggest that c-di-AMP synthesis mutants possess high internal osmotic pressure, which leads to reduced cell stability.

We next examined if there are cell wall peptidoglycan changes in c-di-AMP-defective strains that may be contributing to CEF sensitivity and reduced cell stability. Remarkably, the cdaA-1 strain, which is CEF sensitive and exhibited less stability than the other strains, had a cell wall that was the same thickness as that of the WT and thicker than the cell walls of the gdpP-1 and glnP-1 strains (Fig. S5A). This suggests that a defect in c-di-AMP synthesis in L. lactis does not negatively affect cell wall biosynthesis. In previous work, we found that an L. lactis gdpP mutant contained elevated peptidoglycan precursor UDP-N-acetylglucosamine (UDP-NAG) levels, which were lowered upon mutation of the phosphoglucosamine mutase gene glmM (22). Here, we measured UDP-NAG levels and found that they negatively correlated with cell wall thickness. UDP-NAG levels were higher in mutants with thinner cell walls, indicating that slower cell wall biosynthesis may result in the accumulation of peptidoglycan precursors (Fig. S5B). Peptidoglycan muropeptides and the peptidoglycan cross-linking index of strains were also analyzed (Fig. S5C). Since CEF inhibits peptidoglycan cross-linking (34), cells with reduced cross-linking may exhibit greater sensitivity. However, a small but statistically significant increase in cross-linking was observed in the CEF-sensitive cdaA-1 mutant compared to its parent gdpP-1 strain (Fig. S5C). Taken together, cell wall analyses did not provide an explanation for the variations in CEF resistance and cell integrity observed in the strains examined here.

Mutations in GlnPQ Lower Intracellular Gln, Glu, and Asp levels. While the roles of K+ and the c-di-AMP-binding receptor KupB have been studied previously in the osmoresistance of L. lactis (25, 26), the role of GlnPQ in c-di-AMP-regulated processes has not been reported. GlnPQ has been found to bind and transport 3 different amino acids (Gln, Glu, and Asn) with different affinities and rates (30, 32, 33). GlnPQ transports only the protonated form of Glu, not the anion, which is the dominant species at physiological pH, and its capacity to transport Asn is lower than its capacity to transport Gln (33). It is the sole transport system in L. lactis for the essential amino acids Gln and Glu (32). This work points to Gln being the primary target of GlnPQ.

The CEF-sensitive cdaA-1 strain contained a higher level of Gln than its parent gdpP-1 strain, which had a low level like the glnPQ suppressors (Fig. 5A). Quantitation of all free amino acid levels in WT L. lactis revealed that Asp and Glu were by far the most abundant, present at ∼100-fold-higher concentrations than Gln (Fig. 5B). Interestingly, their levels also varied significantly between the strains tested. The cdaA-1 strain contained significantly higher Glu and Asp levels than its CEF-resistant glnPQ suppressors (Fig. 5A). In addition, the c-di-AMP level had a negative effect on Glu and Asp levels, with cdaA-1 and gdpP-1 mutants having significantly higher and lower levels, respectively, than their parents (Fig. 5A). From this, we hypothesized that following import by GlnPQ, Gln is converted to Glu and Asp, which together form a major anionic solute
FIG 4  CEF-resistant suppressors release less DNA and RNA during growth with CEF or following resuspension in water. (A) Agarose gel electrophoresis of supernatants from strains grown for various times in HI broth with various CEF concentrations. The locations of genomic DNA (gDNA) and rRNA are indicated. (B) Agarose gel electrophoresis analysis of the supernatant following resuspension of cells (grown to mid-log phase in GY medium) in water. (C) Agarose gel electrophoresis analysis of the supernatant following resuspension of cells (grown to mid-log phase in GY medium with different NaCl concentrations) in water. The gradient from left to right for each strain indicates cells grown in GY medium with 0, 0.05, 0.1, 0.15, and 0.2 M NaCl. The M lane indicates the DNA ladder.
FIG 5  GlnPQ mutations lower the levels of Gln, Glu, and Asp in CEF-resistant suppressors. (A) Intracellular levels of Gln, Glu, and Asp in the indicated strains. Data are means ± SD from three independent biological replicate experiments. *** P < 0.001 using one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons. (B) All intracellular free amino acid levels in WT L. lactis. The inset shows amino acids of low abundance. Data are means ± SD from three independent biological replicate experiments. (C) Proposed pathway for the conversion of Gln to Glu and Asp in L. lactis. Note that several other enzymes in L. lactis can convert Gln to Glu during nucleotide, nucleotide-sugar, and amino acid biosynthesis. Pyr, pyruvate; OA, oxaloacetate; α-KG, α-ketoglutarate; GltB and GltD, glutamate synthase large and small subunits, respectively; AspC, aspartate aminotransferase. The red circles depict c-di-AMP binding to pyruvate carboxylase (PC).
pool. A proposed pathway showing the conversion of Gln to Glu and Asp in L. lactis is shown in Fig. 5C. The pathway also includes the c-di-AMP receptor pyruvate carboxylase (PC), which synthesizes the Asp precursor oxaloacetate. From these results, it was of interest to investigate how c-di-AMP levels might also affect GlnPQ activity and conversion of Gln to Glu and Asp.

**Gln uptake by GlnPQ is activated by increased intracellular ionic strength and K⁺, which provides high levels of the counter-ion Glu.** The experiments described above were carried out using cells grown in rich complex media, so intracellular amino acids can derive from both imported peptides as well as free extracellular amino acids. To verify that the changes in Glu and Asp levels seen in strains were specifically due to altered import of Gln by GlnPQ, we carried out Gln feeding assays with resting (non-growing) cells in buffer with glucose (Fig. 6A). In the WT, rapid and large increases in intracellular Gln and Glu levels were observed; however, no increase in Asp was observed even after 60 min with Gln (Fig. 6B; Fig. S6). This suggests that Gln/Glu is not used to generate Asp in resting cells. We therefore used intracellular Glu as a marker for GlnPQ activity since its level continued to rise over 60 min, while the level of Gln fell due to its conversion to Glu. This confirms that following ATP-dependent import of Gln, rapid conversion to Glu occurs. The high-c-di-AMP gdpP mutant strain gdpP-1 was unable to increase its Glu level to high levels, and its levels remained around 7-fold lower than that of the WT after 5 min (Fig. 6B). The restricted increase in Glu in the gdpP-1 strain was the result of reduced activity of GlnPQ since intracellular Gln levels measured after 5 min of feeding with Gln were also much lower (~8-fold) than those of the WT. The cdaA-1 strain contained high initial levels of Glu but further increased this level following Gln feeding to remain higher than that of the WT. The glnP suppressor mutant glnP-1 had a low starting level of Glu and following Gln addition remained low, as expected, since the GlnPQ transporter is defective in this strain.

The finding that the gdpP mutant strain gdpP-1 is unable to strongly increase Glu levels in this assay suggests that GlnPQ activity is inhibited in this strain. The gdpP-1 strain contains a high level of c-di-AMP (22), which leads to inhibition of K⁺ import through direct binding to KupB (25, 26). We hypothesized that GlnPQ activity is affected by ionic strength in L. lactis cells and that the low K⁺ level observed in the gdpP mutant prevents the activation of GlnPQ. Exposing cells to elevated external osmotic conditions, which we predicted would increase the intracellular ionic strength, led to significantly higher intracellular Glu levels (Fig. 6C).

Next, we examined if an increase in intracellular K⁺ in a high-c-di-AMP gdpP mutant could activate GlnPQ. The gdpP-2 strain containing either a single copy or multiple copies of a constitutively active kupBA618V gene variant accumulated significantly higher Glu levels following Gln feeding (Fig. 6D). Levels of Glu and Asp in the gdpP-2 strain expressing kupBA618V were also significantly higher in cells during growth in rich media (Fig. 6E). Therefore, the level of intracellular K⁺, which is inhibited by c-di-AMP binding to KupB, positively affects the accumulation of the major counter-ion amino acids Glu and Asp in L. lactis.

Finally, we were interested in determining if overfeeding of Gln to cdaA-1 cells would trigger greater cell lysis in nongrowing cells. We grew cdaA-1 cells in CDM with low Gln levels to decrease the Glu pool and reduce cell lysis. Cells were then incubated with either glucose only or glucose and Gln (Fig. 6F), washed, and then resuspended in water. It was found that cdaA-1 cells incubated with glucose and Gln lysed more than the same batch of cells incubated with only glucose (Fig. 6G). Since this assay was performed using nongrowing cells, this confirms that lysis is the direct result of Gln uptake (and Glu overaccumulation) and is unrelated to a change in a metabolic process (e.g., cell wall biosynthesis). Gln feeding to the WT did not result in any observable increase in lysis, which suggests that its osmotic pressure is still lower than that in the cdaA mutant. This is likely due to the intact c-di-AMP system in the WT lowering the levels of other osmolytes within the cell, unlike the cdaA mutant.
FIG 6  GlnPQ activity controls intracellular Glu in response to intracellular K⁺, and overaccumulation of Glu leads to greater cell instability in the cdaA-1 strain. (A) Pathway showing ATP-dependent Gln uptake by GlnPQ and conversion to Glu. (B) Intracellular Glu levels in resting cells following 5 min of incubation with glucose with or without Gln. The means from three independent biological replicate experiments are shown as horizontal bars. (C) Effect of increased osmolarity on Gln uptake in resting cells of the WT after 20 min in buffer. Levels are relative to those in cells in buffer with no additional osmolyte added. *, P < 0.001 using one-way ANOVA followed by Tukey’s test for multiple comparisons. (D) Effect of expression of the c-di-AMP-insensitive KupB variant KupBA618V in a single copy (gdpP-2–kupBA618V) and multiple copies (gdpP-2–pGh9–kupBA618V) on Gln uptake by resting cells of the high-c-di-AMP gdpP-2 mutant in buffer. ***, P < 0.001 using one-way ANOVA followed by Tukey’s test for multiple comparisons. (E) Intracellular Glu and Asp levels in gdpP-2 cells expressing the c-di-AMP-insensitive K⁺ importer KupBA618V in (Continued on next page)
c-di-AMP has emerged as a global osmoregulatory signal in numerous bacterial genera (35). Evidence has indicated that the link between c-di-AMP and β-lactam antibiotic resistance is an additional consequence of its regulation of intracellular osmolyte levels (5, 6). This hypothesis proposes that cells possessing higher internal osmotic pressure (i.e., cdaA mutants) will be more susceptible to osmotic lysis, especially when the stress-bearing peptidoglycan layer is compromised upon CEF exposure. Here, in a screen for suppressors that rescued the CEF resistance of partially defective cdaA L. lactis mutants, mutations that lowered the levels of major inorganic or organic ions (K⁺, Glu, and Asp) were found. GlnPQ and KupB suppressors grew poorly on media with elevated salt, demonstrating that these ions play important roles in osmoregulation in L. lactis. In nongrowing cells, Gln uptake (and Glu accumulation) triggered greater lysis of an L. lactis cdaA mutant, providing further evidence for osmotic pressure being an important contributor to cell instability in a strain with defective c-di-AMP synthesis. Reduced ion accumulation has also been observed in CEF-resistant suppressors of an L. monocytogenes cdaA mutant (6). Mutations that restricted PC activity led to lower levels of the citrate anion. With respect to a direct role of c-di-AMP in cell wall homeostasis, differences in peptidoglycan cross-linking or precursor synthesis have been identified previously in high-c-di-AMP mutants (13, 22, 36). Our analysis of peptidoglycan thickness, cross-linking, and precursor levels, however, did not reveal alterations that would appear to render the L. lactis cdaA mutant peptidoglycan more susceptible to CEF. Indeed, the high-c-di-AMP gdpP mutant, which had the thinnest cell wall and the least cross-linked peptidoglycan, was CEF resistant. A role for more subtle cell wall changes in CEF resistance, however, cannot be excluded. Therefore, our findings provide additional support for a model whereby cdaA mutants accumulate unhealthy levels of several different osmolytes, which, when combined, lead to a critical internal osmotic pressure that a normally structured cell wall is unable to fully withstand.

In support of this theory, we identified that the cdaA⁻¹ mutant contains a busAA-AB promoter mutation that destroys the transcription of the transporter for the compatible solute glycine-betaine (see Table S2 and Fig. S7 in the supplemental material). This mutation likely permitted the growth of this suppressor, which contains a severe cdaA frameshift mutation, on normal media. This is similar to that seen in a Streptococcus agalactiae cdaA mutant, which was viable only after the inactivation of its glycine-betaine transporter (8). Although viable, the L. lactis cdaA⁻¹ mutant is still sensitive to CEF, and a subsequent lowering of additional osmolytes through the inactivation of GlnPQ is necessary to restore CEF resistance. Therefore, c-di-AMP-controlled osmolytes likely have an additive effect on internal osmotic pressure whereby moderate osmolyte overaccumulation permits growth but generates a CEF-sensitive phenotype, while extreme osmolyte overaccumulation results in a complete loss of viability. It is well established that the turgor pressure of Gram-positive bacteria is up to 10-fold higher than that of Gram-negative bacteria and therefore needs to be tightly controlled (37). Our findings and those of others (5–9, 11, 25, 38) suggest that phenotypes affecting the growth and cell integrity of low- and high-c-di-AMP mutants can in most part be explained by variations in the levels of turgor-inducing internal osmolytes.

In L. lactis, c-di-AMP negatively regulates Asp, K⁺, and glycine-betaine levels through direct binding to PC, Kup homologs, BusR, and BusAA/OpuAA (25, 26, 39, 40). Its regulatory reach can now be extended to the control of Glu (and Asp) levels through indirect regulation of GlnPQ. Glu is the major anion in most bacteria, and its accumulation allows the cell to balance the charge of significant levels of K⁺ (41, 42). In the L. lactis WT, Glu is present at a high level, but the other anionic amino acid Asp...
is also present at a similar concentration. Together, these two amino acids represent 55% of the total free amino acids in WT L. lactis (Fig. 5B). In L. lactis, Glu (and Gln) is unable to be synthesized due to an incomplete tricarboxylic acid (TCA) cycle and needs to be sourced from peptides or amino acids external to the cell (33). The GlnPQ transporter in L. lactis plays an important role through its ability to efficiently transport Gln with both high and low affinities (33). Studies of L. lactis growing in defined media revealed that out of 20 amino acids provided to cells in defined media, Gln was the most consumed amino acid, accounting for up to 50% of the total nitrogen imported (43). Therefore, Gln uptake and conversion are significant processes in L. lactis and allow the cell to generate large osmolyte pools of Glu and Asp. Here, we provide evidence that in L. lactis, c-di-AMP has a significant influence on GlnPQ transporter activity through its control of K⁺ levels via direct binding to KupB (25, 26, 44). c-di-AMP can therefore modulate the levels of the major K⁺ counter-ions Glu and Asp. The presence of structurally and functionally similar GlnPQ transport systems in streptococci and enterococci (30) suggests that this regulation mechanism likely extends beyond L. lactis. Based on our work and others, a model for c-di-AMP-regulated homeostasis of the major intracellular ions and their effect on CEF resistance in L. lactis can be proposed (Fig. 7).

Recent work in S. aureus and B. subtilis identified suppressor mutations in Gln and Glu transporters, respectively, which rescued the growth of mutants devoid of c-di-AMP (9, 38). These results align well with those found in L. lactis and suggest that cells defective in c-di-AMP production are unable to regulate intracellular levels of major osmolyte amino acids. The mode of regulation of the mutated transporters (AlaT, AimA, and YfKc) is not known; however, it would be of interest to explore if they are controlled indirectly by c-di-AMP, like GlnPQ in L. lactis. In support of our findings, a previous in vitro characterization of L. lactis GlnPQ found that Gln uptake in reconstituted proteoliposomes was activated ~4-fold by an increased salt concentration in the lumen (32). In other work, the ATPase component (GlnQ) of S. agalactiae was found not to bind c-di-AMP (8), therefore making it unlikely that c-di-AMP-regulated Gln uptake occurs via a direct interaction. Indeed, when we overexpressed constitutively active KupBΔ618V in the gdpP-2 strain, which triggers an accumulation of c-di-AMP (25), GlnPQ was found to be more active due to elevated K⁺ import (Fig. 6D). Recent work in B. subtilis has found that Glu availability affects K⁺ import activity by KtrCD (45), further confirming the need for alignment of ionic osmolyte levels. Coordination of K⁺ and anion levels ensures a balancing of the charge within the cell, and by controlling both through a common orchestrator (c-di-AMP), it can occur efficiently and rapidly.

**MATERIALS AND METHODS**

**Strains, media, and chemicals used.** L. lactis strains (see Table S2 in the supplemental material) were routinely grown in nonshaking tubes at 30°C in M17 medium (Difco, USA) supplemented with 0.5% (wt/vol) glucose (GM17). When needed, 3 μg/ml erythromycin (Em) was added to the media for L. lactis. DNA release assays were carried out on cells grown in either heart infusion (HI) medium (Oxoid) or glucose-yeast (GY) medium (1.33% yeast extract [Oxoid], 1.33% glucose, and 1% 0.1 M K₂HPO₄). To characterize the growth of L. lactis under different concentrations of glutamine (Gln), chemically defined minimal medium (CDM) using two types of Dulbecco’s modified Eagle’s medium (DMEM) (Merck) was used as the base (39). The first type of DMEM (catalog no. 5671) contains 4.5 g/liter glucose and sodium bicarbonate, while the second type of DMEM (catalog no. 5546) contains 1 g/liter glucose and 0.11 g/liter pyruvic acid. Both media are devoid of Gln and Glu. These media were supplemented with histidine at 0.13 mg/ml, arginine at 0.72 mg/ml, leucine at 1 mg/ml, valine at 0.6 mg/ml, glucose at 0.15%, sodium acetate at 0.75 mg/ml, morpholinepropanesulfonic acid (MOPS) at 13 mg/ml, guanine at 0.05 mg/ml, xanthine at 0.05 mg/ml, FeSO₄ at 0.1 mg/ml, ZnSO₄ at 0.1 mg/ml, and adenine at 0.2 mg/ml. Unless stated otherwise, cells were washed and resuspended in KPM buffer (0.1 M K₂HPO₄ adjusted with H₂PO₄ acid to pH 6.5 and supplemented with 10 mM MgSO₄) (46). Escherichia coli NEB-5α cells containing pGln derivatives were grown in Hi medium containing 150 μg/ml Em at 30°C with aeration at 250 rpm.

**c-di-AMP extraction and quantification.** c-di-AMP from L. lactis was extracted as previously described (25). c-di-AMP was detected and quantified by liquid chromatography-coupled tandem mass spectrometry (LCMS-8060; Shimadzu, Japan). Chromatographic separation was performed on an ultra-high-pressure liquid chromatography (UHPLC) Nextera X2 instrument using a Shim-pack Velox SP-C₁₈ column (1.8 μm, 2.1 by 150 mm; Shimadzu, Japan). Eluents A and B consisted of 0.05% (vol/vol) formic acid in water and acetonitrile (Merck), respectively. The sample volume was 10 μl with a flow rate of...
Eluent A (95%) was used from 0 to 1 min, followed by a linear gradient from 95% to 50% eluent A until 10 min. The column was then washed with 90% eluent B for 3 min and then reequilibrated with 95% eluent A for 2 min prior to reinjection. The internal standard of azidothymidine (AZT) (Sigma) was used. c-di-AMP was detected with a triple-quadruple mass spectrometer equipped with an electrospray ionization interface.
source using multiple reaction monitoring transitions of m/z 657—124 in negative ionization mode. Data obtained were curated using LabSolutions Insight version 3.2 SP1 and LabSolutions Postrun/QuantBrowser version 5.95 (Shimadzu Corporation).

Isolation of CEF-resistant suppressors and WGS. The cdaA mutant strains cdaA-1 and cdaA-2 were streaked or spread either from mid-log-phase cultures, from broth cultures grown overnight, or directly from frozen glycerol stocks (40% glycerol) onto GM17 agar containing 0.08 μg/ml CEF (Merck) and incubated for 2 days at 30°C. Colonies were picked and restreaked on agar containing the same concentration of CEF, from where they were obtained to ensure purity. CEF resistance confirmation was carried out by serial dilution of mid-log-phase cultures onto GM17 agar with CEF.

CEF disk diffusion assays were carried out by mixing 5 μl of a mid-log-phase culture (optical density [OD] of ~0.7) with 7 ml of 0.75% GM17 agar and pouring the culture onto a 15 ml 1.5% GM17 agar base. Following drying, a sterile 8-mm disk was placed on the top agar, and a 10-μl solution of CEF was added (0.15 μg). Following incubation overnight, zones of inhibition were observed. CEF-resistant suppressors were checked for cdaA back-mutations using PCR (Table S3), as described previously (22), before being analyzed by WGS. Genomic DNA extractions were performed as described previously (47). Sequencing was performed using the Illumina NovaSeq 6000 platform (Macrogen, South Korea). Single nucleotide polymorphisms (SNPs) were analyzed using Geneious Prime (Biomatters Ltd., New Zealand) (22, 25).

Genetic manipulation of strains. Plasmids and primers used in this study are shown in Tables S2 and S3, respectively. Electroporation of L. lactis strains was done as previously described (48), with minor changes for some strains. Following electroporation of pGh9-kupB and pGh9-glnP into the kupB-2 and glnP-1 strains, respectively, cells were plated onto GM17 agar with 3 μg/ml Em supplemented with 0.1 M NaCl. The activities of promoters were determined using pTCV-lac (49). The WT promoter of kupB and the mutated variant from the kupB-2 strain were amplified and cloned into pTCV-lac and assayed for activity in the L. lactis WT using a β-galactosidase assay.

Isolation of L-S-N-hydroxyglutamine-resistant suppressors. The cdaA mutant strain cdaA-1 was streaked onto GM17 agar containing 1 mM the toxic glutamine analog L-S-N-hydroxyglutamine (Merck) and incubated for 2 days at 30°C. Colonies were picked and restreaked on agar containing the same concentration of the analog to ensure purity. Resistance confirmation was carried out by the serial dilution drop plate method as described above.

Cell wall thickness analysis. Cells grown to mid-log phase were fixed with 2.5% glutaraldehyde (ProSciTech) in phosphate-buffered saline (pH 7.4), and after washing in buffer, they were postfixed in 1% osmium tetroxide. They were then gradually dehydrated in ethanol (30 to 100%) and infiltrated with a gradual increase in the concentration of Epon resin, and then polymerized for 2 days at 60°C. Ultrathin (60-nm) sections were collected onto 200-mesh copper grids and stained with uranyl acetate and lead citrate. Grids were examined using a Hitachi HT7700 electron microscope (Hitachi, Japan) operated at 80 kV. Images were acquired with a complementary metal oxide semiconductor (CMOS) camera (Advanced Microscopy Techniques), and peptidoglycan thickness was measured on micrographs.

UDP-NAG quantitation. Mid-log-phase (OD at 600 nm [OD600] of ~0.6) cells were collected by centrifugation at 5,000 × g for 10 min at 4°C and washed 2 times with 1/10 KPM buffer. UDP-NAG was extracted and quantified as previously described (22).

Peptidoglycan muropeptide and cross-linking analysis. Peptidoglycan was extracted from exponential-phase cells (OD600 of ~0.8) and then digested with mutanolysin as described previously (50). The resulting soluble muropeptides were reduced with sodium borohydride and separated by reverse-phase UHPLC (RP-UHPLC) with a 1290 chromatography system (Agilent Technologies) and a Zorbax Eclipse Plus C18 column. Resulting soluble muropeptides were reduced with sodium borohydride and separated by reverse-phase UHPLC (RP-UHPLC) with a 1290 chromatography system (Agilent Technologies) and a Zorbax Eclipse Plus C18 column. The different muropeptides were quantified by integration of the peak areas, and the percentage of each peak was calculated as the ratio of its area over the sum of all muropeptides.

Quantification of amino acid pools in growing L. lactis cells. Cells were grown in 30 ml GM17 medium until an OD600 of ~0.7 was reached, collected by centrifugation at 5,000 × g for 10 min at 4°C, and washed twice in 1/10 KPM buffer. After resuspension in 1.8 ml of 0.75% GM17 agar and pouring the culture onto a 15-ml 1.5% GM17 agar base, the activities of promoters were determined using pTCV-lac (49). The WT promoter of kupB and the mutated variant from the kupB-2 strain were amplified and cloned into pTCV-lac and assayed for activity in the L. lactis WT using a β-galactosidase assay.

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the Glu assay kit (catalog no. MAK004-1KT; Merck) or the Gln and Glu determination kit (catalog no. GLN1-1KT; Merck), with minor adjustments to the protocols. For the Glu assay kit, the dried samples were resuspended in 50 µl of Glu assay buffer, vortexed well, and centrifuged at 16,000 x g for 5 min at 25°C. A portion (2 µl) was added into the kit master mix before incubation and reading of the OD450 using a NanoDrop One instrument (Thermo Fisher Scientific). For quantification, Glu standards were prepared at 1,000 to 31.25 µM in serial 2-fold dilutions.

**REFERENCES**

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**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**FIG S1, DOCX file, 0.02 MB.**

**FIG S2, DOCX file, 0.8 MB.**

**FIG S3, DOCX file, 1.7 MB.**

**FIG S4, DOCX file, 1.5 MB.**

**FIG S5, DOCX file, 0.04 MB.**

**FIG S6, DOCX file, 0.02 MB.**

**FIG S7, DOCX file, 2.1 MB.**

**TABLE S1, DOCX file, 0.01 MB.**

**TABLE S2, DOCX file, 0.03 MB.**

**TABLE S3, DOCX file, 0.01 MB.**

**REFERENCES**


