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## The Ktr Potassium Transport System in *Staphylococcus aureus* and its Role in Cell Physiology, Antimicrobial Resistance, and Pathogenesis

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### Summary

Potassium (K<sup>+</sup>) plays a vital role in bacterial physiology, including regulation of cytoplasmic pH, turgor pressure, and transmembrane electrical potential. Here, we examine the *Staphylococcus aureus* Ktr system uniquely comprised of two ion-conducting proteins (KtrB and KtrD) and only one regulator (KtrA). Growth of Ktr system mutants was severely inhibited under K<sup>+</sup>-limitation, yet detectable after an extended lag phase, indicating the presence of a secondary K<sup>+</sup> transporter. Disruption of both *ktrA* and the Kdp-ATPase system, important for K<sup>+</sup> uptake in other organisms, eliminated regrowth in 0.1 mM K<sup>+</sup>, demonstrating a compensatory role for Kdp to the Ktr system. Consistent with K<sup>+</sup> transport mutations, *S. aureus* devoid of the Ktr system became sensitive to hyperosmotic conditions, exhibited a hyperpolarized plasma membrane, and increased susceptibility to aminoglycoside antibiotics and cationic antimicrobials. In contrast to other organisms, the *S. aureus* Ktr system was shown to be important for low-K<sup>+</sup> growth under alkaline conditions, but played only a minor role in neutral and acidic conditions. In a mouse competitive index model of bacteremia, the *ktrA* mutant was significantly outcompeted by the parental strain. Combined, these results demonstrate a primary mechanism of K<sup>+</sup> uptake in *S. aureus* and a role for this system in pathogenesis.

### Keywords

*Staphylococcus aureus*; potassium homeostasis; alkaline growth; hyperosmotic stress; antibiotic resistance; bacteremia

### Introduction

Despite the limited availability of potassium (K<sup>+</sup>) in the environment, this essential macroelement is the most abundant intracellular cation in all living organisms. Unlike other ions, K<sup>+</sup> can accumulate to very high concentrations in bacteria and plays a primary role in regulation of cytoplasmic pH (Bakker & Mangerich, 1981), transmembrane electrical potential (Booth, 1985), and turgor pressure (Epstein, 1986) in non-halophilic bacteria (for review see (Bakker, 1993)). Bacterial cells must therefore possess the ability to effectively import K<sup>+</sup> against steep transmembrane concentration gradients to maintain homeostatic K<sup>+</sup> concentrations required for growth.

Three major, multicomponent systems have been found to mediate K<sup>+</sup> uptake in bacteria: Trk, Ktr, and Kdp (Epstein, 2003). The K<sup>+</sup>-conducting proteins of these systems (TrkH,

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KtrB, and KdpA, respectively) are members of the superfamily of  $K^+$  transporters (SKT), evolutionarily related to small, two transmembrane  $K^+$  channels (Doyle *et al.*, 1998). For SKT protein function, the channel must be activated (opened) via interaction with its regulatory subunit. Peripheral SKT regulatory proteins, such as KtrA within the Ktr system, contain dinucleotide binding sites which, when bound, induce a conformational shift, opening the  $K^+$ -conducting protein gate (Roosild *et al.*, 2002, Albright *et al.*, 2006, Roosild *et al.*, 2009). As a result, KtrA confers import velocity and ion specificity to KtrB. All well-characterized Ktr systems encode one regulatory protein that specifically activates one channel protein (Nakamura *et al.*, 1998, Zulkifli *et al.*, 2010). For example, *Bacillus subtilis* encodes two complete Ktr systems (KtrAB and KtrCD), and there appears to be no cross talk between these systems, indicating that each regulator is specific for its channel protein (Holtmann *et al.*, 2003).

Ktr systems appear to be constitutively expressed and responsible for moderate-affinity (mM)  $K^+$  uptake requiring sodium ions ( $Na^+$ ) for transport, suggesting  $Na^+/K^+$  symport activity (Matsuda *et al.*, 2004, Tholema *et al.*, 1999). In contrast, Kdp systems are efficient  $K^+$  scavenging pumps, making use of ATP hydrolysis to perform high-affinity  $K^+$  uptake. The KdpFABC system is transcriptionally-regulated by the KdpDE two-component system, which senses extracellular  $K^+$  (Voelkner *et al.*, 1993, Laermann *et al.*, 2013). Kdp systems are utilized during times of  $K^+$  starvation and/or fluctuating ionic conditions when other transporters cannot meet the cellular requirements for  $K^+$  (Heermann & Jung, 2010, Walderhaug *et al.*, 1992). Importantly, bacteria typically contain more than one  $K^+$  uptake system, likely to ensure that adequate levels of this essential element are present in the cell (Corratge-Faillie *et al.*, 2010).

Mechanisms of  $K^+$  transport have been characterized in a variety of bacterial pathogens and implicated in the virulence of these organisms (Alkhuder *et al.*, 2010, Mason *et al.*, 2006, Stingl *et al.*, 2007, Su *et al.*, 2009). However, no studies have clearly elucidated the mechanism of  $K^+$  uptake in any staphylococcal species. *Staphylococcus aureus* is a commensal of the human skin as well as a pathogen known for its ability to cause a wide spectrum of diseases including skin and soft tissue abscesses, bacteremia, and implanted medical device infections (DeLeo *et al.*, 2010). Recent work by Xue *et al.* (Xue *et al.*, 2011) describing a Kdp system encoded by *S. aureus* revealed that this system has no significant role in  $K^+$  transport. Additionally, a KtrA-like protein was recently found to be required for *S. aureus* growth under  $K^+$ -limiting conditions (Corrigan *et al.*, 2013); however, its role in the regulation of Ktr-specific transport proteins was not investigated. In the current study we examined in detail the function of this unique Ktr  $K^+$  transport system, which consists of one regulatory protein and two ion-conducting channels required for  $K^+$ -limited growth of *S. aureus* in alkaline conditions. We also demonstrated that the Ktr system plays a major role in maintaining cell physiology, including regulating membrane potential and cellular osmotic tolerance. Finally, the Ktr system was shown to play a significant role in antimicrobial resistance and fitness within an *S. aureus* bacteremia model of infection.

## Results

### A novel Ktr system in *Staphylococcus aureus*

To identify potential  $K^+$  transport proteins in *S. aureus*, we aligned the amino acid sequences of the ion-conducting proteins, KtrB and KtrD, from *B. subtilis* strain 168 with the *S. aureus* strain FPR3757 proteins in the GenBank database using the Basic Local Alignment Search Tool (<http://blast.ncbi.nlm.nih.gov/>). Two hypothetical *S. aureus* proteins were identified (locus numbers SAUSA300\_1979 and SAUSA300\_0924) that shared amino acid sequence similarity with both *B. subtilis* KtrB and KtrD (43% and 50% identity, respectively; see Table 1). Based on the sequence similarities observed, along with the functional studies

described below, we renamed the SAUSA300\_1979 and SAUSA300\_0924 open reading frames, *ktrB* and *ktrD*, respectively. Each of the putative *S. aureus* KtrB and KtrD proteins contain the four glycine residues that form the selectivity filter in Ktr channel proteins (Zhou *et al.*, 2001, Lu & Miller, 1995, Tholema *et al.*, 1999, Tholema *et al.*, 2005) and lack predicted N-terminal transmembrane segments classically found in Trk ion-conducting proteins (Fig. S1). Interestingly, only one protein in *S. aureus* FRP3757, previously designated KtrA (Corrigan *et al.*, 2013), shared sequence similarity with *B. subtilis* KtrA and KtrC, with a higher level of identity observed with KtrC (Table 1). Analysis of the KtrA protein revealed that it contains one predicted C-terminal dinucleotide-binding site (KTN/RCK domain), similar to other Ktr regulators (Fig. S2).

All three *ktr* genes in *S. aureus* are located separately on the chromosome (Fig. S3) and a number of other *S. aureus* isolates whose genomes have been sequenced contain these genes (data not shown). The *ktrA*, *ktrB*, and *ktrD* genes also appear to be conserved in several other staphylococcal species, with the one exception being *S. lugdunensis*, which contains only one putative Ktr transporter system, with the highest level of similarity to the *B. subtilis* KtrC and KtrD proteins (Table 1). All staphylococcal *ktrD* genes listed in Table 1 are downstream relative to a gene encoding an HtrA-like serine protease, and all *ktrA* genes are located downstream of a gene encoding a cytochrome d ubiquinol oxidase subunit-like protein (data not shown). From these *in silico* analyses, we hypothesize that *S. aureus* encodes a novel one regulator/two ion-conducting domain Ktr system in which KtrA regulates the K<sup>+</sup>-transport activity of both KtrB and KtrD.

### Requirement for Ktr in alkaline conditions

K<sup>+</sup> uptake is important for acid stress survival by compensating for H<sup>+</sup> extrusion and resulting alkalinaization of the cytoplasm (Kitko *et al.*, 2010). Conversely, K<sup>+</sup> efflux (i.e. via K<sup>+</sup>/H<sup>+</sup> antiport) is necessary for alkaline stress survival and acidification of the cytoplasm (Padan *et al.*, 2005, Booth, 1985, Krulwich *et al.*, 2009). Since previous studies in other organisms have demonstrated a role for Ktr-mediated transport at different pH ranges (Zulkifli & Uozumi, 2006, Tholema *et al.*, 2005, Kawano *et al.*, 2000), we initially tested the role of the *S. aureus* Ktr system by generating a  $\Delta ktrA$  mutant and culturing in K<sup>+</sup>-limited medium at a range of starting pH conditions. As shown in figure 1, the  $\Delta ktrA$  mutant exhibited a clear defect in growth in alkaline pH, K<sup>+</sup>-limited medium, a phenotype that was restored by expression of the *ktrA* gene from a plasmid. Based on these results, subsequent experimentation was performed under alkaline conditions.

### Low K<sup>+</sup> growth requires Ktr

To further examine the role of *S. aureus* *ktr* genes in K<sup>+</sup> transport, we tested the growth of isogenic *ktr* mutants in *S. aureus* strain LAC in the presence of various KCl concentrations. As shown in figure 2A, the parental LAC strain grew slightly in K<sup>+</sup>-deprived medium (K<sup>-</sup>CDM), and a 16 hour delay was seen after deletion of the putative Ktr regulator, *ktrA* ( $\Delta ktrA$ ), or both *ktrB* and *ktrD* ( $\Delta ktrB\Delta ktrD$ ). Growth of the  $\Delta ktrA$  and  $\Delta ktrB\Delta ktrD$  strains in K<sup>-</sup>CDM supplemented with 0.1 mM KCl remained undetectable for 17 hours after which the cells began to grow (Fig. 2B). When supplemented with 0.5 mM KCl, a 6-hour lag phase was observed before the  $\Delta ktrA$  and  $\Delta ktrB\Delta ktrD$  strain were able to resume a growth rate and yield similar to the wild-type strain (Fig. 2C). When the culture medium was supplemented with 10 mM KCl, all strains grew equally well (Fig. 2D). Notably, deletion of either *ktrB* or *ktrD* alone had no impact on growth in K<sup>+</sup>-limited medium (Fig. S4), demonstrating that only one ion-transporting protein (KtrB or KtrD) and the KtrA regulator are required for growth in low K<sup>+</sup> conditions.

To exclude the possibility that secondary site mutations were responsible for the phenotypes observed, we tested the ability of complementation plasmids to restore growth under  $K^+$ -limiting conditions. Expression of *ktrA* from the plasmid pCM28 restored growth of the *ktrA* mutant to wild-type levels in all  $K^+$  concentrations tested. Additionally, expression of either *ktrB* or *ktrD* alone fully restored the growth of the  $\Delta ktrB\Delta ktrD$  strain in all  $K^+$  concentrations, further demonstrating the requirement for only one channel protein under these growth conditions. Based on these results, we conclude that KtrA regulates the activity of both KtrB and KtrD in *S. aureus*.

### A role for Kdp in low $K^+$ growth

Considering the observation that growth in low  $K^+$  resumed in the  $\Delta ktrA$  and  $\Delta ktrB\Delta ktrD$  mutants after extended incubation periods, we hypothesized that expression of the *kdpFABC* operon is induced under these conditions, thus, providing an alternate mechanism to import  $K^+$ . To test this hypothesis, we generated *kdpFABC* mutants, with and without the *ktrA* mutation, and tested the ability of these strains to grow under low  $K^+$  conditions. Consistent with the results of Xue *et al.* (Xue et al., 2011), disruption of *kdpFABC* ( $\Delta kdpFABC$ ) had an unremarkable effect on the growth of *S. aureus* (Fig. 3A-D). However, the presence of both *ktrA* and *kdpFABC* mutations ( $\Delta ktrA\Delta kdpFABC$ ) eliminated the growth recovery observed for the *ktrA* mutant in 0 mM and 0.1 mM KCl (Fig. 3A and B) and reduced the growth rate of the recovery observed in the presence of 0.5 mM KCl (Fig. 3C). In contrast, normal growth was observed in 10 mM KCl (Fig. 3D) suggesting that alternate mechanisms exist to transport  $K^+$  under these conditions. As expected, expression of *ktrA* from a plasmid in the  $\Delta ktrA\Delta kdpFABC$  strain restored growth in all conditions tested. Interestingly, plasmid-based expression of *kdpFABC* partially complemented the growth defects of the  $\Delta ktrA\Delta kdpFABC$  strain in low  $K^+$  (Fig. 3A-D), likely due to the multi-copy state of the plasmid and over-expression of *kdpFABC*. Together, these results reveal a function for Kdp in  $K^+$  import, but only in the absence of the Ktr system.

### Ktr contributes to osmotic tolerance

Cellular processes require constant cytoplasmic turgor pressure, necessitating bacteria to cope with changes in external osmolarity for survival. The initial adaptation of bacteria to high osmotic stress is rapid accumulation of  $K^+$  (Matsuda et al., 2004, Holtmann et al., 2003, Whatmore *et al.*, 1990), increasing osmotic tolerance. To examine the role of the *S. aureus* Ktr system in hyperosmotic tolerance, we determined the effects of high concentrations of dissolved solutes on growth of the parental and  $\Delta ktrA$  strains. As shown in figure 4A, growth of the *ktrA* mutant was completely inhibited when cultured in K<sup>-</sup>CDM supplemented with 0.5 mM KCl and 400 mM NaCl. Increasing the available  $K^+$  by supplementing the growth medium with 10 mM KCl recovered growth of the *ktrA* mutant (Fig. 4B).

To ensure that the observed growth defect was due to an osmotic effect of NaCl and not an ionic effect, we examined the growth of our *S. aureus* strains in high concentrations of sorbitol. Cultured in K<sup>-</sup>CDM supplemented with 0.5 mM KCl and 750 mM sorbitol, the  $\Delta ktrA$  strain exhibited a severe growth delay compared to the parental and complemented strains (Fig. 4C). As observed with growth in the presence of 400 mM NaCl, supplementing the growth medium with 10 mM KCl restored growth of the  $\Delta ktrA$  strain (Fig. 4D). Similar results were observed using the  $\Delta ktrB\Delta ktrD$  strain and this phenotype was complementable with expression of either *ktrB* or *ktrD* from a plasmid (data not shown). Consistent with its role in low  $K^+$  growth, disruption of *ktrA* and *kdpFABC* extended the lag phase of the  $\Delta ktrA$  strain in 0.5 mM KCl and 750 mM sorbitol (data not shown). Additionally, the parental and  $\Delta kdpFABC$  strain (data not shown) grew consistently and identically in high osmotic conditions regardless of  $K^+$  concentration, indicating that Ktr-mediated  $K^+$  uptake is a major regulator of cellular osmotic pressure.

### Ktr mutants have increased membrane potential

Efficient uptake of  $K^+$  is essential for regulation of the electrical potential across the bacterial cytoplasmic membrane ( $\Delta\Psi$ ) (Bakker & Mangerich, 1981). As protons are translocated out of the cell, creating a proton motive force, the electro-chemical ion gradient is held constant, primarily as a result of  $K^+$  transport. To investigate the role of the Ktr system in regulation of *S. aureus*  $\Delta\Psi$ , we utilized flow cytometry to assess the fluorescence of cells stained with the carbocyanine dye, diethyloxycarbocyanine (DiOC2(3)) (Novo *et al.*, 2000, Novo *et al.*, 1999). As shown in figure 5A, the  $\Delta ktrA$  and  $\Delta ktrB\Delta ktrD$  strains stained with DiOC2(3), produced an increased red:green fluorescence ratio compared to a similarly stained parental strain, demonstrating that the electrical potential across the plasma membrane of cells lacking a functional Ktr system is higher relative to wild-type cells. This hyperpolarization was restored to wildtype levels by complementation of the  $\Delta ktrA$  strain *in trans* with *ktrA* or complementation of  $\Delta ktrB\Delta ktrD$  with either *ktrB* or *ktrD* (Fig. 5B). These data demonstrate that  $K^+$  uptake mediated by the Ktr system plays an important role in the regulation of  $\Delta\Psi$  in *S. aureus*.

### Ktr is required for antimicrobial resistance

Mediating cellular transmembrane electrical potential has been proposed as a mechanism of bacterial defense to the effects of cationic antimicrobial peptides (Yeaman & Yount, 2003, Hancock, 1997, Yeaman *et al.*, 1998) and is known to play a role in aminoglycoside resistance in *S. aureus* (Mates *et al.*, 1982). To determine the role of the Ktr system in antibiotic resistance, we compared the effective concentration of several aminoglycoside antibiotics, cationic antimicrobials, and immune cationic antimicrobial peptides against wild-type LAC and its isogenic *ktrA* mutant strain.

The  $\Delta ktrA$  strain exhibited markedly lower gentamicin and tobramycin minimum inhibitory concentrations (MIC) than the parental strain in 0.5 mM KCl (Table 2). Supplementation of the medium to 10 mM KCl partially restored the MIC of the *ktrA* mutant. Expression of *ktrA* from a plasmid fully restored the MIC of the  $\Delta ktrA$  strain to wild-type levels in all conditions tested. MIC values of 4-, 16-, and 4-fold lower than wild-type were observed when the *ktrA* mutant was exposed to cationic antimicrobial polymyxin B, epithelial cathelicidin LL-37 and human neutrophil peptide 1 (HNP-1), respectively, in 0.5 mM KCl. We also determined the effects of two ionophores, gramicidin and valinomycin, which form monovalent cation-permeable channels in cell membranes. Gramicidin had a marked effect on the *ktrA* mutant in 0.5 mM KCl with a MIC less than the limit of detection ( $<1 \mu\text{g/mL}$ ) compared to the parental strain, which was greater than the highest concentration tested ( $>64 \mu\text{g/mL}$ ). In 10 mM KCl, gramicidin had a moderate effect on the *ktrA* mutant strain, with a MIC of  $32 \mu\text{g/mL}$ , while the MIC of the parental strain remained  $>64 \mu\text{g/mL}$ . Interestingly, the highly  $K^+$ -selective ionophore valinomycin had no measurable effect on the parental or  $\Delta ktrA$  strains in low  $K^+$  conditions (0.5 mM KCl), but had a substantial inhibitory effect in the presence 10 mM KCl where the mutant exhibited a 2-fold lower MIC. Additionally, we found the  $\Delta ktrA$  strain to have a minor sensitivity to vancomycin (2-fold lower MIC than wildtype), a cationic antibiotic that acts on the cell wall.

To examine the effect of pH on the antimicrobial susceptibility of the  $\Delta ktrA$  strain, MIC assays were also performed in acidic, neutral, and alkaline conditions (Table S3). Consistent with its role in growth under these conditions, the gentamicin, polymyxin B, and HNP-1 MICs for the  $\Delta ktrA$  mutant gradually increased as the pH became less alkaline. However, the determined MIC values for the  $\Delta ktrA$  mutant were consistently less than that observed for the wild-type strain, regardless of the pH of the medium.

## Ktr contributes to fitness during infection

The ability of bacterial pathogens to grow within an animal host depends on their capacity to adapt to and survive within a hostile environment, one that contains limited available nutrients, osmotic stress, and an onslaught of antimicrobial immune components. To determine if the Ktr system contributes to cellular fitness during a *S. aureus* infection, we employed a competitive index model by infecting mice with a 1:1 mixture of *ktrA*, *ktrB*, or *ktrD* mutant and wild-type *S. aureus* cells. The *ktrA::ΦNΣ*, *ktrB::ΦNΣ*, and *ktrD::ΦNΣ* mutants from the Nebraska Transposon Mutant Library (Fey *et al.*, 2013) were utilized for their post-infection selection due to the presence of a selectable erythromycin resistance gene within the transposon. After 9 days, bacterial burden within the kidneys was determined and the relative competitive index (RCI) was calculated. As shown in figure 6, the *ktrA* mutant was significantly outcompeted by JE2 ( $p=0.0001$ ), demonstrating that the presence of a functional Ktr system confers a fitness advantage to *S. aureus* during infection. In contrast, the *ktrB* and *ktrD* mutants were able to compete effectively with the JE2 strain, indicating that these genes are individually dispensable for bacterial fitness within an infection. The RCI of the *ktrA::ΦNΣ* mutant was significantly less than that of the *ktrB::ΦNΣ* and *ktrD::ΦNΣ* mutants and is consistent with the lack of an observable phenotype in the other functional assays described above.

## Discussion

Transport mechanisms of the essential mineral nutrient  $K^+$  have been well-described in a variety of bacterial species; however the specific mechanisms of its uptake in the Gram-positive pathogen, *Staphylococcus aureus*, have yet to be revealed. The data generated by this study demonstrate that *S. aureus* utilizes a unique Ktr  $K^+$  transport system in which two separate ion-conducting proteins, KtrB and KtrD, are regulated by a single protein, KtrA. This type of Ktr system has yet to be described and its occurrence appears to be conserved among many staphylococcal species (Table 1). We have shown that KtrA and either KtrB or KtrD are required for normal growth in medium containing less than 10 mM  $K^+$ , and expression of either *ktrB* or *ktrD* from a plasmid fully complemented the growth defect of the  $\Delta ktrB\Delta ktrD$  mutant strain (Fig. 2). Deletion of either *ktrB* or *ktrD* alone had no effect on  $K^+$ -limited growth (Fig. S4). Furthermore, KtrA appears to have very tight regulation over KtrB and KtrD activity as the  $\Delta ktrA$  and  $\Delta ktrB\Delta ktrD$  strains had identical growth characteristics. These results reveal that, while encoding two separate channel proteins is largely conserved within staphylococcal species, only one channel protein is necessary and sufficient for full Ktr function in *S. aureus*. However, any functional differences between the two  $K^+$  channel proteins were indistinguishable as the growth rates of the *ktrB* and *ktrD* mutants were identical to the wild-type strain, and the  $\Delta ktrB\Delta ktrD$  phenotypes produced were equally complementable with either *ktrB* and *ktrD*, despite the fact that KtrB and KtrD share only 55% overall amino acid identity (data not shown). Moreover, with consideration that KtrA appears to regulate the activity of each of these proteins to a similar extent, it was unexpected that KtrB and KtrD share only 53% identity in the 15 amino acids described as essential for Ktr regulatory protein binding (Hanelt *et al.*, 2010) (data not shown). Thus, additional work will be required to identify functional differences that may distinguish these channel proteins.

Although mutations rendering the *S. aureus* Ktr system nonfunctional resulted in an extended lag phase when grown in a  $K^+$ -limited medium (Figs. 2B and C), the mutant strains were ultimately able to grow at the same rate and yield as the parental strain. These results indicate that, as  $K^+$ -deplete stationary-phase cells are diluted from overnight cultures into fresh culture media, the Ktr system is responsible for the initial uptake of  $K^+$ . Lacking a functional Ktr system, the cells appear to remain  $K^+$ -deplete and are unable to begin growth at the same time as the wild-type strain. However, the Ktr mutant strains were eventually

able to resume apparently normal growth after an amount of time that was dependent on the  $K^+$  concentration within the medium. Based on these results, we propose that the *S. aureus* Ktr system is a major effector in the initial acquisition of  $K^+$ , enabling the cells to effectively reach a threshold intracellular  $K^+$  concentration that is growth permissive. While we have demonstrated a clear role for the Ktr system in low  $K^+$  growth, it is also apparent that *S. aureus* utilizes a second  $K^+$  transport mechanism in the absence of a functional Ktr system. Importantly, repeating the low  $K^+$  growth experiments with  $\Delta ktrA$  cells collected after growth recovery under these conditions resulted in a similar growth delay, indicating that the observed growth was not due to a spontaneous mutation that permitted growth in  $K^+$ -limiting conditions (data not shown). Instead, the delayed growth of the  $\Delta ktrA$  mutant in 0.1 and 0.5 mM KCl appeared to be related to Kdp-ATPase system (Fig. 3B and C), despite having previously been characterized as having a negligible role in  $K^+$  uptake in *S. aureus* (Xue et al., 2011). Growth defects of  $\Delta ktrA\Delta kdpFABC$  were fully complementable by expression of *ktrA* in both conditions, confirming the role of the Kdp system in  $K^+$  acquisition in the absence of Ktr. Thus, it is clear that the Ktr system is the major  $K^+$  uptake system in *S. aureus*, and we propose that Kdp functions as  $K^+$ -scavenger, secondarily to Ktr. Interestingly, the KdpDE two-component system has been shown to regulate the expression of the KdpFABC  $K^+$  transport system in *Escherichia coli* (Heermann & Jung, 2010, Voelkner et al., 1993, Polarek et al., 1992). Under  $K^+$  limitation or high osmolarity, the histidine kinase, KdpD, phosphorylates the response regulator, KdpE, increasing its affinity for the *kdpFABC* promoter and triggering transcription of this operon (Laimins et al., 1981, Epstein, 1992). However, KdpE functions to repress the transcription of *kdpFABC* in *S. aureus* (Xue et al., 2011), and this is likely due to the ability of the Ktr system to satisfy the majority of the  $K^+$  requirements in *S. aureus*.

$K^+$  uptake in the face of acidic stress is important for compensation of  $H^+$  extrusion and resulting alkalization of the bacterial cytoplasm (Bakker & Mangerich, 1981, Booth, 1985). Thus, it was surprising to find that the *S. aureus* Ktr system was essential for growth under alkaline stress, conditions in which efflux of  $K^+$  and/or  $Na^+$  typically occur (Padan et al., 2005, Krulwich et al., 2009), but not at a neutral or acidic pH (Fig. 1). Given that Ktr systems are characteristically dependent on inward  $Na^+$  gradients for  $K^+$  uptake activity (Matsuda et al., 2004, Tholema et al., 1999, Zulkifli et al., 2010), it is tempting to speculate that the *S. aureus* Ktr system may function differently due to the requirement for Ktr under alkaline growth but not in neutral or acidic conditions. Further mechanistic studies will be required to elucidate the  $K^+/Na^+$  transport activity of the *S. aureus* Ktr system. Interestingly, the Ktr system of *Enterococcus hirae* has also previously been shown to be important for alkaline growth (Murata et al., 1996, Kawano et al., 2000). The *E. hirae* KtrB-like channel protein (NtpJ) is encoded as the last gene within the Ntp  $Na^+$ -ATPase operon, implicating the Ktr system in  $Na^+$  cycling. While the *ktr* genes of *S. aureus* are not associated with any known  $Na^+$  efflux genes, it remains possible that cellular  $Na^+$  efflux activity is associated with Ktr activity. Notably, the  $\Delta ktrA\Delta kdpFABC$  mutant displayed similar growth profiles as the  $\Delta ktrA$  single mutant in 0.5 mM KCl at pH 5.21 and 7.02 (data not shown), indicating that a third, yet uncharacterized  $K^+$  transport system is required for low  $K^+$  growth in these conditions.

*S. aureus* is considered a highly osmotolerant organism (Scott, 1953). When cultured in medium of high osmolarity, other bacterial species are known to import  $K^+$  to help maintain the turgor pressure necessary for growth (Csonka, 1989). However, only the contribution of glycine betaine and proline have been examined in detail in *S. aureus* osmoadaptation (Pourkomialian & Booth, 1992, Townsend & Wilkinson, 1992). We observed an inability of the  $\Delta ktrA$  mutant cells to grow in 0.5 mM KCl medium containing 400 mM NaCl (Fig. 4A) suggesting that the Ktr-mediated import of  $K^+$  is also important for overcoming the strong osmotic pressure of NaCl in *S. aureus*. Growth in the presence of 400 mM NaCl could only

be partially rescued by supplementation of the medium with 10 mM KCl (Fig. 4B) indicating that the Ktr system is required for osmotolerance in *S. aureus* at K<sup>+</sup> concentrations beyond 10 mM. Since previous studies have demonstrated that Ktr systems in other organisms are energized by K<sup>+</sup>/Na<sup>+</sup> symport (Matsuda et al., 2004, Tholema et al., 1999), we addressed the possibility that the abundant Na<sup>+</sup> present in the growth medium was complicating the interpretation of these results. Thus, we performed similar experiments using high concentrations of the non-metabolic compound sorbitol. Supplemented with 0.5 mM K<sup>+</sup> and 750 mM sorbitol, a 12.5-hour lag phase was observed before the  $\Delta ktrA$  mutant was able to resume growth (Fig. 4C). Once again, supplementing this medium with 10 mM KCl fully restored growth (Fig. 4D). We conclude from these data that the Ktr system plays an important role in the ability of *S. aureus* to overcome high osmotic pressures by facilitating the uptake of K<sup>+</sup>. The ability to adapt to osmotic fluctuations and grow in high osmotic conditions is essential for bacterial pathogens to infect. Within the intestinal lumen, for example, the osmolarity is very high (around 300 mM NaCl) and the blood stream has an osmolarity equivalent to about 150 mM NaCl.

Because of the role of K<sup>+</sup> in the maintenance of the trans-membrane electrical potential ( $\Delta\Psi$ ) (Bakker & Mangerich, 1981), we also investigated the potential effects of the *ktr* genes on  $\Delta\Psi$ . As protons are translocated out of bacterial cells, forming a proton motive force, a charge gradient develops across the membrane. Importantly, the import of K<sup>+</sup> ions accompanies the export of protons such that the charge differential across the membrane is maintained. Thus, the inability to efficiently import K<sup>+</sup> often accompanies alterations in  $\Delta\Psi$  (Bakker & Mangerich, 1981). Consistent with this, the  $\Delta ktrA$  and  $\Delta ktrB\Delta ktrD$  mutations resulted in a hyperpolarized cell membrane (Fig. 5A) compared to the parental and complemented strains (Fig. 5B), indicative of an inability to maintain a homeostatic ion balance. One consequence of an altered  $\Delta\Psi$  is that it could affect antibiotic susceptibility. For example,  $\Delta\Psi$  in *S. aureus* has been shown to be positively correlated with the uptake of the aminoglycoside antibiotic gentamicin (Mates et al., 1982). Consistent with this was the finding that mutations affecting the Ktr system altered susceptibility to the effects of gentamicin and tobramycin (Table 2). Regulation of  $\Delta\Psi$  is also known to influence resistance to the activity of cationic antimicrobial peptides that target the bacterial lipid bilayer which are a major part of the biological defense arsenal against bacterial pathogens (Peschel & Sahl, 2006) by electrically repelling their entry (Hancock, 1997, Yeaman & Yount, 2003, Yeaman & Yount, 2007, Yount *et al.*, 2006). Our studies demonstrated that the *ktrA* mutant was more susceptible than wild-type to the poly-cationic antibiotic polymyxin B, human cathelicidin LL-37, and human neutrophil peptide-1 (HNP-1) (Table 2). Likewise, susceptibility to the widely used antibiotic, vancomycin, was found to be effected by the *ktrA* mutation, although to a lesser extent. These data are consistent with a model proposing that tight regulation of  $\Delta\Psi$  mediated by K<sup>+</sup> uptake is important to inhibit the insertion of cationic antimicrobials into the membrane (Parra-Lopez *et al.*, 1994).

An alternative hypothesis is that K<sup>+</sup> uptake is required for the reacquisition of K<sup>+</sup> lost during antibiotic treatment (Stumpe & Bakker, 1997). The markedly lower MIC of the  $\Delta ktrA$  strain to the cationic ionophore gramicidin, which eliminated ion gradients (Katsu *et al.*, 1986), suggests that the Ktr system may be required to restore intracellular K<sup>+</sup> levels. However, treatment with the neutrally-charged ionophore, valinomycin (Pressman, 1976), demonstrates that cells with a non-functional Ktr system exhibit similar susceptibility to this antibiotic as wild-type cells. These results indicate that the decreased MIC of the *ktrA* mutant to gramicidin is likely due to its cationic nature (Kelkar & Chattopadhyay, 2007) rather than an inability to acquire K<sup>+</sup>. This is in agreement with previous findings showing *S. aureus* wild-type and  $\Delta ktrA$  strains had a comparable, two-fold MIC difference to the neutrally-charged ionophore, nigericin (Corrigan et al., 2013).



Based on the results of this study, we propose that the *S. aureus* Ktr system functions to maintain ion homeostasis by facilitating K<sup>+</sup> uptake, thereby counterbalancing proton extrusion and neutralizing intracellular hydroxyl anions. Mediating membrane polarization (inside negative), the Ktr system plays a significant role in resisting the actions of a wide variety of antimicrobial agents including cationic antibiotics and antimicrobial peptides, potentially by preventing their membrane insertion and/or uptake. Considering that membrane energetics may serve as a selective mechanism for immune cationic peptide action, targeting these molecules to the invading bacteria (Yeaman & Yount, 2003, Yount & Yeaman, 2013), tight regulation of  $\Delta\Psi$  likely plays an important role in pathogen survival during infection. Furthermore, it has recently been hypothesized that K<sup>+</sup> sequestration from a common limited supply may be a critical host defense mechanism in resistance to bacterial infection (Freeman *et al.*, 2013). Recent work has indicated the potential for K<sup>+</sup> transport in *S. aureus* pathogenesis (Xue *et al.*, 2011, Zhao *et al.*, 2010), however, these studies focused on the less-effective Kdp system. The ability of the *S. aureus* Ktr system to mediate host antimicrobial peptide resistance lead us to propose that Ktr contributes significantly to cellular fitness during an infection. Indeed, interruption of *ktrA* resulted in a significant out-competition by wild-type in a mouse model of bacteremia (Fig. 6), indicating that the combination of limited K<sup>+</sup> availability, osmotic stress, and/or the effects of immune cationic antimicrobials are factors that *S. aureus* must overcome to survive within a host.

## Experimental Procedures

### Bacterial strains and plasmids used

*Escherichia coli* DH5 $\alpha$  was used for cloning and was grown at 37°C in LB medium supplemented with ampicillin (100  $\mu$ g ml<sup>-1</sup>) or spectinomycin (50  $\mu$ g ml<sup>-1</sup>) as needed for selection. Oligonucleotides (Supplementary Table 2) were synthesized by Integrated DNA Technologies (Coralville, IA) and PCR was performed with Mastercycler proS (Eppendorf, Hamburg, Germany) using KOD DNA polymerase (Novagen, Madison, WI). Amplified DNA fragments were confirmed by separation on a 1% agarose gel and extracting the desired band when required. DNA was recovered using the DNA Clean and Concentrator-5 Kit (Zymo Research, Orange, CA). Klenow fragment, DNA ligase, and restriction enzymes were obtained from New England Biolabs (Beverly, MA). Plasmids were purified using the Wizard Plus SV Miniprep DNA purification system (Promega Corporation, Madison, WI), and the nucleotide sequences of the plasmids generated were determined at the High-Throughput DNA Sequencing and Genotyping Core Facility at the University of Nebraska Medical Center (Omaha, NE) and analyzed using Vector NTI (Invitrogen, Carlsbad, CA) to ensure the absence of unintended mutations.

The *Staphylococcus aureus* strains used in this study were derived from a USA300 strain isolated from a skin and soft tissue infection in a detainee from the Los Angeles County Jail (LAC) (Voyich *et al.*, 2005). Plasmids and oligonucleotides used in the manipulation of these strains (Supplementary Table 1) were generated based on the sequence of the highly related *S. aureus* strain FPR\_3757 (NC\_007793.1). Chromosomal changes in *S. aureus* were engineered by allelic exchange using the temperature-sensitive *E. coli*-*S. aureus* shuttle vector, pCL52.2 (Sau *et al.*, 1997) or pJB38 (Bose *et al.*, 2013), containing ~1000 bp of upstream and downstream DNA from the gene of interest. Primers were designed to add restriction enzyme recognition sites to the 3' and 5' ends of the upstream and downstream products and fragments amplified from the chromosome of *S. aureus* LAC. Plasmids were generated in *E. coli* and transferred to the highly-transformable, restriction-deficient *S. aureus* strain RN4220 (Kreiswirth *et al.*, 1983) by electroporation. Transduction into LAC was performed using  $\Phi$ 11 propagated on plasmid-containing RN4220 cells. Allelic exchange was carried out as previously described (Bose *et al.*, 2012). Complementation

fragments were cloned into pCM28 and included 500 bp upstream of the gene of interest to contain the native promoter.

Transposon insertion mutants were acquired from the Nebraska Transposon Mutant Library (Fey et al., 2013) and the transposon insertions were transduced into a fresh wild-type (JE2) strain using  $\Phi$ 11 selected by plating on tryptic soy agar (TSA) medium containing erythromycin (5  $\mu$ g ml<sup>-1</sup>). Confirmation of the mutations was achieved by PCR amplification of DNA surrounding the gene harboring the transposon and pulse-field gel electrophoresis was used to confirm the genetic background prior to experimentation.

### BLAST analysis

The Basic Local Alignment Search Tool (<http://blast.ncbi.nlm.nih.gov>) was used to compare the amino acid sequences of *Bacillus subtilis* strain 168 KtrA (NP\_390987), KtrB (NP\_390988), KtrC (NP\_389334), and KtrD (NP\_389233) to the *Staphylococcus* sp. strains listed in Table 1. An Expected (E) value  $<10^{-10}$  and Query coverage  $>90\%$  was required for protein homology consideration. Table S2 lists the locus and accession numbers for each protein listed. Amino acid alignments were performed using ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>).

### Growth conditions

Cells from overnight cultures were washed twice with K<sup>-</sup>CDM and diluted to OD<sub>600</sub> = 0.05 in 200  $\mu$ L of K<sup>-</sup>CDM supplemented with 0, 0.1, 0.5, or 10 mM KCl in wells of a 96-well cell culture plate (Corning Incorporated, Corning, NY). Growth was monitored by acquiring OD<sub>600</sub> measurements every 30 or 60 minutes with incubation at 37°C and continuous orbital shaking using a TECAN Infinite 200 PRO (Tecan Group Ltd, Männedorf, Switzerland). For osmotolerance assays, cells were cultured in 0.5 and 10 mM KCl as described above except with the addition of either 400 mM NaCl or with 750 mM sorbitol.

Prior to the experiments, *S. aureus* strains harboring pCM28 and pCM28-derived plasmids were streaked for isolation on TSA medium containing chloramphenicol (10  $\mu$ g ml<sup>-1</sup>) then grown overnight in a chemically-defined medium (CDM) as previously described (Hussain et al., 1991) except for the addition of 0.25% glucose and 10 mM KCl, the absence of KH<sub>2</sub>PO<sub>4</sub>, and a final pH of ~8.4. For experiments testing growth in the presence of various K<sup>+</sup> levels, KCl was added to the desired concentration by dilution from a 1M KCl stock solution. It should be noted that the K<sup>+</sup>-deficient medium in which no KH<sub>2</sub>PO<sub>4</sub> or KCl was added (K<sup>-</sup>CDM) was not completely free of K<sup>+</sup> due to trace contamination of commercially available reagents. Compensatory molar amounts of LiCl were added from a 1M stock so that the total ionic strength of the medium contributed from K<sup>+</sup> and Li<sup>+</sup> together was 10 mM at all times. Na<sub>2</sub>HPO<sub>4</sub> was also eliminated during the making of the CDM and added, unless otherwise stated, to 142 mM (10 g/L). pH adjustments were made with addition of either HCl or Triethanolamine. All reagents were purchased from Sigma Aldrich (St. Louis, MO), EMD Chemical Inc (Gibbstown, NJ), or Becton, Dickinson, and Company (Sparks, MD).

### Measurement of membrane potential

The resting membrane potential was determined using the carbocyanine dye 3,3'-diethyloxa-carbocyanine iodide (DiOC<sub>2</sub>(3)) (*BacLight* B34950). Cells from overnight cultures were washed twice with K<sup>-</sup>CDM and diluted to OD<sub>600</sub> = 0.1 in 3 mL of K<sup>-</sup>CDM plus 0.1 mM KCl in a 14 mL polystyrene culture tube (Becton, Dickinson, and Company, Sparks, MD). Cultures were then grown at 37°C with 250 rpm shaking until an OD<sub>600</sub> of 2.0 was reached. 1-mL cell samples were centrifuged and resuspended in 1 mL K<sup>+</sup>-free phosphate-buffered saline. 0.5 mL of the cell suspension was added to a 14 mL flow cytometry tube and subsequently stained with 30  $\mu$ M DiOC<sub>2</sub>(3) for 30 minutes. As a

negative control, one sample was treated with 30  $\mu\text{M}$  CCCP to depolarize the cells.  $1.2-1.3 \times 10^4$  stained bacteria were assayed in a FACS Aria flow cytometer (Becton-Dickinson) using a laser emitting at 488 nm and fluorescence was collected in the green (fluorescein) and red (Texas Red) channels. DiOC2(3) exhibits green fluorescence in all bacterial cells, but shifts to red as the dye molecules self-associate at higher cytosolic concentrations caused by a larger  $\Delta\Psi$ . The ratio of red to green fluorescence intensity was used as a size-independent indicator of membrane potential and plotted on a ratiometric histogram.

### Determination of antibiotic MIC

Broth microdilution assays were performed in a 96-well plate using serial 2-fold dilutions of antibiotics in K-CDM supplemented with either 0.5 or 10 mM KCl. Susceptibility levels were defined based on the Clinical and Laboratory Standards Institute supplement M07-A9. MIC values reported are the concentration of antibiotic in which no growth was seen following 20 hrs of static incubation at 37°C.

Antibiotics were purchased from Sigma Aldrich (St. Louis, MO), Human Neutrophil Peptide-1 (NHP-1) was purchased from Peptides International (Louisville, KY), and LL-37 was synthesized at the University of Nebraska Medical Center by standard solid phase methods (AAPTEC Apex 396 synthesizer) on a pre-loaded Ser Wang resin using the Fmoc (9-fluorenylmethoxy-carbonyl) method of orthogonal synthesis with HBTU [2-(1H-benzotriazol-1-yl)-1,1,3,3-tetra-methyluronium hexafluorophosphate]-activated esters of the amino acids. LL-37 was characterized by confirmation of molecular mass using Electrospray mass spectrometry;  $M_{\text{calc}} = 4493$ ,  $(M+3H)3^+ = 1498.25$ ,  $(M+4H)4^+ = 1123.92$ ,  $(M+5H)5^+ = 899.31$ ,  $(M+6H)6^+ = 749.59$ ,  $(M+7H)7^+ = 642.66$ .

### Mouse bacteremia model

Prior to inoculation, C57BL/6 mice were anesthetized using ketamine and xylazine. 100  $\mu\text{L}$  containing approximately  $3 \times 10^6$  CFU with 50% *S. aureus* JE2 and 50% *ctrB::\Phi\text{N}\Sigma*, *ctrD::\Phi\text{N}\Sigma*, or *ctrA::\Phi\text{N}\Sigma* inoculated via retro-orbital injection. On day 9 following inoculation, the animals were sacrificed and the kidneys were excised, homogenized, and subsequently dilution plated on TSA and TSA+Erm (5  $\mu\text{g}/\text{mL}$ ). Only those kidneys containing greater than 100 CFU/g of tissue were statistically analyzed. Relative competitive index (RCI) values were calculated by dividing the percentage of mutant cells collected from the kidney by the percentage of mutant cells in the original inoculum.

To determine if significant out-competition occurred, pair-wise comparisons were conducted using the Wilcoxon signed-rank test method. Additionally, a two-sided Wilcoxon rank sum test was used to pair-wise compare two RCI values.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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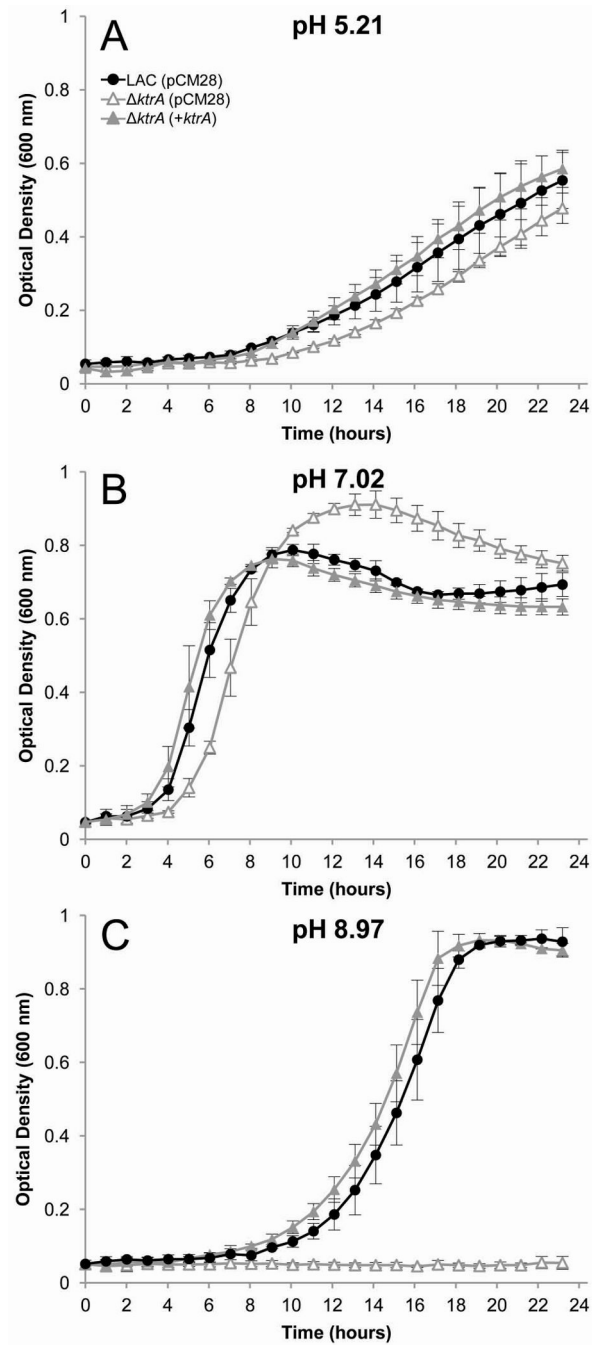
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**Fig. 1.**

Growth of *S. aureus*  $\Delta ktrA$  mutant in 0.5 mM KCl at acidic, neutral, and alkaline pH. Wild-type *S. aureus* (solid circles) and the  $\Delta ktrA$  mutant (open triangles) were inoculated from overnight cultures to  $OD_{600}=0.05$  in K-CDM supplemented with 0.5 mM KCl.

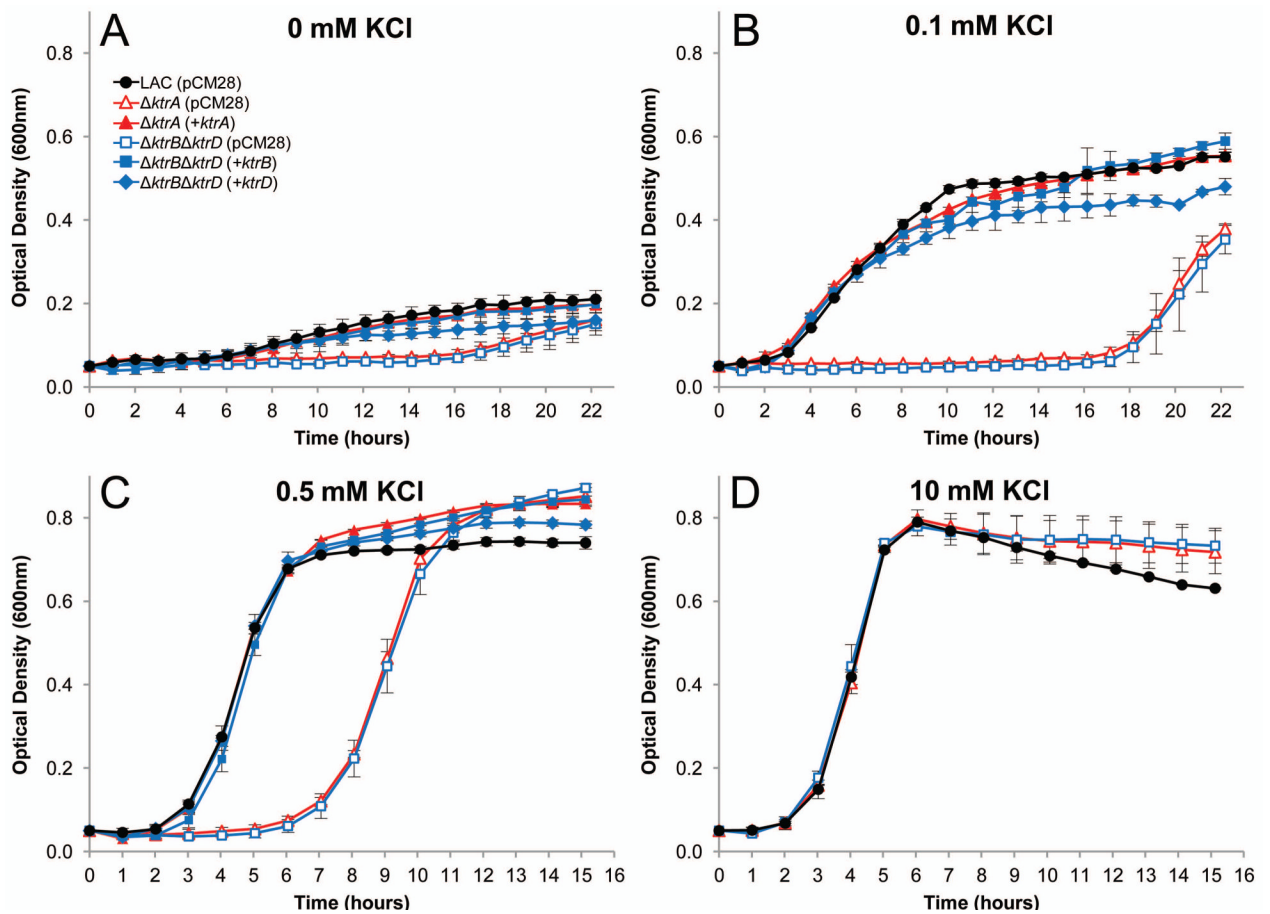
A and B. Growth in acidic (pH 5.21) or neutral (pH 7.02) conditions had minor effects on growth.

C. At pH 8.97, the  $\Delta ktrA$  strain was unable to grow.

A-C. Expression of *ktrA* from plasmid pCM28 in  $\Delta ktrA$  (solid triangles) fully complemented the growth defects in all conditions tested.

Data represents the mean of at least two independent experiments, with  $\pm$  standard deviation.





**Fig. 2.**

Growth of *S. aureus* Ktr mutants in low  $K^+$ . Wild-type *S. aureus* (solid black circles) and mutant strains  $\Delta ktrA$  (open red triangles) and  $\Delta ktrB\Delta ktrD$  (open blue squares) were inoculated from overnight cultures to  $OD_{600}=0.05$  in  $K^-$ CDM supplemented with KCl to the desired concentration.

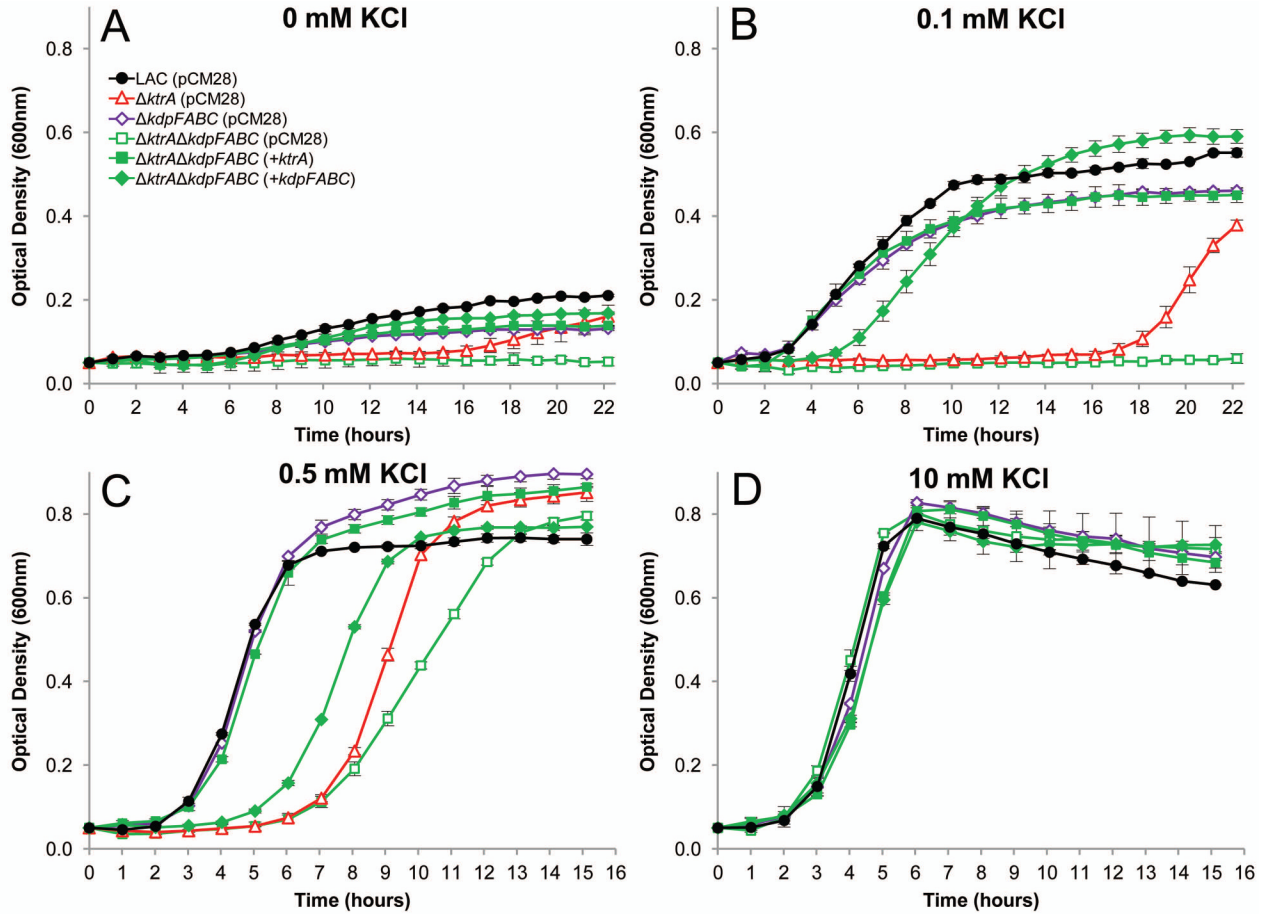
A and B. Without supplemented KCl (A) and in 0.1 mM supplemented KCl (B), the  $\Delta ktrA$  and  $\Delta ktrB\Delta ktrD$  strains had a severe growth delay compared to wild-type.

C. In 0.5 mM KCl, the  $\Delta ktrA$  and  $\Delta ktrB\Delta ktrD$  strains had an extended lag phase before entering a growth rate and yield similar to wild-type.

D. With KCl supplemented to 10 mM, all stains grew equally.

A-D. Expression of *ktrA* from plasmid pCM28 (solid red triangles) fully complemented the  $\Delta ktrA$  mutant growth defects, and expression of either *ktrB* (solid blue diamond) or *ktrD* (solid blue squares) from pCM28 fully complemented growth of the  $\Delta ktrB\Delta ktrD$  strain in all conditions tested.

Data represents the mean of three independent experiments, with  $\pm$  standard deviation.

**Fig. 3.**

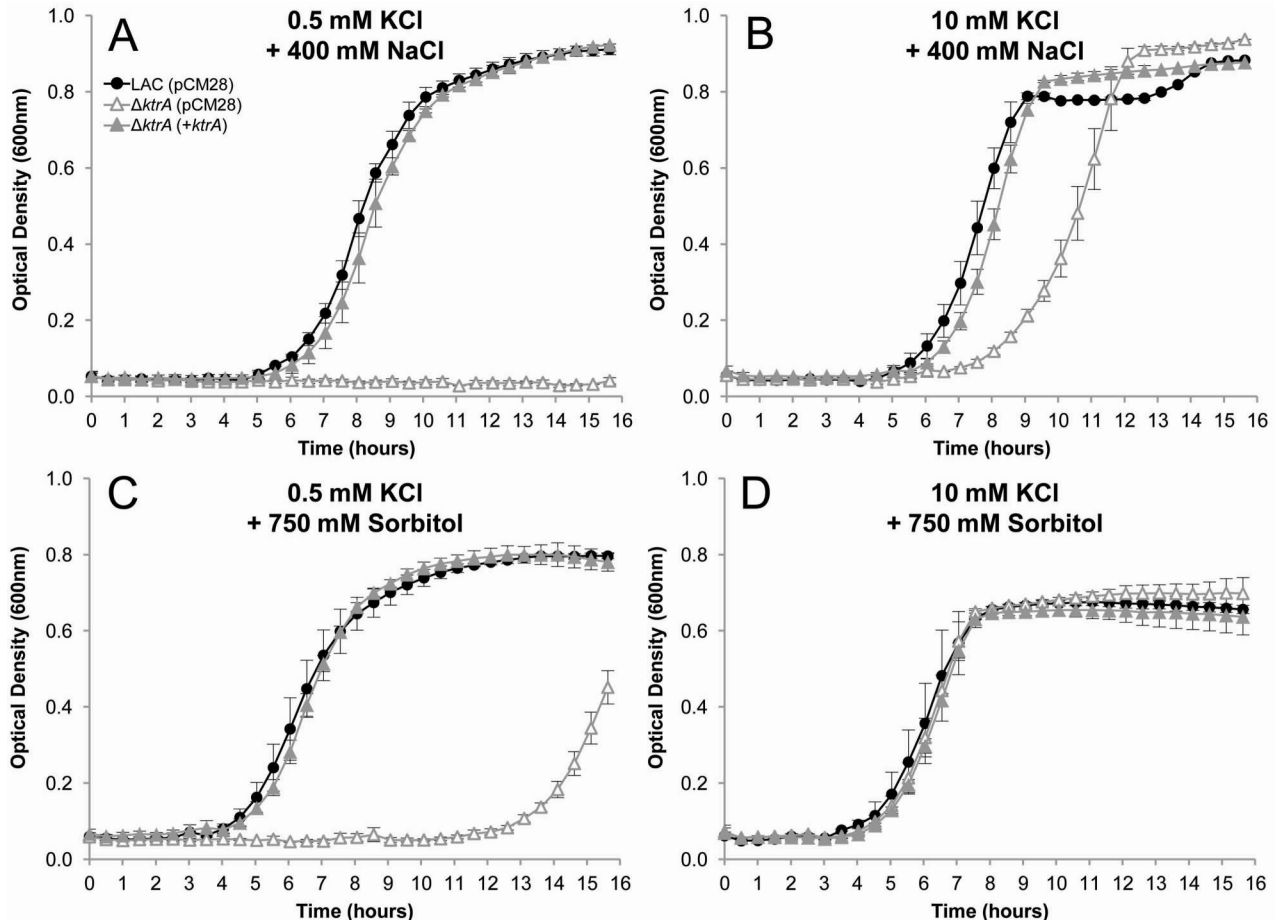
Growth of *S. aureus* Kdp and Ktr mutants in low K<sup>+</sup>. Wildtype *S. aureus* (solid black circles) and mutant strains  $\Delta kdpFABC$  (open purple diamonds) and  $\Delta ktrA\Delta kdpFABC$  (open green squares) were inoculated from overnight cultures to OD<sub>600</sub>=0.05 in K<sup>-</sup>CDM supplemented with KCl to the desired concentration.

A and B. Without supplemented KCl (A) and in 0.1 mM supplemented KCl (B), the  $\Delta ktrA\Delta kdpFABC$  double mutant was unable to grow, while the  $\Delta kdpFABC$  single mutant had only a slight growth defect compared to wild-type. The  $\Delta ktrA$  (open red triangles) mutant is shown for reference.

C. In 0.5 mM added KCl, the  $\Delta ktrA\Delta kdpFABC$  strain had a slower rate of growth than the single  $\Delta ktrA$  mutant.

D. With KCl supplemented to 10 mM, all stains grew similarly.

A-D. Expression of *ktrA* (solid green squares) from plasmid pCM28 fully complemented the  $\Delta ktrA\Delta kdpFABC$  double mutant growth defects, and expression of *kdpFABC* (solid green diamonds) partially complemented growth of the  $\Delta ktrA\Delta kdpFABC$  strain in all conditions. Data represents the mean of three independent experiments, with  $\pm$  standard deviation.



**Fig. 4.**

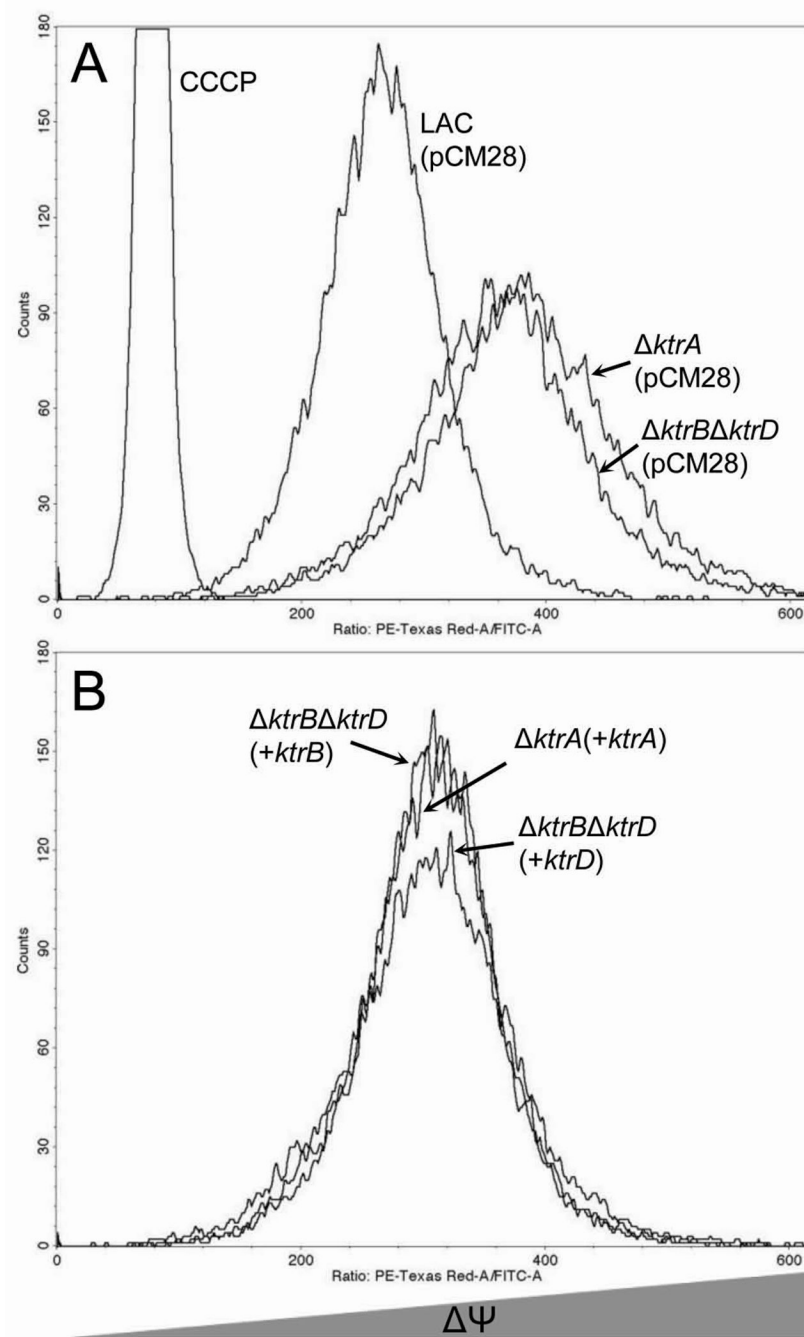
Ktr contributes to high osmotic growth. Wild-type (solid circles),  $\Delta ktrA$  (open triangles), and  $\Delta ktrA(+ktrA)$  (solid triangles) strains were inoculated from overnight cultures to  $OD_{600}=0.05$  in K-CDM supplemented with KCl and either NaCl or Sorbitol to the desired concentration.

A and B. Growth in high NaCl. The  $\Delta ktrA$  strain was unable to grow in K-CDM supplemented with 0.5 mM KCl and 400 mM NaCl (A), but recovered growth to near wild-type levels with added 10 mM KCl (B).

C and D. Growth in high sorbitol. As an alternative solute, 750 mM sorbitol also severely delayed the growth of the  $\Delta ktrA$  mutant in 0.5 mM KCl (C), but was fully restored in 10 mM KCl (D).

Expression of *ktrA* from a pCM28 fully complemented the growth defects of the  $\Delta ktrA$  mutant in all conditions.

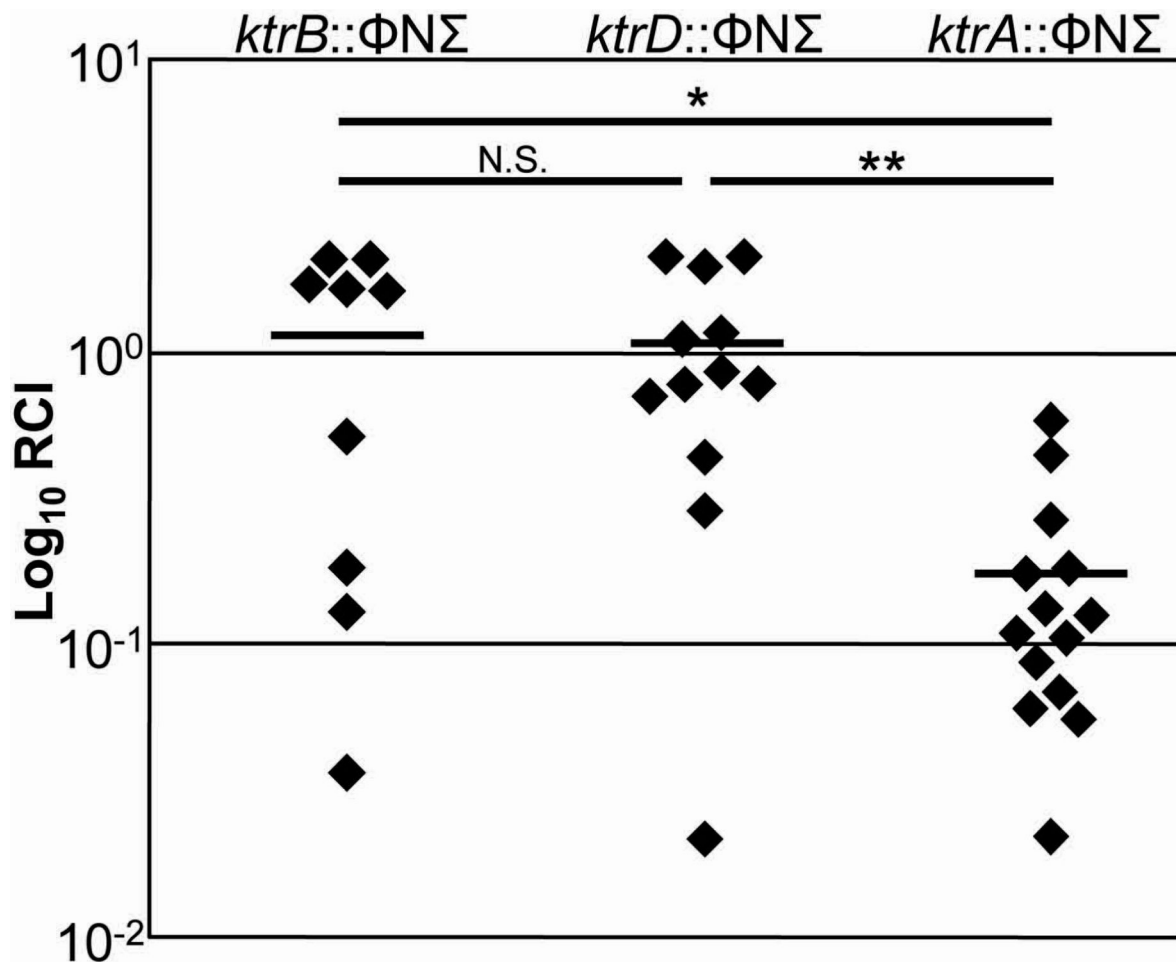
Data presented represent the mean of at least two independent experiments, with  $\pm$  standard deviation.



**Fig. 5.** Ktr-mediated regulation of membrane potential. The cyanine dye DiOC2(3) was utilized to determine the role of the Ktr system in regulation of *S. aureus* resting electrical membrane potential.

A. The  $\Delta ktrA$  and  $\Delta ktrB\Delta ktrD$  mutant strains were hyperpolarized compared to wild-type.

B. Hyperpolarization was decreased to near wild-type levels with pCM28-based expression of *ktrA* in the  $\Delta ktrA$  mutant and either *ktrB* or *ktrD* in the  $\Delta ktrB\Delta ktrD$  strain. Results are representative of three independent experiments.



**Fig. 6.**

Ktr contributes for fitness in a mouse bacteremia model. C57BL/6 mice were infected with  $\sim 3 \times 10^6$  CFU of  $\sim 50\%$  *S. aureus* JE2 and  $\sim 50\%$  *ktrB*::ΦNΣ (n=5 mice), *ktrD*::ΦNΣ (n=6 mice), or *ktrA*::ΦNΣ (n=7 mice). After 9 days, both kidneys were individually homogenized and bacterial burden of JE2 and ΦNΣ mutants were determined through viable cell count on TSA and TSA+Erm, respectively. RCI values were calculated by dividing the percentage of the ΦNΣ strain within each kidney by percentage of the ΦNΣ strain within the initial inoculum and plotted as an individual data point. Horizontal lines represents the RCI mean. The probability that significant differences in RCI are noted between *ktrB*::ΦNΣ and *ktrA*::ΦNΣ (\*p=0.0128), *ktrD*::ΦNΣ and *ktrA*::ΦNΣ (\*\*p=0.008), but not *ktrB*::ΦNΣ and *ktrD*::ΦNΣ (p=0.9716).

Table 1

## Identification of staphylococcal Ktr proteins

	Loc#	KtrB	KtrD	Loc#	KtrA	KtrC
<i>S. aureus</i> USA300_FPR3757	1979	<b>43%</b>	33%	0988	54%	<b>65%</b>
	0924	33%	<b>50%</b>			
<i>S. epidermidis</i> ATCC 12228	0571	<b>42%</b>	33%	0786	53%	<b>64%</b>
	0724	33%	<b>50%</b>			
<i>S. haemolyticus</i> JCS1435	2567	<b>41%</b>	33%	1867	53%	<b>64%</b>
	1935	33%	<b>52%</b>			
<i>S. lugdunensis</i> HKU09-01	1845	33%	<b>50%</b>	1788	33%	<b>66%</b>
<i>S. saprophyticus</i> ATCC 15305	2353	<b>41%</b>	32%	1703	53%	<b>64%</b>
	1762	33%	<b>49%</b>			
<i>S. carnosus</i> TM300	0441	<b>42%</b>	31%	0709	54%	<b>65%</b>
	0626	32%	<b>49%</b>			
<i>S. pseudintermedius</i> HKU10-03	0870	<b>43%</b>	32%	0795	51%	<b>66%</b>
	0719	33%	<b>48%</b>			

<sup>a</sup> Loc# represents locus ID for the given *Staphylococcus* sp.

<sup>b</sup> Percentage represents the percent amino acid identity to the respective *B. subtilis* Ktr protein.

Table 2

Contribution of Ktr to antimicrobial resistance.

Antibiotic	MIC Values					
	0.5 mM KCl			10 mM KCl		
	LAC (pCM28)	$\Delta ktrA$ (pCM28)	$\Delta ktrA$ (+ktrA)	LAC (pCM28)	$\Delta ktrA$ (pCM28)	$\Delta ktrA$ (+ktrA)
Gentamicin	0.5	<0.0625	0.5	0.5	0.25	0.5
Tobramycin	1	0.125	1	1	0.5	1
Polymyxin B	32	8	32	32	16	32
LL-37	256	16	256	512	64	512
HNP-1	6	1.5	6	6	3	6
Vancomycin	2	1	2	2	1	2
Gramicidin	>64	<1	>64	>64	32	>64
Valinomycin	>64	>64	>64	4	2	4

<sup>a</sup>MIC values reported are the concentration of antibiotic in which no growth was seen following 20 hrs of static incubation at 37°C.

<sup>b</sup>Results are representative of at least two independent experiments.