c-di-AMP-Regulated K⁺ Importer KtrAB Affects Biofilm Formation, Stress Response, and SpeB Expression in *Streptococcus pyogenes*

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ABSTRACT The second messenger cyclic di-AMP (c-di-AMP) controls biofilm formation, stress response, and virulence in *Streptococcus pyogenes*. The deletion of the c-di-AMP synthase gene, *dacA*, results in pleiotropic effects including reduced expression of the secreted protease SpeB. Here, we report a role for K⁺ transport in c-di-AMP-mediated SpeB expression. The deletion of *ktrB* in the Δ *dacA* mutant restores SpeB expression. KtrB is a subunit of the K⁺ transport system KtrAB that forms a putative high-affinity K⁺ importer. KtrB forms a membrane K⁺ channel, and KtrA acts as a cytosolic gating protein that controls the transport capacity of the system by binding ligands including c-di-AMP. SpeB induction in the Δ *dacA* mutant by K⁺ specific ionophore treatment also supports the importance of cellular K⁺ balance in SpeB production. The Δ *dacA* Δ *ktrB* double deletion mutant not only produces wild-type levels of SpeB but also partially or fully reverts the defective Δ *dacA* phenotypes of biofilm formation and stress responses, suggesting that many Δ *dacA* phenotypes are due to cellular K⁺ imbalance. However, the null pathogenicity of the Δ *dacA* mutant in a murine subcutaneous infection model is not restored by *ktrB* deletion, suggesting that c-di-AMP controls not only cellular K⁺ balance but also other metabolic and/or virulence pathways. The deletion of other putative K⁺ importer genes, *kup* and *kimA*, does not phenocopy the deletion of *ktrB* regarding SpeB induction in the Δ *dacA* mutant, suggesting that KtrAB is the primary K⁺ importer that is responsible for controlling cellular K⁺ levels under laboratory growth conditions.

KEYWORDS *Streptococcus pyogenes*, c-di-AMP, DacA, SpeB, KtrAB, Kup, KimA

Most organisms produce cyclic nucleotide second messengers that regulate cellular activities by binding to effector molecules such as proteins or RNAs. Cyclic nucleotide-involved signaling pathways sense environmental changes, such as temperature, nutrition, pH, and other stressors, and transmit the signals to effector molecules (1–4). Many bacteria have been shown to produce several different cyclic dinucleotides, such as c-di-AMP (cyclic di-AMP), c-di-GMP (cyclic di-GMP), and cGAMP (cyclic GMP-AMP). c-di-AMP, produced by many bacteria and archaea (5), is involved in controlling diverse cellular processes, such as fatty acid biosynthesis (6), DNA integrity detection (7–9), and cell wall homeostasis (10–13), in a bacterium-specific manner. However, more than a decade of research has shown that a major role of c-di-AMP in most bacteria is in maintaining proper turgor pressure by controlling the activity of ion and/or osmolyte transporters (14–16).

Many important pathogens, including *Streptococcus* species (*S. pyogenes*, *S. agalactiae*, and *S. mutans*), *Staphylococcus aureus*, *Mycobacterium tuberculosis*, and *Listeria monocytogenes*, appear to export c-di-AMP into the environment. When these pathogens are internalized into their host cells, the secreted c-di-AMP is detected by the host cell's STING (STimulator of INTERferon Gene) (5, 17). STING also senses 2'3'-cGAMP

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(2'3' cyclic GMP AMP), another cyclic dinucleotide produced by the host enzyme cGAS (cyclic GMP AMP synthase) that is activated by bacterial DNA in the host cytosol (5, 17). When sensing these c-dinucleotides, STING activates type I interferon production. Thus, c-di-AMP regulates not only bacterial but also host cellular processes during infection. However, the detailed role of c-di-AMP during infection is largely unknown.

S. pyogenes, also known as group A *Streptococcus* (GAS), is a Gram-positive pathogen that generally causes noninvasive diseases, such as strep throat and impetigo. However, these superficial infections sometimes develop serious diseases, such as rheumatic heart disease, streptococcal toxic shock syndrome, poststreptococcal glomerulonephritis, and necrotizing fasciitis. A minimum global burden by *S. pyogenes* infection is estimated at over 18 million cases of severe diseases, resulting in over half a million annual deaths (18). Despite the dire consequences of this pathogen, commercial vaccines are not yet available.

Our recent study has revealed that c-di-AMP regulates diverse cellular traits and virulence of *S. pyogenes* (18). When *dacA*, the only c-di-AMP synthase gene in the *S. pyogenes* chromosome, is deleted, the $\Delta dacA$ mutant strain displays many defective phenotypes, including increased lag time for growth, reduced biofilm formation and SpeB expression, and increased sensitivity to stressors such as high salt concentrations, low pH, reactive oxygen, and the cell wall-targeting antibiotic ampicillin. Here, we show that the null mutation of a high-affinity K⁺ channel protein KtrB in the $\Delta dacA$ mutant reverts most of these defective phenotypes, suggesting that the majority of the $\Delta dacA$ phenotypes result from KtrAB malfunction caused by the absence of c-di-AMP. However, the deletion of *ktrB* in the $\Delta dacA$ mutant was unable to restore virulence in a murine model, suggesting that c-di-AMP also plays roles in cell physiology and/or virulence other than K⁺ transport in *S. pyogenes*.

RESULTS

Mutation of the K⁺ importer subunit KtrB restores the SpeB expression of $\Delta dacA$ mutant. We have previously reported that the deletion of the c-di-AMP synthase gene, *dacA*, abolishes the ability of *S. pyogenes* to produce the secreted cysteine protease SpeB (18). Unexpectedly, however, one of the 18 $\Delta dacA$ strains created by the *dacA* in-frame deletion process showed SpeB⁺. Whole-genome sequencing of this mutant revealed a single base pair deletion in *ktrB* (Spy_0327 based on the SF370 reference; L897_01540 in HSC5). KtrB is a subunit of the KtrAB system, known to be a high-affinity K⁺ importer (19–21). KtrB is a membrane-integrated protein that forms a K⁺ channel and interacts with the cytosolic protein KtrA (Spy_0326; L897_01535 in HSC5) that controls the transport activity of KtrB (22). KtrA orthologs, KtrA in *S. aureus*, CabP in *S. pneumoniae*, and CapPA in *S. mutans*, are known c-di-AMP-binding proteins (23–25). In *S. pyogenes*, *ktrA* and *ktrB* form a bicistronic operon in the order of *ktrBA* (Fig. 1), and their coexpression was confirmed by total RNA sequencing (26). The $\Delta dacA \Delta ktrB^{sup}$ suppressor mutant contained a single-nucleotide deletion within a homopolymeric stretch of eight thymines in *ktrB*, resulting in a frameshift mutation (Fig. 1).

To confirm that *ktrB* is responsible for the suppression of the SpeB phenotype observed in the $\Delta dacA \Delta ktrB^{sup}$ strain, an in-frame deletion of *ktrB* was generated in the $\Delta dacA$ mutant, and the SpeB activity of the $\Delta dacA \Delta ktrB$ mutant was examined. As expected, the $\Delta dacA \Delta ktrB$ mutant produced SpeB, similar to the $\Delta dacA \Delta ktrB^{sup}$ mutant and the wild type (Fig. 2). To further confirm the role of the KtrAB system in regulating c-di-AMP-mediated SpeB regulation, the *ktrA* gene was subsequently deleted in the $\Delta dacA$ mutant, and the resulting $\Delta dacA \Delta ktrA$ mutant also produced SpeB, although to a lesser extent than the wild type (Fig. 2C). SpeB activity was examined in $\Delta ktrA$ and $\Delta ktrB$ single-gene deletion mutants, and their SpeB activity was similar to that of the wild type (Fig. 2D). Quantitative reverse transcription-PCR (qRT-PCR) analysis measuring *speB* transcript levels in the cells demonstrated that the SpeB activity change in the $\Delta dacA \Delta ktrB$ and $\Delta dacA \Delta ktrA$ mutants occurs at the transcriptional level (Fig. 3).

FIG 1 *ktrB* genomic context and the mutation site in *ktrB* of the $\Delta dacA$ $\Delta ktrB^{sup}$ suppressor mutant. Each arrow indicates an individual open reading frame and its orientation. *ktrB* is coexpressed with the downstream gene *ktrA*, which is shown with a dotted arrow under the *ktrB* and *ktrA* genes. The predicted proteins encoded by these open reading frames and their putative functions are shown below the gene organization. The asterisk shows the location of the mutation site in *ktrB* in the $\Delta dacA$ $\Delta ktrB^{sup}$ suppressor mutant. In the mutant, one thymidine out of eight consecutive thymidines is deleted.

Mutation of *ktrB* reverts the defects of biofilm formation and stress response of $\Delta dacA$ mutant to levels equal to or near that of the wild type. The deletion of *dacA* results in various defective growth characteristics, such as increased lag phase, decreased biofilm formation, and increased sensitivity to low pH, high-salt concentrations, oxidative stress, and cell wall-targeting antibiotics (18). When *ktrB* was mutated in the $\Delta dacA$ mutant, these defective phenotypes were partially or fully reverted to those of the wild type (Fig. 4). The lag time of the $\Delta dacA$ mutant is almost twice that of the wild type (4.5 h versus 2.5 h in Todd-Hewitt medium supplemented with 0.2% yeast extract [THY medium]), and the $\Delta dacA$ $\Delta ktrB$ mutant decreased its lag time to that of the wild type (2.5 h). The $\Delta dacA$ mutant cannot form a biofilm, but the $\Delta dacA$ $\Delta ktrB$ mutant partially regained the ability to form a biofilm (50% as much biofilm as the wild type) (Fig. 4A). The $\Delta dacA$ mutant cannot grow in THY or C medium whose initial pH is adjusted to 6.0. In contrast, the $\Delta dacA$ $\Delta ktrB$ mutant reached an optical density at 600 nm (OD₆₀₀) of approximately 66% of wild-type levels in acidified THY medium but still did not grow in acidified C medium (Fig. 4B). The $\Delta dacA$ mutant cannot grow or grows very poorly in THY medium containing additional 0.1 M KCl or NaCl. However, the $\Delta dacA$ $\Delta ktrB$ mutant grew to nearly wild-type levels under both conditions (Fig. 4C). The $\Delta dacA$ mutant shows higher sensitivity to reactive oxygen species than the wild type under both short-term and long-term exposure conditions. In contrast, the $\Delta dacA$ $\Delta ktrB$ mutant showed decreased sensitivity to reactive oxygen species compared to the $\Delta dacA$ mutant (Fig. 4D). The $\Delta dacA$ mutant is more resistant to PlyC, as seen by a decrease in cell lysis compared to that of the wild type, whereas the $\Delta dacA$ $\Delta ktrB$ mu-

FIG 2 Deletion of *ktrB* restores the *speB* activity of $\Delta dacA$ mutant. The activity of the secreted protease SpeB is shown on protease indicator plates. Strains were grown overnight and spotted (2 μ l) onto protease indicator agar plates after serial dilution. Protease activity displays a clear zone around the spotted cells after incubation. Strain names are shown above the images, and dilution degrees of the spotted cultures are indicated at the left side of the images. Plates were incubated for 24 h.

FIG 3 SpeB activity variations of the mutants were caused at the transcriptional level. The relative abundance of the *speB* transcript during the stationary-phase growth in mutants was determined using qRT-PCR and compared to that of the wild type. Each column represents the *speB* transcript abundance in a mutant relative to that in the wild type. Shown are the means and standard deviations from three independent experiments. Asterisks indicate the significance of the difference between a mutant and the wild type, as calculated by one-way analysis of variance (ANOVA) followed by Dunnett's multiple-comparison test (ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$).

tant displayed wild-type levels of resistance to PlyC (Fig. 4E). The sensitivity of the $\Delta dacA$ mutant to sublethal levels of ampicillin treatment is much higher than that of the wild type, whereas the sensitivity of the $\Delta dacA \Delta ktrB$ mutant was comparable to that of the wild type (Fig. 4F).

Mutation of *ktrB* does not restore the attenuated virulence of the $\Delta dacA$ mutant. The $\Delta dacA$ mutant displays no virulence in a murine subcutaneous infection model, as seen by the absence of a lesion (18). The virulence of the $\Delta dacA \Delta ktrB$ mutant was examined using the same soft-tissue infection model, and, surprisingly, none of the mice injected with the $\Delta dacA \Delta ktrB$ mutant developed lesions (Fig. 5). Thus,

FIG 4 KtrB mutation either fully or partially reverts defective growth phenotypes caused by *dacA* deletion. (A) Biofilm formation ability of *S. pyogenes* strains. (B) The growth of *S. pyogenes* (OD_{600}) at 8 h postinoculation in acidified THY or C medium (pH 6.0). (C) The growth of *S. pyogenes* (OD_{600}) at 8 h postinoculation in THY medium added with 0.1 M KCl or NaCl. (D) Relative viability of *S. pyogenes* strains after being exposed to oxidative stress caused by methyl viologen (MV; 50 mM for 3 h in short-term exposure, 1.7 mM for 18 h in long-term exposure). (E) Cell lysis by the cell wall-hydrolyzing enzyme PlyC. Each strain was incubated with PlyC (3,000 U/ml) at 37°C. The turbidity (OD_{600}) of each strain at 6 h postincubation was measured to determine the degree of cell lysis. (F) Relative cell growth (OD_{600}) by comparing the growths with or without sublethal levels of ampicillin (40 ng/ml). The horizontal bar in each data set is the mean value of the data points. The significance of the difference between values from two different strains was calculated by one-way ANOVA with Tukey's multiple-comparison test (ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$).

FIG 5 Deletion of *ktrB* does not revert the severely attenuated virulence of the $\Delta dacA$ mutant. The ability of wild-type HSC5 (A), $\Delta dacA$ (B), and $\Delta dacA \Delta ktrB$ (C) strains to cause lesions in a murine subcutaneous infection model is shown. The virulence of each strain was evaluated by measuring the area of the lesion formed on day 3 postinfection when lesion sizes were maximal. Each circle represents the size of a lesion formed by an injection of the wild-type, $\Delta dacA$, or $\Delta dacA \Delta ktrB$ strain. Each solid bar indicates the mean value of ulcer sizes. The asterisk marks above each bracket indicate the significance of difference (****, $P < 0.0001$) between each mutant and the wild type as calculated by the Mann-Whitney U test statistic.

even though many phenotypes of the $\Delta dacA$ mutant were reverted to those of the wild type by *ktrB* mutation (Fig. 4), the virulence loss of the $\Delta dacA$ mutant was not reverted by the same mutation.

Valinomycin and gramicidin ionophore treatment mimics the effect of *ktrB* mutation on the restoration of SpeB activity of the $\Delta dacA$ mutant. Ionophores are small hydrophobic lipid-soluble molecules that transport ions across cell membranes. Because ionophores are not coupled to energy sources, they only transport ions down their electrochemical gradient and are used as antibiotics due to their ability to collapse ion gradients across cellular membranes. Since the deletion of *ktrB* restores SpeB production in the $\Delta dacA$ mutant, we examined the effect of the ionophores that disturb cellular K^+ balance, such as valinomycin and gramicidin, on SpeB production (27). Valinomycin is a carrier ionophore that transports K^+ across the membrane. Gramicidin is a channel-forming ionophore that transports small inorganic monovalent cations, such as K^+ and Na^+ , across the membrane (27). Since both of these ionophores have growth inhibition effects, sub-growth-inhibitory concentrations of these ionophores were determined by monitoring *S. pyogenes* growth (OD_{600}) at different concentrations. A concentration of 1 $\mu g/ml$ valinomycin or 0.5 $\mu g/ml$ gramicidin has little to no effect on the growth of wild-type HSC5 in liquid culture. When tested on solid media, these conditions also minimally affected the growth and SpeB activity of the wild type (Fig. 6). When the $\Delta dacA$ mutant was treated with the ionophores, it regained the ability to produce SpeB (Fig. 6), mimicking the effect of *ktrB* mutation on SpeB activity of the $\Delta dacA$ mutant (Fig. 2). When the $\Delta dacA \Delta ktrB$ mutant was treated with ionophores, it did not significantly change SpeB activity (Fig. 6).

GdpP and Pde2 are two c-di-AMP phosphodiesterases identified in *S. pyogenes* (18). Similar to the $\Delta dacA$ mutant, the $\Delta pde2$ mutant does not produce SpeB (18). However, unlike the $\Delta dacA$ mutant, the $\Delta pde2$ mutant did not show SpeB activity when treated with the ionophores (Fig. 6). This result indicates that the underlying mechanism causing the SpeB null phenotype of the $\Delta pde2$ mutant is different from that of the $\Delta dacA$ mutant.

Deletion of putative K^+ importer gene *kup* or *kimA* does not phenocopy *ktrB* deletion. The *S. pyogenes* genome encodes homologs of at least two additional K^+ importers other than KtrAB, which include a Kup homolog (SPy_1414 in SF370, L897_05730 in HSC5) and a KimA homolog (SPy_2088 in SF370, L897_08830 in HSC5). In-frame deletion mutants of each of these K^+ transporter homologs were generated in the $\Delta dacA$ mutant, and SpeB activity of the resulting $\Delta dacA \Delta kup$ and $\Delta dacA \Delta kimA$ mutants was examined. The $\Delta dacA \Delta kup$ mutant showed very low SpeB activity on a protease agar plate following extended incubation (Fig. 7). Similarly, the $\Delta dacA \Delta kimA$ mutant did not exhibit SpeB activity. These results indicate that the role of KimA and Kup in the $\Delta dacA$ mutant under the *in vitro* growth conditions was not as crucial as that of KtrAB. SpeB activity of $\Delta kimA$ and Δkup single-gene deletion mutants was

FIG 6 Ionophores specific to K⁺ (valinomycin) and monovalent cations (gramicidin) restore SpeB activity of the $\Delta dacA$ mutant to the wild-type level. The effect of ionophores on SpeB activity of *S. pyogenes* strains was observed. Overnight cultures of *S. pyogenes* strains were serially diluted with THY medium and spotted onto protease indicator plates containing no ionophore, valinomycin (1 µg/ml), or gramicidin (0.5 µg/ml). Culture dilution degrees were 10¹, 10², 10³, and 10⁴ from the top.

comparable to that of the wild type (Fig. 7). qRT-PCR analysis measuring the *speB* transcript levels in the cells demonstrate that the SpeB activity change in these mutants occurred at the transcriptional level (Fig. 3).

DISCUSSION

The potassium ion (K⁺) is the most abundant intracellular cation and has a primary role in turgor compensation and osmotic adjustment (28, 29). Thus, controlling cellular K⁺ levels through K⁺ transporters is crucial for the survival of cells in diverse osmotic environments. It has been shown in Gram-positive bacteria that c-di-AMP inhibits transporters that maintain turgor pressure and osmotic adjustment, such as potassium transporters and/or glycine-betaine transporters (14–16, 30, 31). For example, c-di-AMP inhibits the activity of the multiple potassium transporters, the KtrAB system, the KtrCD system, and KimA in *B. subtilis* and the KupA and KupB potassium transporters in *Lactococcus lactis* (30, 31).

In this study, KtrB, the channel component of the K⁺ importer KrtAB system, was identified as a suppressor of the SpeB null phenotype of the $\Delta dacA$ mutant, suggesting

FIG 7 Deletion of a putative K⁺ importer gene, *kup* or *kimA*, does not phenocopy *ktrB* deletion in the $\Delta dacA$ mutant. Unlike the deletion of *ktrB*, the deletion of another K⁺ importer gene, *kup* or *kimA*, did not restore the SpeB activity of the $\Delta dacA$ mutant to the wild-type level. Strain names are shown above the pictures, and the dilution degrees of the cultures are indicated at the left side of the images. The protease indicator plates were incubated for 48 h in this experiment (most strains developed zones of clearance in 24 h, but the $\Delta dacA \Delta kup$ strain took 48 h to become visible).

FIG 8 Model explaining the relationship between the activity of the KtrAB system and SpeB production in *S. pyogenes*. Interactions between c-di-AMP and the KtrAB system and their effect on SpeB expression in the wild type (WT), $\Delta dacA$, $\Delta dacA \Delta ktrB$, and $\Delta dacA \Delta ktrA$ strains are shown.

that the control of cellular K^+ concentration connects c-di-AMP signal transduction and SpeB production. KtrAB is a member of the Trk/Ktr/HKT K^+ transporter superfamily that consists of uniporters and symporters (K^+/Na^+ or K^+/H^+) (32). KtrAB appears to be a high-affinity K^+ importer, as the K_m value of the KtrAB system in *B. subtilis* for K^+ is in the micromolar range (19–21). The KtrAB system is composed of a homodimeric KtrB membrane protein complex and a cytosolic KtrA octameric ring (22). Each KtrB subunit forms a potassium channel, so deletion of *ktrB* abolishes the K^+ transport ability of the KtrAB system. KtrA is a gating component regulating KtrB activity (22). KtrA contains regulator of conductance of potassium (RCK) domains at the amino terminus (RCK_N) and carboxy terminus (RCK_C). ATP and ADP bind to the RCK_N domain. When ATP binds to the domain, the K^+ transport ability of KtrAB increases, but when ADP binds, the ability decreases. It has been shown that KtrA orthologs in *S. aureus* (KtrA), *S. pneumoniae* (CabP), and *S. mutans* (CapPA) are c-di-AMP-binding proteins (23–25). c-di-AMP binds to the RCK_C domain of KtrA and inhibits the K^+ transport ability of the KtrAB system (23). KtrA bound by c-di-AMP does not interact with KtrB in *S. pneumoniae* (25). KtrB without KtrA has almost half of its full K^+ transport activity in *B. subtilis* (32).

A model for the interaction of c-di-AMP and K^+ and SpeB expression is shown in Fig. 8. As the deletion of *dacA* abolishes the ability of *S. pyogenes* to synthesize c-di-AMP (18), the KtrA octameric ring in the absence of c-di-AMP likely interacts with the KtrB dimer, thereby promoting K^+ import (Fig. 8, $\Delta dacA$ mutant) (25). Alternative K^+ transporters and glycine-betaine transporters may import additional K^+ and glycine-betaine in the absence of c-di-AMP (14–16). This increased cellular K^+ concentration may trigger repression of SpeB expression via a decrease in *speB* transcription through a currently undefined mechanism. Removal of the K^+ channel KtrB in a c-di-AMP-devoid strain likely leads to a decrease in intracellular K^+ and subsequent restoration of SpeB expression via an increase in *speB* transcription (Fig. 8, $\Delta dacA \Delta ktrB$ mutant). This model is further supported through exposure to ionophores, as a sublethal treatment of the $\Delta dacA$ mutant with valinomycin or gramicidin presumably leads to an efflux of K^+ from the cells and restoration of SpeB production in the $\Delta dacA$ mutant (Fig. 6). However, ionophore treatment of the $\Delta dacA \Delta ktrB$ mutant has no effect on SpeB production, because the $\Delta dacA \Delta ktrB$ mutant presumably already has low cellular K^+ (Fig. 6). The $\Delta dacA \Delta ktrA$ mutant appears to import more K^+ than the wild type, since SpeB activity of the strain is less than that of the wild type (Fig. 8, $\Delta dacA \Delta ktrA$ mutant). Lower K^+ import than that of the wild type does not appear to influence SpeB expression, because all of the single K^+ transporter gene deletion mutants, $\Delta ktrA$, $\Delta ktrB$, Δkup , and $\Delta kimA$, showed the same SpeB activity as the wild type (Fig. 2, 3, and 7).

Three putative K^+ importers were identified in the *S. pyogenes* genome that could be involved in maintaining intracellular K^+ concentration. These importers are the

KtrAB, Kup, and KimA systems. *S. pyogenes* Kup is an *Escherichia coli* Kup ortholog with 31.7% amino acid identity. *E. coli* Kup is a low-affinity K⁺ importer and is believed to be the major K⁺ importer system under acidic conditions (28, 29, 33). In *L. lactis*, the Kup transporters are c-di-AMP binding proteins, and their potassium transport activities are inhibited upon binding to c-di-AMP (31). Gundlach et al. revealed that membrane protein YdaO in *B. subtilis* is a high-affinity K⁺ importer, so they renamed the importer KimA (K⁺ importer A) (20). KimA in *B. subtilis* is inhibited by c-di-AMP at both the transcriptional and protein activity levels (30). A recent structural study revealed that *B. subtilis* KimA is a K⁺/H⁺ symporter and also a member of the Kup family (34). *S. pyogenes* encodes a homolog of KimA, Spy_2088, with 20% amino acid identity. In-frame deletion of *ktrA*, *ktrB*, *kimA*, or *kup* in the Δ *dadA* mutant revealed that only the deletion of *ktrB* restored SpeB production equal to the wild-type level (Fig. 2). This suggests that the KtrAB system is the main K⁺ importer maintaining cellular K⁺ concentration under the *in vitro* growth conditions. Kup and KimA may have functions under different growth conditions, such as lower or higher osmotic or pH conditions than those tested in this study.

Even though *ktrB* deletion in the Δ *dadA* mutant reverted many defective phenotypes of the Δ *dadA* mutant, the *ktrB* deletion did not revert the loss of virulence of the Δ *dadA* mutant, indicating that other virulence traits regulated by c-di-AMP beyond SpeB production and resistance to stressors cannot be recovered by *ktrB* deletion alone. Thus, c-di-AMP in *S. pyogenes* appears to regulate multiple cellular pathways in addition to K⁺ transport.

MATERIALS AND METHODS

Bacterial strains and media. *S. pyogenes* HSC5 (*emm* genotype 14) (35, 36) was employed for all experiments, including strain construction. SF370 locus numbers (SPy_####) are used as references for genes in HSC5 (37). Molecular cloning experiments utilized *Escherichia coli* DH5 α or TOP10 (Invitrogen), which was cultured in Luria-Bertani broth. The routine culture of *S. pyogenes* employed Todd-Hewitt medium (BBL) supplemented with 0.2% yeast extract (Difco) (THY medium), and cells were grown at 37°C in sealed tubes without agitation. Unless otherwise indicated, C medium (38) was used to grow *S. pyogenes* for SpeB activity assay and RNA preparation for real-time qRT-PCR. Bacto agar (1.4%, wt/vol; Difco) was added to make solid media. Cultures on solid media were incubated under the anaerobic condition created by a commercial product (GasPak; catalog no. 260678; BBL). When appropriate, antibiotics were added to the media at the following concentrations if they are not specified: kanamycin, 50 μ g/ml for *E. coli* and 500 μ g/ml for *S. pyogenes*; erythromycin, 500 μ g/ml for *E. coli* and 1 μ g/ml for *S. pyogenes*.

Manipulation of DNA. Plasmid DNA was isolated via a commercial kit (Gene Elute plasmid miniprep kit; Sigma) and used to transform *S. pyogenes* or *E. coli* as described previously (39). Enzymes for DNA cloning and PCR were used according to the recommendations of the manufacturers. Chromosomal DNA was purified from *S. pyogenes* by using a standard kit (Wizard genomic DNA purification kit [Promega] or GenElute bacterial genomic DNA kit [Sigma]).

Strain construction. In-frame deletion mutations on chromosomal loci were generated by employing the shuttle vector with a temperature-sensitive replication origin, pJRS233 (40, 41). Briefly, PCR products immediately upstream and downstream of the deletion target gene were generated. The primers used to create the PCR products are listed in Table 1. These two PCR products were inserted into pJRS233 using the Gibson assembly (New England BioLabs) to create a deletion allele. A plasmid with a deletion allele, p Δ *ktrB*, p Δ *ktrA*, p Δ *kimA*, or p Δ *kup*, was used to replace each target gene by the gene deletion method that employs the temperature-sensitive replication origin, as described previously (40, 41). The fidelity of all molecular constructs and gene deletions was confirmed by PCR and/or DNA sequencing (www.Psomagen.com).

Whole-genome sequencing. The whole-genome sequencing of the Δ *dadA* Δ *ktrB*^{SUP} suppressor mutant was performed as described previously (18).

SpeB activity measurement using protease indicator plates. Strains were grown overnight in THY medium. The overnight cultures were serially diluted with fresh THY medium, and the diluted cells (2 μ l) were spotted onto protease indicator agar plates (C medium agar plates containing 2% skim milk). The protease indicator plates then were incubated anaerobically at 37°C for 24 h (or 48 h for extended incubation), and SpeB activity, which displays a clear zone around the spotted cells, was observed.

Measurement of biofilm formation degree. The degree of biofilm formation by strains was measured as described previously, with some modifications (42). Overnight cultures in C medium at 37°C were diluted at a ratio of 1:10 with fresh C medium, and 200 μ l of each diluted strain was added to the wells of flat-bottom 96-well polystyrene plates. Each plate was sealed with an optical tape and incubated for 48 to 72 h at room temperature. After incubation, the unbound cells were removed by rinsing the wells with distilled water four times, and then 200 μ l of crystal violet solution (3%, wt/vol) was added to each well and incubated for 15 min at room temperature to stain the biofilm. The plates were rinsed four times with distilled water, and 200 μ l of ethanol (100%) was added to each well to extract crystal

TABLE 1 Primers used

Name	Sequence ^a	Remark
Mutagenesis primers ^b		
To create pΔktrB		
5KtrBifVector	CATCCTTG-GACGTTGTAAAAACGACGGCCAG	For vector amplification (6,000 bp)
3KtrBifVector	GTGATTTGAAG-GCACATCCCCCTTCGCC	For upstream fragment amplification (721 bp)
5KtrBifF1	GGGATGTGC-CTTCAATCACTAGTGGAATGGAACAC	For downstream fragment amplification (725 bp)
3KtrBifF1	CATGTTTCTCAATATCTCC-TACTATAAACAACAAATAAGTTAAAAAGCTAAG	
5KtrBifF2	GTAGGAGATATTGGAGAAAAACATG-TTAAACGTAAACTGTCCG	
3KtrBifF2	GTTTACAACGTC-CAAGGATGTTTTTTTATTATTAAGATAGCCCAAGATAATC	
To create pΔktrA		
5KtrAifVector	CTACTAAAAC-CCCACTCACGACGTTGTAAAAACGACG	For vector amplification (6,020 bp)
3KtrAifVector	GAAAGTTGCC-CGCTTGCAGCACATCC	For upstream fragment amplification (720 bp)
5KtrAifF1	GCAAGGCG-GGCAACTTTTCTTAATATCCAAATCAGTCTC	For downstream fragment amplification (722 bp)
3KtrAifF1	CAATGATAGTATTCGGTTTCTCTTAACCCACTAGAATATCAGTAG	
5KtrAifF2	GTTAAGGAGAAACCGAATACTATCATTTGTGGCCATCGC	
3KtrAifF2	CGTGACTGGG-GTTTTAGTAGGAACAATGCTTTTGTCCG	
To create pΔKimA		
5p7INT-FC2	CCTGTGTGAAATTGTTATCCGCTC	For vector amplification (5,799 bp)
3p7INT-FC2	GTCGTGACTGGGAAAACCCCTGG	
5KimAup	GGGTTTCCAGTCACGAC-AACTGACTTTACAGTGACTATTAGCAACCT	For upstream fragment amplification (1,032 bp)
3KimAup	ATAGTTTTTCTCAATAATGCTCTC-CTTTTGTGCAAAATGC	
5KimAdown	GAGAGCATATTGAGAAAAAACTAT-GACAAGAGTGATTAATTAGATGGC	For downstream fragment amplification (1,034 bp)
3KimAdown	GCGGATAACAATTTACACAGG-CCGTGGAGTTACCTCTGAAATAAC	
To create pΔkup		
5p7INT-FC2	CCTGTGTGAAATTGTTATCCGCTC	For vector amplification (5,799 bp)
3p7INT-FC2	GTCGTGACTGGGAAAACCCCTGG	
5Kupup-2	GGGTTTCCAGTCACGAC-CTACCTCTCAATGATTGAGAACTTAACGAAAAAAC	For upstream fragment amplification (734 bp)
3Kupup-2	CTCAATGATTTTCTTAATCTCTC-CTAATTTTTAAAAATTATAACAAAAATACTG	
5Kupdown-2	GAGGAGTATAAGAAAAATCATTTGAG-ATGATAAGTCTCAATGATTTTCTTTTC	For downstream fragment amplification (732 bp)
3Kupdown-2	GCGGATAACAATTTACACAG-GTCCCCCTTACTTTAAAAAGTCACGAAAGTTCTAAG	
qRT-PCR primers		
RTspeB-F	TGTCGGTAAAGTAGGGGAC	
RTspeB-R	GAGCTGAAGGGTTTAGTCGG	
RTgyrA-F	AACAACCTCAACACAGGTCGGG	
RTgyrA-R	CTCCTTCACGGCTAGATTCC	

^aSequence is shown 5' to 3'. Hyphens indicate junctions between contiguous DNA regions.
^bMutagenesis primers were used for PCRs to amplify DNA segments used to construct plasmids for gene deletion.

violet from cells. After 5 to 10 min of incubation, the solution (100 μ l) was transferred to new plates, and color intensity (OD₆₀₀) was measured with a 96-well plate reader (800 TS Absorbance Reader; BioTek). This experiment was performed in triplicate. The Ω emm strain that does not express M protein and is unable to form a biofilm (42) was used as a negative control.

Susceptibility to stress caused by salts, pH, or oxygen radicals. The susceptibility of *S. pyogenes* to salt, pH, and oxidative stress was assessed as described previously (18). These stress conditions were created by adding KCl or NaCl (0.1 M) into THY medium, adjusting the initial pH of THY or C medium to 6.0 with 2 N HCl, or adding methyl viologen into THY medium. For measuring the susceptibility to stress caused by salts or pH, the optical density at 600 nm (OD₆₀₀) of the cultures was measured at 8 h postinoculation, when *S. pyogenes* typically reaches the stationary phase. For measuring the susceptibility to oxidative stress, *S. pyogenes* cells were exposed to 50 mM methyl viologen (MV) for 3 h in short-term exposure or 1.7 mM for 18 h in long-term exposure. The survival rates were determined by comparing viable cell counts with and without MV treatment.

Susceptibility to PlyC. The susceptibility of *S. pyogenes* to the cell wall-hydrolyzing enzyme PlyC was examined as described previously (18). Briefly, *S. pyogenes* cells grown overnight were washed and resuspended in phosphate-buffered saline (PBS) (OD₆₀₀ of 1.0). PlyC (3,000 U/ml) and the cysteine protease inhibitor E-64 (20 μ M) were added into 1 ml of the cell suspension. The cell suspension was incubated for 6 h at 37°C, and then its turbidity (OD₆₀₀) was measured. This experiment was performed in duplicate.

Susceptibility to a sublethal concentration of ampicillin. The susceptibility of *S. pyogenes* to a sublethal concentration of the cell wall-targeting antibiotic ampicillin was examined as described previously (18). C medium containing ampicillin (0.04 μ g/ml) was inoculated with a strain and incubated at 37°C overnight. The OD₆₀₀ of the overnight culture (~18 h postinoculation) was measured to determine the final cell density. This experiment was performed in triplicate.

Murine subcutaneous infection assay. The ability of *S. pyogenes* strains to cause disease in soft tissues was evaluated using 6- to 8-week-old SKH1 hairless mice (Charles River Labs) as described previously (41). Briefly, each mouse was subcutaneously injected with approximately 1×10^7 CFU of *S. pyogenes* in 100 μ l into the right flank. The area of the lesion that formed was measured every 24 h by digital photography. Any differences in the areas of lesions between experimental groups were tested for significance by the Mann-Whitney U test. This study was carried out in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health (43). This animal study was approved by the Institutional Animal Care and Use Committee (IACUC) of Indiana State University (ISU).

The effect of ionophores on SpeB expression. *S. pyogenes* strains were grown overnight in THY medium. The cultures were then 10-fold serially diluted with THY medium, and 2 μ l of each dilution was spotted on protease indicator plates containing 1 μ g/ml valinomycin (V0627; Sigma) or 0.5 μ g/ml gramicidin (G5002; Sigma) (stock solution: 10 mg/ml valinomycin in dimethyl sulfoxide and 10 mg/ml gramicidin in ethanol). The plates were incubated at 37°C anaerobically for 24 or 48 h, and the clear zones by SpeB activity around the spotted cultures were observed.

Real-time qRT-PCR. Real-time qRT-PCR was conducted as described elsewhere (41). The primers for qRT-PCR are listed in Table 1. The gyrase A subunit gene (*gyrA*) was used as the internal reference gene to normalize the expression level of a specific transcript between samples (44). The reported data represent the means and standard errors from three independent assays, each performed on a different day with newly extracted RNA.

Statistical testing. All statistical tests were performed using GraphPad Prism. Each statistical test applied to the experiments is described in the figure legends.

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