RESEARCH ARTICLE

Transcriptional Profiling of *Staphylococcus aureus* During Growth in 2 M NaCl Leads to Clarification of Physiological Roles for Kdp and Ktr K^+ Uptake Systems

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ABSTRACT ***Staphylococcus aureus*** **exhibits an unusually high level of osmotolerance and Na^+ tolerance, properties that support survival in various host niches and in preserved foods. The genetic basis of these traits is not well understood. We compared the transcriptional profiles of *S. aureus* grown in complex medium with and without 2 M NaCl. The stimulon for growth in high-osmolality media and Na^+ included genes involved in uptake of K^+, other compatible solutes, sialic acid, and sugars; capsule biosynthesis; and amino acid and central metabolism. Quantitative PCR analysis revealed that the loci responded differently from each other to high osmolality imposed by elevated NaCl versus sucrose. High-affinity K^+ uptake (*kdp*) genes and capsule biosynthesis (*cap5*) genes required the two-component system KdpDE for full induction by osmotic stress, with *kdpA* induced more by NaCl and *cap5B* induced more by sucrose. Focusing on K^+ importers, we identified three *S. aureus* genes belonging to the lower-affinity Trk/Ktr family that encode two membrane proteins (KtrB and KtrD) and one accessory protein (KtrC). In the absence of osmotic stress, the *ktr* gene transcripts were much more abundant than the *kdpA* transcript. Disruption of *S. aureus kdpA* caused a growth defect under low-K^+ conditions, disruption of *ktrC* resulted in a significant defect in 2 M NaCl, and a Δ*ktrC* Δ*kdpA* double mutant exhibited both phenotypes. Protective effects of *S. aureus* Ktr transporters at elevated NaCl are consistent with previous indications that both Na^+ and osmolality challenges are mitigated by the maintenance of a high cytoplasmic K^+ concentration.**

IMPORTANCE **There is general agreement that the osmotolerance and Na^+ tolerance of *Staphylococcus aureus* are unusually high for a nonhalophile and support its capacity for human colonization, pathogenesis, and growth in food. Nonetheless, the molecular basis for these properties is not well defined. The genome-wide response of *S. aureus* to a high concentration, 2 M, of NaCl revealed the upregulation of expected genes, such as those for transporters of compatible solutes that are widely implicated in supporting osmotolerance. A high-affinity potassium uptake system, KdpFABC, was upregulated, although it generally plays a physiological role under very low K^+ conditions. At higher K^+ concentrations, a lower-affinity and more highly expressed type of K^+ transporter system, Ktr transporters, was shown to play a significant role in high Na^+ tolerance. This study illustrates the importance of the K^+ status of the cell for tolerance of Na^+ by *S. aureus* and underscores the importance of monovalent cation cycles in this pathogen.**

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Human commensals and opportunistic pathogens belonging to the genus *Staphylococcus* tolerate exceptionally low water activities (1). Plating on 7.5% (1.3 M) NaCl has been a routine method for selective enrichment of staphylococci for decades (2), and it is often posited that the osmotolerance of *Staphylococcus aureus* and *S. epidermidis* supports their growth and survival in association with human skin and mucous membranes. This is consistent with the association of *S. aureus* infection with the inherited disease cystic fibrosis, in which bacteria colonize and grow in the viscous sputum that accumulates within the lung (3). *S. aureus* also grows well at low osmolality and must thus have osmoregulation capacities over a very large range (4). Nonetheless, staphylococci have not been major models for studies in which genetic loci required for osmotolerance have been identified. Rather, molecular mechanisms that support resistance to osmotic stress have been characterized most extensively in prokaryotes with relatively low tolerance, such as *Escherichia coli* and *Bacillus subtilis*, and at the other extreme in halophiles, which grow optimally at osmolalities and Na^+ concentrations that would inhibit almost all other organisms.

Diverse organisms cope with osmotic stress by accumulating solutes that increase the osmolality of the cytoplasm, thereby helping the cells retain water while minimally interfering with protein function. K^+ is often accumulated upon an upshift in external osmolality and is naturally the most abundant cation in most bacteria (5, 6). There is a correlation between medium osmolality and intracellular K^+ concentration (6–9). Many small organic compounds can also enhance osmotolerance. These compatible solutes are often zwitterions such as glutamine, proline, and glycine betaine, and cells can increase their intracellular concentration via increased biosynthesis, decreased degradation, or increased uptake (10). Measurements of intracellular K^+, amino acids, and other compatible solutes during growth in media with various osmolalities have revealed properties that distinguish *S. aureus* from other bacteria. Christian and Waltho found that the intracellular K^+ concentration in *S. aureus* grown in a complex medium was much higher than that of a *Leuconostoc* spp. (another firmicute; 700 mM versus 140 mM). They found that this concentration increased when *S. aureus* was incubated in medium containing added sucrose, NaCl, and KCl but was maintained at concentrations approximately equal to or higher than internal Na^+ in all cases (6). Other studies have reported constitutively high levels of intracellular K^+ in *S. aureus* that presumably make further increases unnecessary to mitigate the stress of high osmolality (4). However, increased K^+ uptake might be required to maintain the high constitutive level of cytoplasmic K^+ under such stress. *S. aureus* can tolerate concentrations of internal Na^+ as high as 900 mM (11), an unusual tolerance that is consistent with findings that the cytotoxicity of Na^+ is mitigated by increased K^+ (12). Similarly, key metabolic enzymes from *S. aureus*, with its especially high cytoplasmic K^+ concentration, are less sensitive to inhibition by Na^+ than those of *E. coli* and *B. subtilis* (1).

With respect to specificities for organic compatible solutes, there is variation among different species, with Gram-negative bacteria generally showing large increases in intracellular glutamate during osmotic stress while Gram-positive bacteria maintain constitutively high levels of glutamate and increase proline concentrations at least modestly during osmotic stress (1, 9). In *S. aureus*, glycine betaine, proline, choline, and taurine have all been noted as compatible solutes that accumulate intracellularly and enable the organism to grow in high-osmolality media (4, 13). Several transport activities have been reported as potential contributors to compatible-solute uptake, but the responsible genes and proteins have not been identified in most cases (14, 15). Mutants with transposon insertions in the *S. aureus* genes *brnQ3* and *arsR* have defects in growth in high-osmolality media, but the mechanisms involved are not known (16–18).

To gain a broader understanding of the molecular basis of *S. aureus* osmotolerance and Na^+ tolerance, we conducted a microarray experiment that compared the transcriptome during growth in the presence and absence of 2 M NaCl. Among a diverse group of genes that exhibited at least 10-fold induction, the most upregulated gene during growth in high Na^+ was part of an operon that encodes a Kdp complex, a high-affinity ATP-dependent K^+ importer. This led to assessment of the conditions under which physiological roles could be demonstrated for the Kdp transporter, which was positively regulated by the two-component system KdpDE, and for a lower-affinity Ktr-type K^+ transporter, for which genes were identified.

RESULTS AND DISCUSSION

The *S. aureus* transcriptional response to growth in 2 M NaCl. To identify genes whose upregulation is associated with growth at elevated salt concentrations, we conducted a microarray experiment comparing *S. aureus* USA300 LAC grown in LB0, a complex medium, with and without the addition of 2 M NaCl. This concentration of NaCl was chosen because it is sufficiently high to completely inhibit the growth of most cultivable bacteria but has only a moderate effect on the growth of *S. aureus* (see Fig. S1 in the supplemental material). The contaminating Na^+ content of LB0 was measured by flame photometry and was approximately 14 mM. Cultures were inoculated at a starting optical density at 600 nm (OD_{600}) of 0.01 and grown in Erlenmeyer flasks to a density of 0.7, which corresponds to late exponential phase (see Fig. S1). The culture grown without added NaCl showed a doubling time of 25 min, while the culture grown with NaCl had a longer doubling time of 45 min.

At the parallel time points shown in Fig. S1, culture samples were transferred immediately to an ice-cold acetone-ethanol solution and frozen at −80°C before subsequent RNA extraction. cDNA samples were prepared and hybridized to commercially available Affymetrix GeneChips containing probes representing 3,300 open reading frames (ORFs) and 4,800 intergenic regions from four different *S. aureus* genomes. We found that 267 genes or intergenic regions were induced (see Table S1 in the supplemental material) while 194 genes or intergenic regions were repressed (see Table S3) during growth in 2 M NaCl compared to growth in the absence of stress. *S. aureus* COL numbers are shown for most of these loci unless otherwise noted.

When the transcriptional profile of cells grown in 2 M NaCl was compared to that of cells grown in the absence of this stress, the most upregulated locus was the *kdpFABC* operon, with a range of 35.1- to 102.4-fold increases among the *kdp* genes. This operon is predicted to encode an ATP-driven, high-affinity K^+ transport system called Kdp. Kdp systems have been implicated in osmotolerance in *E. coli*. Transcription of *kdp* operons is strongly induced by osmotic stress and/or K^+ limitation in many bacterial species (19–21), and *kdp* operon expression is induced by the two-component system KdpDE in *E. coli* and *Leptospira interrogans* (22–24). We observed that the *kdpDE* operon was also significantly induced in *S. aureus* cells grown in the presence of 2 M NaCl, by 21.4- and 8.7-fold for *kdpD* and *kdpE*, respectively. This suggested that KdpDE acts to activate *kdpFABC* expression in *S. aureus* although there had been an earlier report to the contrary (25).

Additional loci that encode proteins with diverse or unknown functions were induced more than 10-fold by growth in 2 M NaCl. The *cap5* operon, which had been reported to be regulated by KdpDE (25, 26), was among them. This locus encodes the biosynthetic enzymes for production of the capsule (serotype 5), a virulence factor that helps protect *S. aureus* from phagocytosis (27). Other highly induced loci that are involved in central metabolism could be contributing to reorientation of these major pathways to support biosynthesis of the capsule, which could constitute a major carbon sink. Such loci include those that encode tricarboxylic acid cycle proteins (e.g., the *gltA*, *suc*, and *sdh* genes), phosphoenolpyruvate carboxykinase, lactate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, dihydroxyacetone kinase, and fructose-1,6-bisphosphatase. One or more represen-

tative genes from four different phosphotransferase system (PTSs), which are involved in sugar uptake, were also induced at least 2-fold and could contribute substrates for central metabolic pathways.

Loci identified with products involved in central metabolism were also related to amino acid transport, biosynthesis, and degradation, which could contribute to compatible-solute uptake and production and/or energy generation under a stressful condition. One of the most upregulated genes during growth in a high NaCl concentration was *rocD*, a component of an alternative pathway for proline biosynthesis by arginine conversion (28), which exhibited 14.4-fold induction by 2 M NaCl. The genes *rocA* and *rocF* were also upregulated, suggesting that this alternative pathway, which has been implicated in osmotolerance in *B. subtilis* (29), facilitates proline accumulation in response to osmotic stress in *S. aureus*. Additional loci that are candidates for support of accumulation of amino acids or chemically related compatible solutes and that were induced by growth in 2 M NaCl are indicated in Table S1 in the supplemental material.

We also noted three other genes that were significantly upregulated, one of which had been found earlier to be induced by NaCl. This was the *sceD* gene, which encodes a putative lytic transglycolase and was induced 21.4-fold by 2 M NaCl in this study. SceD is required for nasal colonization in a cotton rat model, suggesting that osmotic stress can act as a signal for genes that facilitate survival during association with the host (30). *geh*, a large gene that encodes a lipase, is also very strongly upregulated during growth in a high NaCl concentration. The physiological advantages of the overproduction of these transcripts in response to NaCl stress are not known, but their activities are suggestive of cell surface remodeling roles. The third gene, *nanT*, is a sodium-coupled transporter of sialic acid (*N*-acetylneuraminic acid) that has recently been described (31). The *nanT* gene is coexpressed with one of the metabolic genes and could provide energy during stress. So far, the use of a sodium solute symporter family protein for the NanT function is restricted to firmicutes and the noted homology between this transporter and sodium/proline transporters (32) raises the testable question of whether this type of NanT might exhibit a secondary compatible-solute uptake activity, which would directly enhance osmotolerance. The genes *brnQ3* and *arsR*, which had previously been implicated in Na^+ resistance after their identification by mutant screening (16–18), were not affected under our study conditions.

Genes downregulated 10-fold or more in cells grown in 2 M NaCl compared to cells grown in the absence of this stress included those that encode several virulence factors (see Table S3 in the supplemental material). The most downregulated gene, at 39.8-fold downregulation, was SACOL1164, which encodes a fibrinogen binding protein-like protein. Another gene that encodes a fibrinogen binding-related protein, SACOL1169, was downregulated 12.7-fold and 12.1-fold, according to two different probes. *efb*, which also encodes a fibrinogen-binding protein, was downregulated 12-fold. SACOL0857, a gene that encodes a staphylocoagulase precursor, was also represented by two different probes, which reported 14.2-fold and 11.9-fold downregulation. *hlY*, which encodes an alpha-hemolysin precursor, was downregulated 17.1-fold. SACOL0478, which encodes exotoxin 3, was represented by three different probes and was downregulated 17.1-fold, 16.2-fold, and 9.6-fold. Finally, SACOL0024, which encodes a 5′-nucleotidase, was also represented by two different probes and was downregulated 11.2-fold and 9.7-fold. This gene was also represented by a probe that reported 8.5-fold downregulation. Collectively, these hits suggest that *S. aureus* downregulates a virulence program associated with bacteremia and endocarditis during growth in high-osmolality media. This behavior is consistent with the asymptomatic colonization by *S. aureus* in the high-osmolality environment of the anterior nares of more than 20% of the human population (33).

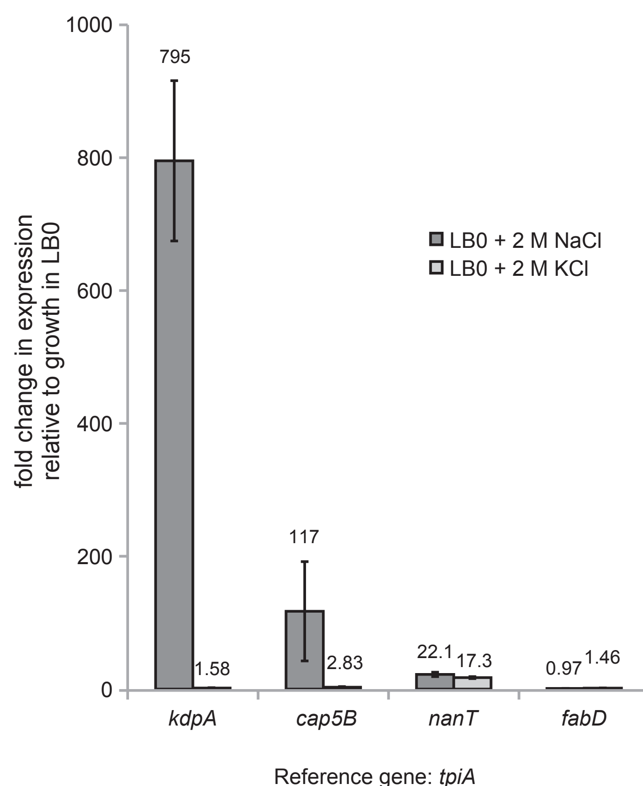


FIG 1 Fold changes in the expression of specific loci induced by growth in 2 M NaCl as assessed by qPCR. *S. aureus* LAC cultures were grown to late exponential phase in LB0 with or without 2 M NaCl or 2 M KCl. Data represent the averages of biological triplicates. Error bars represent standard deviations. *fabD* and *tpiA* were used as reference genes (54).

Major loci induced by growth in 2 M NaCl respond differentially to 2 M KCl. Although *S. aureus* is Na^+ tolerant, it is still sensitive to the toxicity of elevated Na^+ and thus less tolerant of elevated Na^+ concentrations than of comparable concentrations of K^+ (34) (see Fig. S2 in the supplemental material). It was therefore of interest to test whether the response to these two ions was also different at the transcriptional level. We focused on the *kdpA*, *cap5B*, and *nanT* genes and used real-time quantitative PCR (qPCR) to assess changes in the relative abundances of the corresponding transcripts when cultures were grown with 2 M NaCl, 2 M KCl, or no addition. As shown in Fig. 1, induction of *kdpA*, *cap5B*, and *nanT* in response to growth in 2 M NaCl was more pronounced when detected by qPCR than when detected by microarray. Only *nanT*, and not *kdpA* or *cap5B*, was still induced to a similar extent when *S. aureus* was grown in 2 M KCl.

Evaluation of the response to isosmotic concentrations of NaCl and sucrose. The difference in the responses of *kdpA* and *cap5B* transcript levels to Na^+ and K^+ raised the possibility that

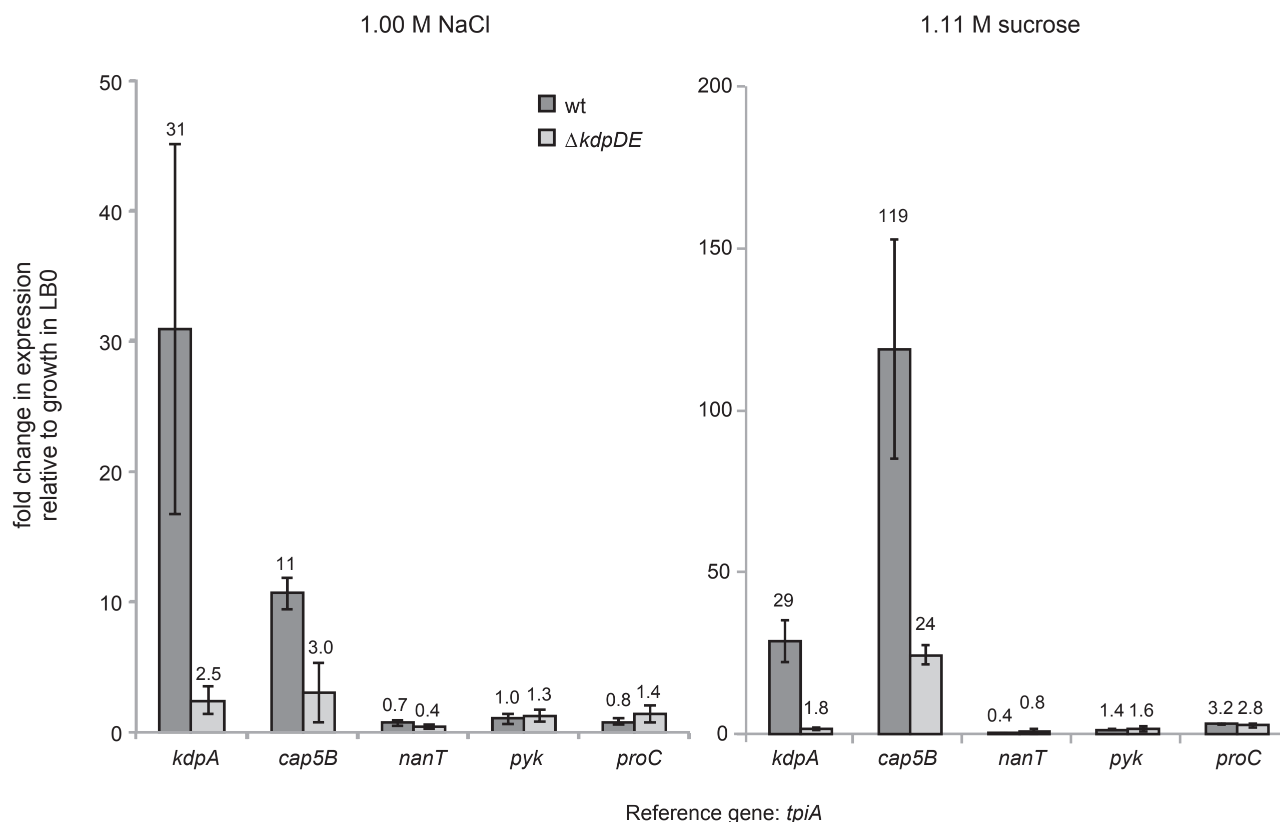


FIG 2 Fold changes in the expression of specific loci in response to growth in isosmotic concentrations (1 and 1.11 M, respectively) of NaCl and sucrose and *kdpDE* dependence of induction. *S. aureus* LAC and mutant cultures were grown to late exponential phase in LB0 with or without 1 M NaCl or 1.11 M sucrose. Data represent the averages of biological triplicates. Error bars represent standard deviations. *pyk*, *proC*, and *tpiA* were used as reference genes (54).

these genes are induced specifically by Na^+ and not by other solutes. To test this, we modified our protocol to allow the addition of isosmotic concentrations of NaCl or sucrose to the culture medium. This required the use of a lower concentration of NaCl (1 M instead of 2 M) to allow the use of sucrose at a soluble concentration that would not make the medium noticeably viscous. Isosmotic concentrations of NaCl and sucrose in LB0 medium were established by measuring standards of media containing these osmolytes at known concentrations using a vapor pressure osmometer and plotting the relationship between concentration and osmolality (see Fig. S3 in the supplemental material). The values we obtained for LB0 containing NaCl and sucrose at concentrations of 0.2 to 1.5 M were comparable to the values for similar standards reported previously (4). We found that the levels of *kdpA* induction at isosmotic concentrations of NaCl and sucrose (1 M and 1.11 M, respectively) were comparable (Fig. 2), though they were more than 10-fold lower than the levels seen with 2 M NaCl. The fold induction of *cap5B* was significantly higher in sucrose than in the isosmotic concentration of NaCl, suggesting that additional regulatory mechanisms induce *cap5* operon expression under this condition. The low level of NaCl used for this experiment, however, was not sufficient to induce the expression of *nanT*. The induction of *kdpA* and *cap5B* by sucrose suggests that induction of the *kdpFABC* and *cap5* loci may occur as part of a generic osmotic stress response.

Full *kdpA* induction requires functional KdpDE. Using isosmotic concentrations of NaCl and sucrose, we tested the dependence of *kdpA* and *cap5B* induction on the presence of a functional KdpDE two-component system. A mutant lacking the *kdpDE* operon (Table 1) was grown under the same high-NaCl or -sucrose conditions as the parent strain. We did not observe a growth defect in the Δ*kdpDE* mutant under these conditions. In the *kdpDE* mutant background, the significant induction of *kdpA* observed in a wild-type control during growth in both high-osmolality media was abolished (Fig. 2). Induction of *cap5B* was also abolished in NaCl but was only partially diminished during growth in sucrose, further supporting the hypothesis that an additional mechanism of induction acts on the *cap5* locus specifically during growth in media containing this osmolyte. The effects of *kdpDE* deletion on *kdpA* and *cap5B* expression in high NaCl and sucrose concentrations, and the lack of *kdpA* and *cap5B* induction during growth in high KCl, raise the possibility that activity of the KdpDE system in controlling the *kdpFABC* and *cap5* operons is modulated by multiple environmental cues, e.g., osmotic strength and K^+ availability.

The *S. aureus* genome encodes both high- and low-affinity K^+ importers. We observed the induction of a high-affinity K^+ importer, KdpFABC, during the growth of *S. aureus* in LB0 medium, which was shown by flame photometry to contain approximately 7.4 mM contaminating K^+. This raised the possibility that at its highly increased levels of expression, the KdpFABC transporter might make a modest contribution to K^+ homeostasis by using the contaminating K^+ but would play a more prominent role at an even lower K^+ concentration. It was further expected

TABLE 1 Bacterial strains used in this study

Species and strain	Genotype and/or description	Source or reference(s)
S. aureus		
LAC	Wild type, USA300	59
SH1000	*S. aureus* 8325-4 with repaired *rsbU*	60, 61
LAC Δ*kdpDE*		This study
SH1000 Δ*kdpA*		This study
SH1000 Δ*ktrC*		This study
JE2		40
JE2 *kdpA*::ϕNΣ		40
JE2 *ktrB*::ϕNΣ		40
JE2 *ktrC*::ϕNΣ		40
E. coli		
DH5α		62
DH5α/pJMB168	*E. coli* DH5α containing plasmid pJMB168, which is pJB38 plus an insert designed for allelic recombination and deletion of *kdpDE*; Cm^r	This study
DH5α/pCKP47	*E. coli* DH5α containing plasmid pCKP47, which is pMAD plus an insert designed for allelic recombination and deletion of *kdpA*; Amp^r	This study
DH5α/pCKP67	*E. coli* DH5α containing plasmid pCKP67, which is pMAD plus an insert designed for allelic recombination and deletion of *ktrC*; Amp^r	This study

that a distinct low-affinity K^+ importer, still to be identified, would be a major contributor to the ability of *S. aureus* to accumulate K^+ at high levels (0.7 to 1.1 M) during growth in rich, complex media, even in the absence of osmotic stress (4, 11). We searched *S. aureus* genomes for homologues of low-affinity K^+ uptake systems in other bacteria and found proteins with sequence similarity to subunits of Ktr systems, which have been studied in *B. subtilis*. Ktr systems typically consist of two types of subunits: a transmembrane protein, required for K^+ transport, and a membrane-associated, nucleotide-binding (KTN/RCK domain) regulatory protein (34–36). While *B. subtilis* genomes contain genes for two transmembrane and two regulatory components (37), *S. aureus* genomes contain genes for two transmembrane components, which we will call *ktrB* (SACOL2011) and *ktrD* (SACOL1030) on the basis of sequence identity at the amino acid level to the *B. subtilis* counterparts, and only one gene that encodes a regulatory component, which we have designated *ktrC* (SACOL1096), on the basis of the closer similarity of the encoded protein to KtrC than to the second homologue, KtrA, found in *B. subtilis* (see Table S2 in the supplemental material). Ktr systems differ markedly from Kdp systems. *kdp* operons in diverse bacteria are regulated at the transcriptional level, and Kdp systems are powered by ATPase activity. In contrast, Ktr systems are typically constitutively expressed, show a lower affinity for K^+, have ATP-activated channel-like properties, and are powered by electrochemical ion gradients across the membrane rather than by ATPase activity (34, 38, 39).

Low-affinity K^+ import is critical for Na^+ tolerance in a complex medium. To evaluate the relative importance of the Kdp and Ktr K^+ import systems in Na^+ resistance in *S. aureus*, we generated strains with markerless deletions of *kdpA* and *ktrC* in *S. aureus* SH1000, a strain that is more genetically tractable than USA300 LAC. The individual mutant phenotypes described in this and the following sections were similar to those observed for transposon insertion mutants in USA300 LAC acquired from the Nebraska Transposon Mutant Library (data not shown) (40). Deletion of *kdpA* and/or *ktrC* had no measurable effect on the growth of SH1000 in LB0 with no added salts (Fig. 3A). In LB0 with 2 M NaCl added, the *kdpA* mutant showed a decline in stationary phase in some experiments that was not reproducible enough for its significance to be assessed. Both the Δ*ktrC* and Δ*kdpA* Δ*ktrC* mutants showed significant growth defects in exponential phase, with the Δ*kdpA* Δ*ktrC* mutant exhibiting a slightly more severe defect at the transition from the exponential to the stationary phase of the growth curve (Fig. 3B). This small difference suggests a minor, but perhaps meaningful, physiological role of *S. aureus* Kdp during osmotic stress that is largely masked by the activity of the Ktr system(s) in the wild type. After this report was drafted, Corrigan et al. (41) reported the identification of the single KTN (RCK) Ktr protein, for which they propose the name KtrA, as well as KdpD of *S. aureus* as receptors for the secondary signaling molecule cyclic di-AMP (c-di-AMP). In our present work, sodium stress, but not sucrose, caused a large elevation in KdpD-dependent expression. Together, the results here and those of Corrigan et al. (41) suggest sodium stress as a potential candidate for mediation of c-di-AMP production in *S. aureus.*

High-affinity K^+ import is critical for growth in a defined medium with limiting K^+. To test the expectation that the *S. aureus* Kdp system plays its most significant role in K^+ import under conditions under which K^+ is extremely limiting, we designed a medium, Tris-CDM (T-CDM), that would allow us to control the added concentrations of K^+ and Na^+ without contamination from complex ingredients. When K^+ was added to this medium at 1,000 μM, both the single and double *kdpA* and *ktrC* mutants grew similarly to the wild type (Fig. 3C). When K^+ was added to this medium at a low concentration (10 μM), mutants with *kdpA* deleted did not grow, while the *ktrC* mutant showed a longer lag phase than the wild type (Fig. 3D). Xue et al. recently examined the growth of Kdp-defective *S. aureus* mutants and *kdp* gene expression. They did not find a growth defect in these mutants and reported evidence that KdpDE acts to repress, rather than activate, the expression of *kdpFABC* in *S. aureus* (25). The development of a defined medium without significant contaminating Na^+ or K^+ allowed us to precisely control the amounts of these ions and uncover a growth defect in the Δ*kdpA* mutant when K^+ was limiting. Differences in the KdpDE dependence of *kdpA* induction as detected by qPCR and relative quantification may have arisen from our adoption of the recommendation that more than one

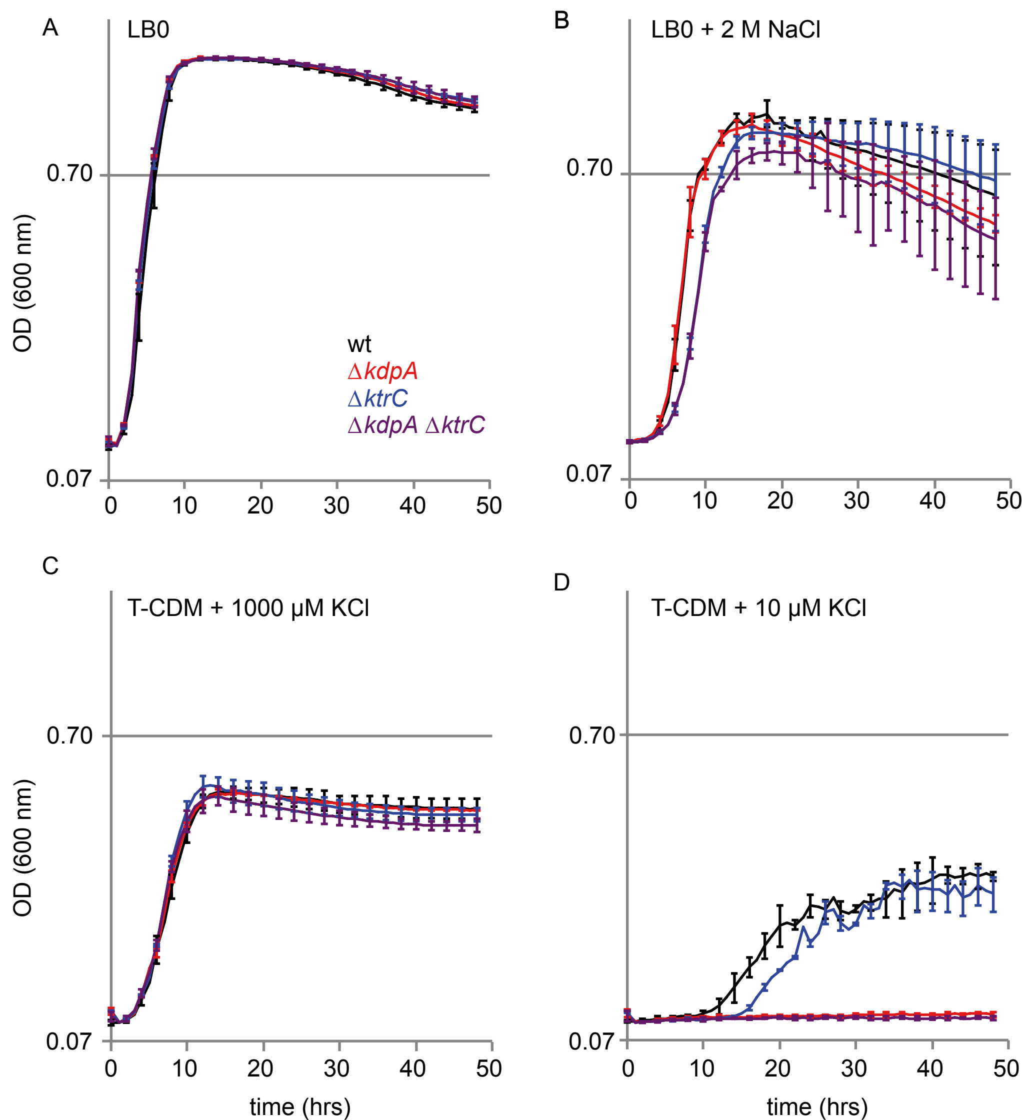
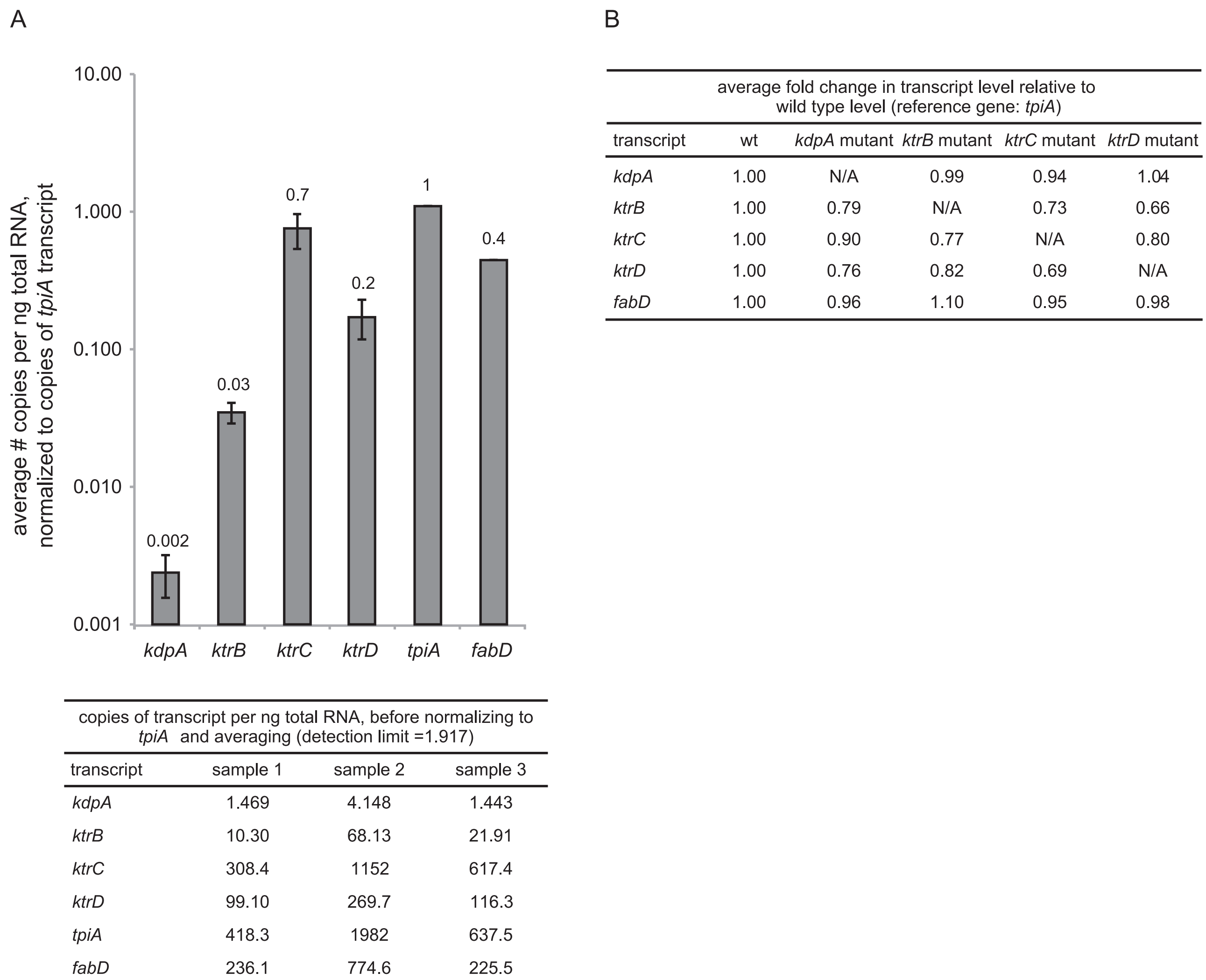


FIG 3 Growth of *S. aureus* SH1000 *kdpA* and *ktrC* mutants in complex and defined media. Panels show growth in LB0 (A), LB0 with 2 M NaCl added (B), T-CDM with 1,000 μM KCl added (C), and T-CDM with 10 μM KCl added. Data represent the averages of biological triplicates. Error bars represent standard deviations and are given for every other time point to improve visibility. wt, wild type.

reference gene be used for normalization and that use of the 16S rRNA gene be avoided (42, 43).

***ktr* genes are constitutively expressed at high levels, and *ktr* gene disruptions do not affect the expression of remaining, intact *ktr* genes.** In *B. subtilis*, Ktr activity is induced by osmotic stress but the expression levels of the *ktr* genes do not change under this condition, suggesting that Ktr systems are constitutively expressed and that Ktr activity is regulated posttranscriptionally, e.g., by c-di-AMP (41). We evaluated the expression levels of the *S. aureus kdp* and *ktr* genes by absolute quantification qPCR and found that *ktr* gene transcripts were present at levels 1 to 2 orders of magnitude higher than *kdpA* gene transcripts when cultures were grown in LB0 without any additional osmolytes added (Fig. 4A). In *B. subtilis*, it has been reported that disruptions in *ktr* genes lead to compensatory induction of the remaining intact *ktr* genes (37). We tested this model in *S. aureus* USA300 LAC by using qPCR and examined mutants with disruptions in *ktrB*, *ktrC*, *ktrD*, and *kdpA* (see Table S1 in the supplemental material). No significant changes were observed in the expression of remaining intact *ktr* or *kdp* genes in response to the disruption of these genes (Fig. 4B).

Previous reports have emphasized the unique ability of *S. aureus* to maintain relatively high intracellular K^+ levels in both high- and low-osmolality environments and postulated that this is an adaptation that supports osmotolerance (4, 6, 11). The results of this study indicate roles for diverse transporters in supporting growth in the presence of 2 M NaCl but highlight contributions of K^+ importers, since high cytoplasmic K^+ levels would mitigate the potential cytotoxicity of the high Na^+ concentration, as well as its challenge to osmoregulation. However, more specific strategies are probably also in place to export Na^+ from the cytoplasm under conditions under which the large induction of *nanT*, for example, would result in Na^+ cotransport along with the sialic acid substrate. The genomes of *S. aureus* and *S. epidermidis* both encode at



copies of transcript per ng total RNA, before normalizing to *tpiA* and averaging (detection limit =1.917)			
transcript	sample 1	sample 2	sample 3
kdpA	1.469	4.148	1.443
ktrB	10.30	68.13	21.91
ktrC	308.4	1152	617.4
ktrD	99.10	269.7	116.3
tpiA	418.3	1982	637.5
fabD	236.1	774.6	225.5

average fold change in transcript level relative to wild type level (reference gene: *tpiA*)					
transcript	wt	*kdpA* mutant	*ktrB* mutant	*ktrC* mutant	*ktrD* mutant
kdpA	1.00	N/A	0.99	0.94	1.04
ktrB	1.00	0.79	N/A	0.73	0.66
ktrC	1.00	0.90	0.77	N/A	0.80
ktrD	1.00	0.76	0.82	0.69	N/A
fabD	1.00	0.96	1.10	0.95	0.98

FIG 4 Expression of K^+ importer genes in LB0 in the absence of osmotic stress. (A) Absolute quantification by qPCR of transcripts from K^+ importer genes. *S. aureus* LAC cultures were grown to late exponential phase in LB0. *tpiA* and *fabD* were used as reference genes (54). The graph at the top shows data representing the averages of biological triplicates after *fabD* normalization. Error bars represent standard deviations. The table at the bottom lists values for individual replicates before *tpiA* normalization. (B) Relative quantification by qPCR of transcripts from K^+ importer genes in the *S. aureus* JE2 wild-type (wt) and K^+ importer mutant backgrounds. *tpiA* and *fabD* were used as reference genes (54).

least eight putative Na^+/H^+ antiporters that are expected to be important contributors to this activity (12). The loci that encode these proteins are apparently not induced by growth in the high-osmolality medium employed here, raising the possibility that one or more key Na^+/H^+ antiporters is constitutively expressed in a manner similar to that found here for the Ktr transporters.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and mutants used in this work are listed in Table 1. Routine growth was carried out with LB0 medium (lysogeny broth [44] without added NaCl, i.e., 10 g tryptone and 5 g yeast extract per liter). Experimental cultures were inoculated at a normalized starting OD_{600} of 0.01, unless otherwise noted, from 3-ml precultures grown in screw-cap tubes. For the microarray and qPCR experiments, incubation was at 37°C at 225 rpm in a rotary shaker.

For experiments examining growth with defined concentrations of Na^+ and K^+, a medium (T-CDM) was developed that was based on that of Pattee and Neveln (45). The Na^+ phosphate used as a buffer in the original medium was replaced with 50 mM Tris, and 1 mM phosphoric acid was added as a phosphorus source. The pH was set to 7.5 with HCl.

For growth experiments examining mutant phenotypes, a Bio-Tek Powerwave plate reader was used. Strains were inoculated at a normalized starting OD_{600} of 0.005 in a total of 200 μl in individual wells of 96-well plates. Plates were incubated with continuous shaking on the low setting at 37°C.

Sampling for GeneChip and qPCR experiments and RNA isolation. RNA was isolated by a modified method that incorporates reagents from the Qiagen RNeasy kit (catalog no. 74104). Culture volumes of 30 ml were grown in 250-ml Erlenmeyer flasks to an OD_{600} of 0.5 to 0.7. At sampling time, 20 ml of culture was transferred to a prechilled tube containing 20 ml of a 50% ethanol–50% acetone solution and mixed by inversion. Samples were then placed immediately at −80°C for at least 16 h. Samples were thawed on ice and then centrifuged at 3,600 × *g* for 10 min at 4°C. Supernatants were poured off, and pellets were left to dry upside down on a Kimwipe for 15 min. Pellets were resuspended in 500 μl RLT buffer (Qiagen) and transferred to tubes containing a lysing matrix (Fisher cat-

TABLE 2 Plasmids and primers used in this study

Plasmid or primer	Description or sequence	Source or reference
Plasmids		
pJB38		55
pJMB168	pJB38 plus an insert designed for allelic recombination and deletion of *kdpDE*	This study
pMAD		56
pCKP47	pMAD plus an insert designed for allelic recombination and deletion of *kdpA*	This study
pCKP67	pMAD plus an insert designed for allelic recombination and deletion of *ktrC*	This study
Primers		
kdpA 1 f	CCTTCGCCACCAAATACAAC	
kdpA 1 r	TGGAGCAGGTTTGTCAGCAC	
cap5B f	GCGATATGCGTAAGCCAACA	
cap5B r	CAGATGGATTTGGAGGTACAGG	
SACOL0311 f (for *nanT*)	GCAGCTGCCGCAGTATTTAG	
SACOL0311 r (for *nanT*)	CGGTTTCGGCACTGTCTTT	
ktrB f	AGGTGGTCTGGGTATCGTGA	
ktrB r	TAACACCACCAGGTTCGTCA	
ktrC f	TTGGAGCAGATACGGTTGTG	
ktrC r	AGAATGCTCGTCTGCCAACT	
ktrD f	AAGAAGTGCGGGTCTTCAAA	
ktrD r	GTACGAATACCGCCACCAAC	
tpiA f	GGTGAAACAGACGAAGAG	54
tpiA r	TTACCAGTTCCGATTGCC	54
fabD f	CCTTTAGCAGTATCTGGACC	54
fabD r	GAAACTTAGCATCACGCC	54
pyk f	GCATCTGTACTCTTACGTCC	54
pyk r	GGTGACTCCAAGTGAAGA	54
proC f	GGCAGGTATTCCGATTGA	
proC r	CCAGTAACAGAGTGTCCAAC	
2035 up 5 EcoRI	GGGGAATTCCCCCATAAATCCATTAAATGCCAGAAAATGTTTGAC	
2035 up3 NheI	ACGCGTGGTACCGCTAGCGCTAGCGCGATTCAGTGTTTGACATAACCTTCACCTCG	
2035 down 5 MluI	GCTAGCGGTACCACGCGTACGCGTGGCTATGTTAATAAGACTGAAATGCCTAGTTTAAG	
2035 down 3 SalI	CCCGTCGACCGGTAAACCAAGTGGTTCTCGTAACAGAAATAGT	
kdpA AQ std. 1	TGTCGCAATGTTTTTCATTTTT	
kdpA AQ std. 2	GCAGCAGCTGATGTCATTTC	
ktrB AQ std. 1	TTACTGGCTTGTCCCCAGTT	
ktrB AQ std. 2	TCACGACAAAATGTCCAATACC	
ktrC AQ std. 1	TGATGAACTCTTTGCCTCGTT	
ktrC AQ std. 2	TATCGCTACTCATGCGGTTG	
ktrD AQ std. 1	CCATGCGTTCAAAGGTTTAAG	
ktrD AQ std. 2	GGTTCTCGACGTCCTGCTAT	
tpiA AQ std. 1	CGAAGATAATGGTGCGTTCA	
tpiA AQ std. 2	TGATGCGCCACCTACTAATG	
fabD AQ std. 1	ATTAATGGCGCAAGCATTTC	
fabD AQ std. 2	CTTTTCCAGGACCAATTTCAA	
kdpA 1–1 b	ATATAGAATTCTCACTCATCAAGTCGGCAAC	
kdpA 1–2	ACGATTAGTGATACGCCAAAATACTCTTGACGATTGCACCAA	
kdpA 2-1	TTGGTGCAATCGTCAAGAGTATTTTGGCGTATCACTAATCGT	
kdpA 2-2	ATATAGGATCCGCGATTCGATTGCCATAAGT	
ktrC 1-1	ATATAGAATTCCCCAGTTTGGGAAGTTACGA	
ktrC 1–2	TTTGCCTCGTTTAATTGCAAATGCATTCAACTCACGAACG	
ktrC 2-1	CGTTCGTGAGTTGAATGCATTTGCAATTAAACGAGGCAAA	
ktrC 2-2	ATATAGTCGACGGCATGGTTCTCAAGGTGAT	

alog no. NC9875968). Tubes were processed in a bead beater (Biospec) for three rounds of 10 s each alternating with 1-min incubations on ice and then centrifuged at 16,000 $\times$ *g* for 15 min at 4°C. A 250-μl volume of the upper liquid phase was transferred to a fresh tube. After mixing with 500 μl RLT and 500 μl ethanol, the sample was applied to an RNeasy column and the RNeasy protocol was followed, including on-column DNase digestion (Qiagen RNase-free DNase set, catalog no. 79254). After RNA elution with 40 μl water, an additional DNase digestion was performed with 5 μl RQ1 buffer and 1 μl DNase (reagents from the Promega RQ1 RNase-free DNase kit [catalog no. M6101]) per sample. After a final round of the Qiagen RNeasy cleanup protocol, RNA was eluted into 30 μl of water. RNA quality was checked by agarose gel electrophoresis according to the protocol described by Sambrook et al. (46). RNA concentrations were measured with a Bio-Tek Powerwave XS2 plate reader equipped with a Take3 plate adapter.

For qPCR, cDNA was generated with the Bio-Rad iScript kit (catalog no. 170-8891) after normalizing the input RNA. One microgram of input RNA was used in the reverse transcriptase reaction. Control reactions with no reverse transcriptase added were run for representative samples and checked for DNA contamination by qPCR. Any amplifications observed in these control reactions occurred at a higher cycle number than those obtained with cDNA samples.

RNA labeling and GeneChip analysis. RNA samples were labeled, hybridized to commercially available *S. aureus* Affymetrix GeneChips (part number 900514), and processed in accordance with the manufacturer's instructions for prokaryotic arrays (Affymetrix, Santa Clara, CA). Briefly, 10 μg of each RNA sample was reverse transcribed with Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). The resulting cDNA was purified with QIAquick PCR purification kits (Qiagen, Germantown, MD), fragmented with DNase I (Ambion, Carlsbad, CA), and 3′ biotinylated with Enzo Bioarray terminal labeling kits (Enzo Life Sciences, Farmingdale, NY). Two micrograms of a labeled cDNA sample was hybridized to an *S. aureus* microarray for 16 h at 45°C, processed, and scanned in an Affymetrix GeneChip 3000 7G scanner as previously described (47, 48). Signal intensity values for all of the ORFs and intergenic regions represented on the microarray were normalized to the average signal of the microarray to reduce sample labeling and technical variability, and the signals for the biological replicates ($n = \geq 2$) were averaged by using GeneSpring 7.2 software (Agilent Technologies, Redwood City, CA) (48–51). Differentially expressed transcripts were identified as those RNA species that generated a 2-fold increase or decrease in 2 M NaCl-treated cells in comparison to a no-NaCl sample (*t* test, $P = 0.05$). All related GeneChip data files were deposited in the NCBI Gene Expression Omnibus repository in the MIAME-compliant format.

qPCR assays. qPCR experiments were conducted according to the standard protocols developed by the Mount Sinai qPCR Shared Resource Facility. These protocols rely on SYBR green-based fluorescence detection of double-stranded DNA—specificity is conferred by the primers added—and are very similar to those described by Yuen et al. (52), with the adjustment that the final reaction volume was 10 μl. Each reaction was conducted in triplicate in 384-well plates with an Applied Biosystems ABI PRISM 7900 HT sequence detection system. The PCR program consisted of an initial stage of 2 min at 95°C; 40 repeats of 15 s at 95°C, 15 s at 55°C, and 30 s at 72°C; 15 s at 95°C; 15 s at 60°C; and 15 s at 95°C. Results were analyzed using Applied Biosystems SDS 2.2.1 software with a threshold value of 3.0 and automatic baseline calculation.

For relative quantification, cycle threshold (C_T) values were used to calculate fold changes in expression using the $2^{-2\Delta\Delta CT}$ method (53). Two or three reference genes were used for normalization in each experiment, selected from the less-affected genes reported for *S. aureus* treated with berberine (54) and were checked against each other to verify that the relative differences in their expression were between 0.5 and 2 (representing a <2-fold change in expression) (42, 43). For absolute quantification, standards of transcripts of interest were generated by dilution of conventional PCR products to concentrations ranging from 10^1 to 10^8 copies/μl. The sequences of the primers used to generate these products are listed in Table 2. These standards were run alongside samples and used to generate standard curves from which the concentrations of unknowns were calculated.

Construction of markerless deletions by allelic replacement. To generate the *kdpDE*-deficient *S. aureus* USA300 LAC mutant, approximately 1,000-bp sequences upstream and downstream of the *kdpDE* gene pair (SAUSA300_2035-2036) were amplified by PCR with *S. aureus* USA300 LAC chromosomal DNA as the template and primers 2035up5EcoRI and 2035up3NheI and primers 2035down5MluI and 2035down3SalI. Amplicons were gel purified and joined by PCR with primers 2035up5EcoRI and 2035down3SalI. The PCR product was gel purified, digested with EcoRI and SalI, and ligated into similarly digested pJB38 (55). The ligation was transformed into *E. coli* DH5α and selected on ampicillin, and colonies were screened for the correct insert (final plasmid, pJMB168). Plasmid pJMB168 was isolated and transformed into RN4220 and selected on tryptic soy agar (TSA) containing chloramphenicol at 30°C. Plasmid pJMB202 was transduced into AH1263, and single colonies were used to inoculate 5 ml tryptic soy broth (TSB) containing chloramphenicol. Cultures were grown at 42°C overnight to select for single recombinants. Single colonies were used to inoculate 5 ml of TSB and grown overnight, and cultures were diluted 1:25,000 before plating on TSA-anhydrotetracycline to screen for loss of pJMB168. Chloramphenicol-sensitive colonies were screened for the double recombination event by PCR.

Deletions of target genes in *S. aureus* SH1000 were generated with pMAD (56) as previously described (57). Briefly, ~1-kb PCR products on either side of the sequence to be deleted were generated and fused by gene splicing by overlap extension (SOEing) (58). The primers used for these PCRs are listed in Table 2. The 2-kb gene SOEing product was ligated into pMAD and transformed into *E. coli*. After plasmid isolation and sequence verification, the construct was moved into *S. aureus* RN4220 by electroporation. After isolation from RN4220, the construct was electroporated into the target *S. aureus* SH1000 wild-type or mutant strain. The plasmid was recombined into the genome by incubating a liquid culture for 2 h at the permissive temperature (30°C), followed by 4 h at the restrictive temperature (42°C), and plating dilutions on LB0 agar containing erythromycin. Merodiploid clones (containing the plasmid recombined into the chromosome) were verified by PCR. To resolve the plasmid out of the chromosome and generate candidate deletion mutants, liquid cultures of merodiploids were incubated at 30°C without selection and transferred by 1:100 dilutions for 3 days before plating on LB0 agar. Candidate mutants were screened for loss of erythromycin resistance (confirming loss of plasmid), and PCR was used to confirm the exclusive presence of the deleted allele.

Microarray data accession number. The microarray protocols and metafiles determined in this study have been deposited in the NCBI Gene Expression Omnibus under accession number GSE46383.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00407-13/-/DCSupplemental.

Figure S1, EPS file, 0.9 MB.
Figure S2, EPS file, 0.9 MB.
Figure S3, EPS file, 1 MB.
Table S1, DOCX file, 0.1 MB.
Table S2, DOCX file, 0.1 MB.
Table S3, DOCX file, 0.2 MB.

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