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2010. Staphylococcus aureus AI-2 Quorum Sensing Associates with the KdpDE Two-Component System To Regulate Capsular Polysaccharide Synthesis and Virulence. Infect. Immun. 78(8):3506-3515. doi:10.1128/IAI.00131-10.**

INFECTION AND IMMUNITY, Aug. 2010, p. 3506–3515 Vol. 78, No. 8
0019-9567/10/$12.00 doi:10.1128/IAI.00131-10

Staphylococcus aureus AI-2 Quorum Sensing Associates with the KdpDE Two-Component System To Regulate Capsular Polysaccharide Synthesis and Virulence▽†‡

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Received 9 February 2010/Returned for modification 23 March 2010/Accepted 11 May 2010

Autoinducer 2 (AI-2) is widely recognized as a signal molecule for intra- and interspecies communication in Gram-negative bacteria, but its signaling function in Gram-positive bacteria, especially in *Staphylococcus aureus*, remains obscure. Here we reveal the role of LuxS in the regulation of capsular polysaccharide synthesis in *S. aureus* NCTC8325 and show that AI-2 can regulate gene expression and is involved in some physiological activities in *S. aureus* as a signaling molecule. Inactivation of *luxS* in *S. aureus* NCTC8325 resulted in higher levels of transcription of capsular polysaccharide synthesis genes. The survival rate of the *luxS* mutant was higher than that of the wild type in both human blood and U937 macrophages. In comparison to the *luxS* mutant, a culture supplemented with chemically synthesized 4,5-dihydroxy-2,3-pentanedione (DPD), the AI-2 precursor molecule, restored all the parental phenotypes, suggesting that AI-2 has a signaling function in *S. aureus*. Furthermore, we demonstrated that the LuxS/AI-2 signaling system regulates capsular polysaccharide production via a two-component system, KdpDE, whose function has not yet been clarified in *S. aureus*. This regulation occurred via the phosphorylation of KdpE binding to the *cap* promoter.

Quorum sensing (QS) is a cell-cell communication mechanism in which bacteria secrete and sense small diffusible molecules called autoinducers (AIs) to coordinate social activities, such as bioluminescence, biofilm formation, swarming behavior, antibiotic production, and virulence factor secretion (7, 23, 38, 59). Many QS mechanisms have evolved among bacteria. In general, Gram-negative bacteria use acylated homoserine lactones (AHLs) as AIs, and Gram-positive bacteria use oligopeptide AIs, which act through two-component phosphorelay cascades. Studies have shown that one QS mechanism is shared by both Gram-positive and Gram-negative bacteria and involves the production of autoinducer 2 (AI-2) (4, 38, 59, 60), which is synthesized by the LuxS enzyme in a metabolic pathway known as the activated methyl cycle (50, 57, 61). AI-2 is not a single compound but a family of interconverting compounds derived from 4,5-dihydroxy-2,3-pentanedione (DPD), which cyclizes spontaneously to form two epimeric furanoses, (2*R*,4*S*)- and (2S,4*S*)-2,4-dihydroxy-2-methyldihydrofuran-3-one (*R*- and *S*-DHMF), respectively. Hydration of *R*- and *S*-DHMF produces (2*R*, 4*S*)- and (2*S*, 4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (*R*- and *S*-THMF), respectively (40). In contrast to the other autoinducers that are usually involved in intraspecies communication, AI-2 is widely present in bacteria, leading to the suggestion that it is a universal language for interspecies communication (50, 64).

The LuxS/AI-2 system is a more recently described QS system which was first identified in *Vibrio harveyi*, where it functions as part of a complex multilayered QS system to regulate bioluminescence (9, 10). LuxS plays a metabolic role in the activated methyl cycle, and one molecule of DPD is formed as a by-product every one cycle. This gives DPD the ability of being an ideal signal to connect the metabolic activity to population density (12, 61–64). The LuxS/AI-2 QS system is known to be involved in the regulation of a range of behaviors in diverse bacteria, such as biofilm formation in *Escherichia coli*, virulence-associated traits in *Vibrio cholerae*, antibiotic susceptibility in *Streptococcus anginosus*, and motility in enterohemorrhagic *E. coli* (1, 9, 29, 39, 57). However, in many *luxS*-containing bacteria, the functions that are controlled by LuxS and whether LuxS is involved in the QS pathway still remain matters of debate.

Staphylococcus aureus is a major nosocomial pathogen with the ability to cause a variety of infectious diseases, from relatively benign skin infections to potentially fatal systemic disorders (3, 32). The pathogenicity is determined by surface-associated adhesins, superantigens, exoenzymes, and exotoxin, which are regulated by a wide range of regulatory systems (14, 18, 41). Among these regulatory elements, the Agr (the accessory gene regulator) system is the only characterized QS system in *S. aureus* and controls the expression of approximately 150 genes (20). Interestingly, *S. aureus* also possesses a functional *luxS* gene and has the ability to produce AI-2, and purified LuxS protein from *S. aureus* exhibited the catalytic activity of AI-2 production (19, 61). However, no potential AI-2 receptor has been found by searching for established AI-2 receptors (i.e., the LuxPQ receptor of *V. harveyi* and the Lsr ABC transporter of *Salmonella enterica* serovar Typhimurium)

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† Supplemental material for this article may be found at http://iai.asm.org/.

▽ Published ahead of print on 24 May 2010.

‡ The authors have paid a fee to allow immediate free access to this article.

in *S. aureus* by genomic analysis (47). Due to the dual function of LuxS and the absence of genomic evidence of established AI-2 receptors, the AI-2 quorum-sensing function in *S. aureus* has been intangible, until now. In contrast, the function of the LuxS/AI-2 system in *Staphylococcus epidermidis* was investigated more clearly. The *luxS* gene is functional in *S. epidermidis* and impacts biofilm formation via transcriptional regulation of the polysaccharide intercellular adhesin-synthesized gene *ica*, and AI-2 has a signaling function controlling virulence-associated gene expression (30, 65). It is of great importance to explore whether AI-2 can function as a QS signal to regulate physiological functions in *S. aureus*.

Among the many virulence factors that are produced by *S. aureus*, capsular polysaccharide (CP) is an important cell wall component that can interact with the host immune system during the invasive process, allowing the organism to resist uptake and killing by phagocytes. More than 90% of *S. aureus* strains produce 1 of 11 CPs, and most strains colonizing and infecting humans produce either CP5 or CP8 (13, 27, 42, 56). CP5 and CP8 have been used as targets for vaccine development, and specific antibodies against CP5 and CP8 have been shown to be protective against *S. aureus* infections (21, 28). The capsular polysaccharide produced in *S. aureus* NCTC8325 is CP5, which is encoded by the *cap* operon which contains 16 closely linked genes, *cap5A* through *cap5P*, transcribed in one orientation (49). Existing experimental evidence shows that the transcription of the *cap* operon in *S. aureus* NCTC8325 can be modulated by a range of regulatory elements, such as *yabJ-spoVG*, *arlRS*, *agr*, *sbcDC*, *ccpA*, *mgr*, *sae*, and *sarA* (17, 33–36, 51, 53). In addition, it has been indicated that the expression of CP in *S. aureus* can also be regulated by various environmental cues (27).

In bacteria, there are many two-component systems involved in the regulation of gene expression. In general, these two-component systems consist of two proteins, a sensor histidine kinase and a response regulator, and function by sensing the environmental signals and initiating phosphorelay cascades (6, 45). KdpD together with KdpE constitutes a two-component signal transduction system, which was first characterized in *E. coli*. In this organism, proteins KdpD and KdpE regulate the production of Kdp-ATPase, which is an inducible high-affinity K^+ transporter that is synthesized under conditions of severe K^+ limitation or osmotic upshift (2, 26). A BLAST search with Kdp protein sequences shows that the Kdp-ATPase system is widely distributed among bacteria (5). Recently, several lines of evidence have shown that the KdpDE system is involved in virulence in some bacteria. For instance, in *Mycobacterium tuberculosis*, deletion of *kdpDE* resulted in increased virulence. Mice infected with the *M. tuberculosis kdpDE* mutant died more rapidly than those infected with wild-type bacteria (44). Although several reports have shown that in *S. aureus* the transcript level of *kdpDE* changes under certain environmental stresses (exposure to neutrophil microbicides or growth under biofilm conditions) (11, 43), information about the role of KdpDE in *S. aureus* and how it functions remains incomplete.

In the present study, we investigated the role of the *S. aureus* LuxS/AI-2 system by construction and analysis of an allelic replacement mutant of *S. aureus* NCTC8325. Our results show that *luxS* regulates the gene transcription of CP5 in *S. aureus* NCTC8325 by the AI-2 QS pathway. In addition, we demonstrate that KdpE regulates the transcription of *cap* and acts as an important connector linking AI-2 quorum sensing to CP5 production and virulence.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are described in Table 1. *S. aureus* and *E. coli* were grown in Luria-Bertani (LB) or tryptic soy broth (TSB; soybean-casein digest medium USP; Oxoid) medium with the appropriate antibiotics for plasmid selection or maintenance (ampicillin at 100 mg liter^{-1} and kanamycin at 50 mg liter^{-1} for *E. coli*; erythromycin at 2.5 mg liter^{-1} and chloramphenicol at 15 mg liter^{-1} for *S. aureus*).

DNA manipulation. Genomic DNA of *S. aureus* NCTC8325 was prepared by a standard protocol for Gram-positive bacteria. Plasmid DNA was extracted using a plasmid purification kit (Promega), according to the manufacturer's instructions. *Taq* and *Pfu* DNA polymerases were purchased from Promega, and PrimeSTAR DNA polymerase was purchased from TaKaRa. Restriction enzymes were obtained from New England Biolabs. *S. aureus* was transformed by electroporation, as described previously (24).

Construction of *S. aureus* mutant strains. To construct the *luxS* and *kdpDE* deletion mutants from *S. aureus* NCTC8325, two sets of primers (Table 2) were used to amplify the upstream and downstream fragments (about 600 bp) of each target gene. The primer pairs were as follows: up-luxS-f-BamHI/up-luxS-r-SalI and down-luxS-f-PstI/down-luxS-r-HindIII for *luxS* deletion and up-kdpDE-f-BamHI/up-kdpDE-r-SalI and down-kdpDE-f-HindIII/down-kdpDE-r-XbaI for *kdpDE* deletion. The sequences of the amplified fragments were verified and cloned into shuttle plasmid pBT2, such that the upstream and downstream fragments flanked the erythromycin resistance gene amplified from pEC1 with the relevant primers (Em-f-PstI/Em-r-SalI for *luxS* deletion and Em-f-HindIII/Em-r-SalI for *kdpDE* deletion) and had the same orientation as they do in the chromosome to create pBTluxS and pBTkdpDE. For *kdpE* deletion, the *kdpE* gene was amplified from *S. aureus* NCTC8325 with primers DEL-kdpE-f-XbaI and DEL-kdpE-r-BamHI, and the fragment was inserted into a gene for Erm^r amplified from pEC1 with primers Em-f-NdeI and Em-r-HindIII and cloned into pBT2 to create pBTkdpE. The resulting plasmids were used for allele replacement, as described previously (15). All PCR primers used in this study were generated using the Primer (version 5.0) software and are listed in Table 2.

Complementation of mutants. The deleted genes and their promoters from *S. aureus* NCTC8325 were amplified by PCR with primers c-luxS-f-HindIII and c-luxS-r-EcoRI for *luxS* and primers c-kdpDE-f-EcoRI and c-kdpDE-r-BamHI for *kdpDE*. The PCR products were cloned into pLI50 (Addgene) to create plasmids pLIluxS and pLIkdpDE. For pLIkdpE construction, before it was cloned into pLI50, the *kdpE* gene fragment was amplified with primers g-kdpE-f-SmaI and g-kdpE-r-BamHI and ligated to the *kdpDE* promoter fragment (amplified with primers p-kdpDE-f-EcoRI and p-kdpDE-r-SmaI). Site-directed mutagenesis by PCR was used to delete the Asp phosphorylation site of the KdpE protein. For example, to create plasmid pLIkdpEM, plasmid pLIkdpE was used as the template for PCR, and the primers for constructing a deletion mutation were M-kdpEAsp-f and M-kdpEAsp-r. The DNA fragment (6.5 kb, containing the full length of pLIkdpE except the 3-bp nucleotide bases) was amplified by PCR with PrimeSTAR DNA polymerase. The PCR products were digested with DpnI, phosphorylated, self-ligated, and transformed into *E. coli* DH5α. The positive clones with mutational plasmids yielded pLIkdpEM. The plasmids were transformed by electroporation into *S. aureus* RN4220 and subsequently transferred to their mutant strains. The AI-2 precursor molecule, DPD, was purchased from Omm Scientific Inc., TX.

Measurement of AI-2 activity. The AI-2 activity of the culture supernatants was determined using the *V. harveyi* reporter strain BB170 as described previously (8, 55). Briefly, the supernatants were diluted 1:10 in fresh autoinducer bioassay medium containing *V. harveyi* BB170 (inoculated 1:5,000 from an overnight culture) to give a final volume of 200 μl. The culture was shaken (180 rpm) at 30°C, and the luminescence was determined at 30-min intervals for at least 8 h. AI-2 activity is reported as the fold induction of bioluminescence of the reporter strain over the bioluminescence of the background. The reported values represent the average bioluminescence stimulated by three independent preparations of each strain's supernatant.

Total RNA isolation, cDNA generation, and microarray processing. Overnight cultures of *S. aureus* were diluted 1:100 in LB medium and grown to the late exponential phase (optical density at 600 nm [OD_{600}] = 2.1). Cells were collected and resuspended in TE (Tris-EDTA) buffer (pH 8.0) containing 10 g liter^{-1} lysozyme and 40 mg liter^{-1} lysostaphin. After incubation at 37°C for 5 min, *S.*

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype	Reference or source
Strains		
S. aureus		
NCTC8325	Wild type	NARSA[a]
RN4220	8325-4 r^-	NARSA
SX1	8325 *luxS*::*ermB*	This study
SX2	8325 *luxS*::*ermB* pLIluxS	This study
SX8	8325 *kdpDE*::*ermB*	This study
SX9	8325 *kdpDE*::*ermB* pLIkdpDE	This study
SX10	8325 *kdpE*::*ermB*	This study
SX11	8325 *kdpE*::*ermB* pLIkdpE	This study
SX12	8325 *kdpE*::*ermB* pLIkdpEM	This study
E. coli		
DH5α	Clone host strain, *supE44* Δ*lacU169* (ϕ80d*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Laboratory stock
BL21	F^- *ompT* $hsdS_B(r_B^- m_B^-)$ *gal dcm*(DE3)	Laboratory stock
V. harveyi BB170	Sensor 1 negative, sensor 2 positive	B. L. Bassler
Plasmids		
pEasy-TB	Clone vector, Kan^r Ap^r	TransGen
pET28a(+)	Expression vector with hexahistidine tag, Kan^r	Novagen
pGkdpE	pET28a(+) with *kdpE*, Kan^r	This study
pEC1	pBluescript derivative, source of *ermB* gene, Ap^r	Bruckner
pBT2	Shuttle vector, temp sensitive, Ap^r Cm^r	Bruckner
pBTluxS	pBT2 containing 500-bp upstream and 500-bp downstream fragments of *luxS* and *ermB* gene, for *luxS* mutagenesis, Ap^r Cm^r Em^r	This study
pBTkdpDE	pBT2 containing 600-bp upstream and 600-bp downstream fragments of *kdpDE* and *ermB* gene, for *kdpDE* mutagenesis, Ap^r Cm^r Em^r	This study
pBTkdpE	pBT2 containing 600-bp upstream and 700-bp downstream fragments of *kdpE* and *ermB* gene, for *kdpE* mutagenesis, Ap^r Cm^r Em^r	This study
pLI50	Shuttle cloning vector, Ap^r Cm^r	Addgene
pLIluxS	pLI50 with *luxS* and its promoter, Ap^r Cm^r	This study
pLIkdpDE	pLI50 with *kdpDE* and its promoter, Ap^r Cm^r	This study
pLIkdpE	pLI50 with *kdpE* and the promoter of *kdp* operon, Ap^r Cm^r	This study
pLIkdpEM	pLIkdpE with a deletion mutation	This study

[a] NARSA, Network on Antimicrobial Resistance in *Staphylococcus aureus*.

aureus cells were prepared for total RNA extraction using the Trizol method (Invitrogen), and residual DNA was removed with DNase (RNase free; TaKaRa). cDNAs were synthesized and labeled according to the manufacturer's suggestions for *S. aureus* antisense genome arrays (Affymetrix Inc., Santa Clara, CA). Further preparation, hybridization, and scanning were conducted by Biochip Company of Shanghai, China. Real-time reverse transcription-PCR (RT-PCR) was performed with a PrimeScript 1st Strand cDNA synthesis kit and SYBR Premix Ex *Taq* (TaKaRa) using a StepOne real-time PCR system (Applied Biosystems). The quantity of cDNA measured by real-time PCR was normalized to the abundance of 16S cDNA. Microarray data were analyzed with Microarray Suite software, version 5.1 (Affymetrix Inc.), and a four-comparison survival method.

Purification of KdpE. The His_6-tagged KdpE was cloned and purified using standard procedures. The full-length *kdpE* gene fragment was amplified by PCR with primers g-kdpE-f-NcoI and g-kdpE-r-XhoI from *S. aureus* NCTC8325 genomic DNA, cloned into expression vector pET28a(+) (Novagen), and transformed into *E. coli* BL21. The transformant was grown in LB medium at 37°C to an OD_{600} of 0.4, transferred to 16°C, and induced overnight with 0.1 mM isopropyl-β-D-1-thiogalactopyranoside. Cells were harvested and lysed by sonication in lysis buffer (20 mM Tris-HCl, pH 8.0, 1 M NaCl). The His_6-tagged KdpE protein was purified with nickel-nitrilotriacetic acid agarose solution (Invitrogen). The column was washed with 100 volumes of washing buffer (20 mM Tris-HCl, pH 8.0, 1 M NaCl) containing a linear gradient of imidazole (5 mM to 100 mM). Bound protein was eluted with elution buffer (250 mM imidazole, 20 mM Tris-HCl, pH 8.0, 1 M NaCl, 10% glycerol). The imidazole in the eluant was removed by using a Centrifuge Biomax-5 column (Millipore), and then the KdpE protein solution was stored at −80°C until use. The purity of the protein was analyzed by SDS-PAGE, and the protein concentration was determined by the Bradford assay with bovine serum albumin as the standard.

Gel-shift assay. The DNA fragment containing the *cap* promoter was amplified from the *S. aureus* NCTC8325 chromosome with primers p-cap-f and p-cap-r by PrimeSTAR DNA polymerase (TaKaRa). The PCR products were purified and labeled using a digoxigenin (DIG) gel-shift kit (second generation; Roche), according to the manufacturer's instructions. The labeled fragment was incubated at 25°C for 15 min with various amounts of purified KdpE in 10 μl of incubation buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 3 mM magnesium acetate, 0.1 mM EDTA, 0.1 mM dithiothreitol). After incubation, the mixtures were added into 2.5 μl of gel loading buffer and were then electrophoresed in a 4% native polyacrylamide gel in 0.5× TBE (Tris-borate-EDTA) buffer. The band shifts were detected and analyzed according to the manufacturer's instructions.

***S. aureus* survival in human blood and in U937 monocytic cells.** Heparinized venous blood samples were collected from healthy donors who provided written consent to participate in the study. A previous study showed that *S. aureus* cells grown on solid medium generated more CP5 than *S. aureus* cells grown in broth (56). The strains were harvested from the TSB plates after they were cultured at 37°C for 16 h, washed twice in phosphate-buffered saline (PBS), and suspended to an optical density at 600 nm of 0.8. Heparinized human blood (1 ml) was inoculated with 1×10^6 CFU of *S. aureus* and was incubated at 37°C with shaking (250 rpm). A total of 5×10^6 U937 monocytic cells were mixed with 2×10^6 CFU of *S. aureus* opsonized with 10% normal human serum and incubated at 37°C under an atmosphere of 5% CO_2 with intermittent shaking. Bacterial cells were diluted to the appropriate concentration for testing at the required intervals, and the number of CFU was calculated from the plate counts determined in duplicate on TSB agar. The percentage of *S. aureus* CFU that survived was determined by comparing the bacterial burden in each sample after the indicated time with the bacterial burden at the start of the assay (0 h).

TABLE 2. Oligonucleotide primers used in this study

Primer name	Oligonucleotide (5′–3′)[a]
up-luxS-f-BamHI	GCGgatccTTGTTCGACTGCTTTTCTGA
up-luxS-r-SalI	GCGgtcgacGAATGTTGAAAGTTTCAATT
down-luxS-f-PstI	GCGctgcagCGCTTATGATTTGTTGCATTATAA
down-luxS-r-HindIII	GCGaagcttTATAGCTGGCCCGTCAAGTC
up-kdpDE-f-BamHI	GCGgatccTCTACTCTTGACGATTGCAC
up-kdpDE-r-SalI	GCGgtcgacATCTAAATATT
down-kdpDE-f-HindIII	ATTCaagcttTAGATAACGG
down-kdpDE-r-XbaI	GCGtctagaCACCAAATGTGGTGAGTATA
DEL-kdpE-f-XbaI	GCGtctagaTCAATTTGATTGAAAATGCA
DEL-kdpE-r-BamHI	GCGgatccAACGCATTTCTACAGAGTTG
Em-f-NdeI	GCGcatatgGATACAAATTCCCCGTAGGC
Em-f-PstI	GCGctgcagGATACAAATTCCCCGTAGGC
Em-f-HindIII	GCGaagcttGATACAAATTCCCCGTAGGC
Em-r-HindIII	GCGaagcttGAAATAGATTTAAAAATTTCGC
Em-r-SalI	GCGgtcgacGAAATAGATTTAAAAATTTCGC
c-luxS-f-HindIII	GCGaagcttAAAGCATCGTATTCTGCTAA
c-luxS-r-EcoRI	GCGgatccTGAAAAATACAATCAATCTA
c-kdpDE-f-EcoRI	GCGgaattcCATTGTTAGAAACAAAATTATTTC
c-kdpDE-r-BamHI	GCGgatccCCACCAAATGTGGTGAGTAT
p-kdpDE-f-EcoRI	GCGgaattcATATTTTCAAATCTAGTAAATATTA
p-kdpDE-r-SmaI	GCGcccgggAACCTTCACCTCGATAGCAA
g-kdpE-f-SmaI	GCGcccgggATGCAATCTAAAATATTGATAATT
g-kdpE-r-BamHI	GCGgatccTTATTTCTCTTTCCACTGCA
M-kdpEAsp-f	TTAGGTTTACCAGATAAAGATGGAT
M-kdpEAsp-r	TAATAAAATGACATCTGGTTTATCA
p-cap-f	TCTATCTGATAATAATCATCTAACT
p-cap-r	TATTTACCTCCCTTAAAAAT
g-kdpE-f-NcoI	GCGccatggTGCAATCTAAAATATTGATAATTGAAG
g-kdpE-r-XhoI	GCGctcgagTTTAATATCATCTCATAAGT
rt-16S-f	CGTGGAGGGTCATTGGA
rt-16S-r	CGTTTACGGCGTGGACTA
rt-capA-f	CAGTTAAAGTCGCACCAA
rt-capA-r	GAACCCAATACAGGCAAT
rt-capC-f	CGTCATTAGCGGGTATTT
rt-capC-r	TCTTGTTGTGGCATTCGT
rt-capE-f	TTTCCAGTTGAGGCAGTG
rt-capE-r	TTCTTGATTTGGCTACGA
rt-capG-f	TTGAAGTTCCCTGGTGTC
rt-capG-r	CATCGGTTCGTTATTGTT
rt-capI-f	AAGGAAGCGAATGTTAT
rt-capI-r	AGCTGATGAAGCAATAA
rt-capK-f	GCTGCCCTATGTTTAGTC
rt-capK-r	ATGTTATTGATGCGTGTT
rt-capN-f	GACCTCGTTCAAAGATTA
rt-capN-r	CATAGTTGGTCGTAGGGT
rt-kdpA-f	ATTGTTCGGTTTATTGTCC
rt-kdpA-r	CATCATACTGCCCATTTCT
rt-kdpD-f	AGTCCAGGTGTTGGTAAA
rt-kdpD-r	TATTGTCGGCGATTCTTC
rt-kdpE-f	CTACAGCCGACAATGCCACA
rt-kdpE-r	TGCCCGAAGCTCATCAACA
rt-rna3-f	GGTTATTAAGTTGGGATGG
rt-rna3-r	GAGTGATTTCAATGGCACA
rt-agrA-f	AAAGTTGCAGCGATGGATTT
rt-agrA-r	ATGGGCAATGAGTCTGTGAG
rt-sarA-f	GACATACATCAGCGAAAA
rt-sarA-r	TACGTTGTTGTGCATTAA
rt-saeR-f	AAGTGGCGACCATTACAT
rt-saeR-r	CATTATTGCCTCAAATACGT
rt-mgrA-f	AGTACAATCTAACATACC
rt-mgrA-r	TTGCGATAAAGAAGAAGC
rt-ccpA-f	TCGTGGACTTGAAGATAT
rt-ccpA-r	CCATTTGTTCCTGATACT
rt-arlR-f	GCTGGGCTTGATTACGGT
rt-arlR-r	GCGCCATTTACCGTCACT
rt-sbcD-f	TTTTATACACTCCCTTATGC
rt-sbcD-r	GATGTCTTTCCACCTTGA
rt-spoVG-f	ACTCTGGCTTGTTCGTTG
rt-spoVG-r	CTTCTGATGTAGCGTTTT

[a] The sequences in lowercase letters refer to the restriction endonuclease recognition sites.

Microarray data accession number. The microarray data were submitted to the CIBEX database (http://cibex.nig.ac.jp) with accession number CBX128.

RESULTS

The *luxS* gene is functional and is required for AI-2 production in *S. aureus*. *S. aureus* NCTC8325 genome sequence analysis indicated that the strain contained a putative *luxS* gene. To determine its ability to produce the signal molecule AI-2, supernatants from the *S. aureus* wild type and the *luxS* mutant were tested for AI-2 activity using *V. harveyi* reporter strain BB170. The AI-2 activity in the culture supernatant of the wild type was detectable from the mid-exponential phase onward, reaching maximum levels during the beginning of the stationary phase and then decreasing, whereas no AI-2 activity was detectable in the supernatant of the *luxS* mutant during any phase (Fig. 1). Meanwhile, the abolished AI-2 production pathway could be restored by a complementary plasmid containing *luxS* (data not shown). This suggests that LuxS is a key determinant in the AI-2 production process in *S. aureus*, and these results are consistent with those of a previous study of AI-2 production in *S. aureus* (19).

Identification of genes under LuxS/AI-2 regulation. To characterize the gene transcriptional profiles influenced by LuxS/AI-2, DNA microarray assays were performed using parental strain NCTC8325, the *luxS* mutant, and the *luxS* mutant with exogenous AI-2. Cells were grown in LB medium to an OD_{600} of 2.1. A 1.6-fold induction ratio as a cutoff limit was used to compare the transcriptional profiles between the wild type and the *luxS* mutant. Microarray data indicated that 34 genes were induced and 24 genes were repressed. Among these regulated genes, several major classes were associated with the metabolism, signal transduction, and virulence of *S. aureus*. Table 3 shows the main genes influenced by LuxS. Genes regulated by *luxS* are mainly associated with metabolism, which is in accord with the findings of a previous study, suggesting that LuxS plays a role in sulfur metabolism in *S. aureus* (19). Interestingly, genes related to the synthesis of the virulence determinant CP, composed of a *cap* gene cluster, were regulated by LuxS and AI-2. Meanwhile, the two-component system gene *kdpDE* was upregulated by *luxS* deletion and was restored by the addition of exogenous AI-2. We further analyzed the transcription of a set of genes by real-time RT-PCR, and the results corre-

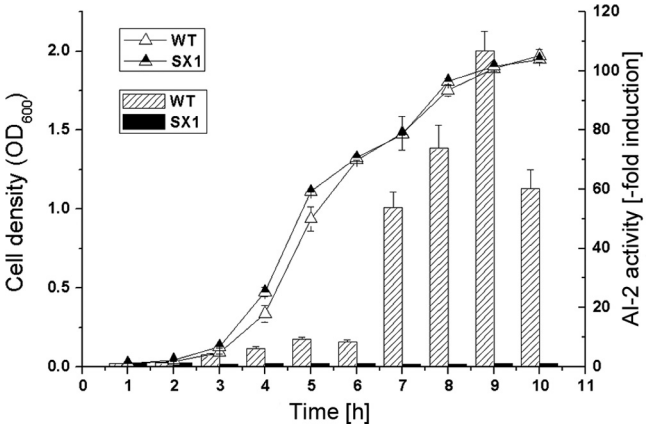

FIG. 1. Growth and extracellular AI-2 production. Cultures of *S. aureus* NCTC8325 wild type (WT) and the *luxS* mutant (SX1) were inoculated into LB medium at time zero and at the indicated intervals. Growth was monitored, and aliquots were taken and filtered to remove cells. AI-2 activity in the supernatant was measured using the *V. harveyi* BB170 bioassay. The data shown represent the mean values of four parallel AI-2 bioassays. The fold induction of AI-2 in the wild type exceeded 100 when the OD_{600} was 1.8.

TABLE 3. Main genes affected by LuxS and AI-2

Gene function and identifier	Gene product	Log_2 ratio	
		Mutant/WT	Mutant + AI-2/WT
Metabolism genes			
SAOUHSC_00898	Argininosuccinate lyase	−0.7	−0.6
SAOUHSC_01540	Prophage L54a, HNH endonuclease family protein	−1	−0.1
SAOUHSC_00311	Ascorbate-specific PTS[a] enzyme IIB	1	2.5
SAOUHSC_01532	SLT open reading frame 110-like protein	0.8	−0.1
SAOUHSC_01536	Scaffolding protease	1	0.4
SAOUHSC_02311	Potassium-translocating P-type ATPase, B subunit, putative	1.2	0.8
SAOUHSC_02374	HmrA, aminobenzoyl-glutamate utilization protein B, putative	2	1.9
SAOUHSC_02468	Acetolactate synthase	0.8	0
SAOUHSC_00310	Ascorbate-specific PTS enzyme IIC	2.5	2.7
SAOUHSC_00312	PTS IIA component	2.6	2.8
SAOUHSC_02933	Betaine aldehyde dehydrogenase	0.9	0.5
SAOUHSC_02173	Amidase, lytic enzyme	1.5	1.3
Membrane component genes			
SAOUHSC_00062	Integral membrane domain-containing protein	−0.9	−0.3
SAOUHSC_02821	Membrane-spanning protein, putative	−0.7	−0.6
SAOUHSC_01386	Phosphate ABC transporter, permease protein, putative	−0.9	−0.1
SAOUHSC_01084	Cell wall surface anchor protein	−0.7	−0.2
SAOUHSC_02310	KdpC, potassium-transporting ATPase, C subunit	0.9	0.6
SAOUHSC_02312	KdpA, potassium-transporting ATPase, A subunit	1.4	0.8
SAOUHSC_02864	Ferrous iron transport protein B	0.8	0.6
Regulator genes			
SAOUHSC_00915	Sua5/YciO/YrdC/YwlC family protein	−0.9	0.2
SAOUHSC_03046	Helix-turn-helix domain-containing protein	−0.7	−0.4
SAOUHSC_03046	Helix-turn-helix domain-containing protein	−0.7	−0.4
SAOUHSC_03046	Helix-turn-helix domain-containing protein	−0.7	−0.4
Virulence genes			
SAOUHSC_00399	Superantigen-like protein	−1	0.1
SAOUHSC_00433	Pathogenicity island protein	−0.8	−1.3
SAOUHSC_00384	Superantigen-like protein	−0.9	−0.7
SAOUHSC_00389	Superantigen-like protein	−0.9	−1.1
SAOUHSC_00121	CapH	0.9	0.7
SAOUHSC_00122	CapI	1.0	0.8
SAOUHSC_00123	CapJ	0.8	0.8
SAOUHSC_00124	CapK	1	0.9
SAOUHSC_00119	CapF	1	0.7
SAOUHSC_00120	CapG	1	0.6
SAOUHSC_00125	CapL	0.8	0.8
SAOUHSC_00126	CapM	0.9	0.9
SAOUHSC_00127	CapN	1	0.6
SAOUHSC_00117	CapD	0.7	0.3
SAOUHSC_00118	CapE	0.7	0.6
SAOUHSC_01705	Enterotoxin family protein	0.7	0.9
Hypothetical protein genes			
SAOUHSC_00817	Hypothetical protein	−0.8	−0.2
SAOUHSC_01295	Hypothetical protein	−0.7	−0.2
SAOUHSC_01296	Hypothetical protein	−0.7	−0.3
SAOUHSC_01297	Hypothetical protein	−0.9	−0.3
SAOUHSC_01533	Hypothetical protein	−1.2	0.8
SAOUHSC_02141	Hypothetical protein	−0.7	−0.7
SAOUHSC_00966	Hypothetical protein	−0.8	−0.2
SAOUHSC_02688	Hypothetical protein	−0.8	−0.5
SAOUHSC_00323	Hypothetical protein	−0.7	−0.1
SAOUHSC_00830	Hypothetical protein	−0.8	−0.3
SAOUHSC_02596	Hypothetical protein	−0.9	−0.6
SAOUHSC_00043	Hypothetical protein	−0.7	−0.5
SAOUHSC_01548	Hypothetical protein	0.9	1.3
SAOUHSC_02933	Hypothetical protein	0.8	1.1
SAOUHSC_02761	Hypothetical protein	0.8	1
SAOUHSC_01921	Hypothetical protein	0.7	0.5
SAOUHSC_00971	Hypothetical protein	0.7	0.8
SAOUHSC_02787	Hypothetical protein	0.7	0.3
SAOUHSC_02865	Hypothetical protein	0.7	0.3

[a] PTS, phosphotransferase system.

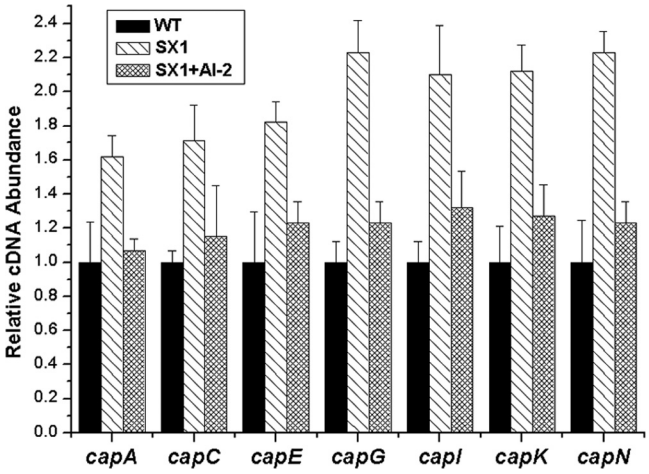

FIG. 2. Comparative measurement of transcription of CP synthesis-related genes. The relative transcript levels of the CP synthesis-related genes were determined in the *S. aureus* NCTC 8325 wild type (WT), the *luxS* mutant (SX1), and the *luxS* mutant with 39 nM AI-2 complementation (SX1 + AI-2). Strains were grown in LB medium with shaking at 37°C to an OD_{600} of 2.1. AI-2 was added to the *luxS* mutant at the inoculation time to a final concentration of 39 nM. The RNA was extracted and transcription was quantified by real-time RT-PCR for the CP synthesis-related genes (represented by *capA*, *capC*, *capE*, *capG*, *capI*, *capK*, and *capN*). The quantity of *cap* cDNA measured by real-time PCR was normalized to the abundance of 16S cDNA within each reaction mixture. Error bars indicate the variance between triplicate samples within the real-time PCR. The relative cDNA abundance in the wild-type samples was arbitrarily assigned a value of 1.

sponded to the microarray data (see Fig. S1 in the supplemental material).

***S. aureus* AI-2 signaling associated with the KdpDE system to regulate CP expression.** Both microarray and real-time RT-PCR data indicated that the transcript levels of the CP synthesis-related genes from *capA* to *capP* were increased in the *luxS* mutant and were restored by exogenous AI-2 (Fig. 2). However, the transcript levels of various regulatory elements known to modulate CP synthesis in *S. aureus*, such as *agrA*, *sarA*, *sbcDC*, *rnaIII*, *arlRS*, *ccpA*, *mgrA*, *saeRS*, and *spoVG*, displayed no apparent changes (see Fig. S2 in the supplemental material), suggesting that LuxS/AI-2 modulated *cap* gene transcription through another mechanism. Interestingly, the transcript levels of the two-component system genes *kdpD* and *kdpE* displayed the same tendency as *cap* gene transcription in these strains (see Fig. S2 in the supplemental material). To further determine whether this alteration was achieved by AI-2 signaling, the pre-AI-2 molecule DPD was used to complement the *luxS* mutant over a range of concentrations from 3.9 nM to 39 μM. The DPD threshold concentrations were 39 nM to 3.9 μM, and the transcript levels of both the *cap* gene and the *kdpDE* genes were restored in the *luxS* mutant under the threshold concentrations (Fig. 3A and B). These results strongly indicated that the lower transcription level of the *cap* and *kdpDE* genes in the wild type was a consequence of AI-2-mediated signaling. Meanwhile, these results indicated that the optimal DPD concentration has a direct link to the expression of KdpDE and CP in *S. aureus*. The correct concentrations of autoinducer may be critical for cells to make a proper

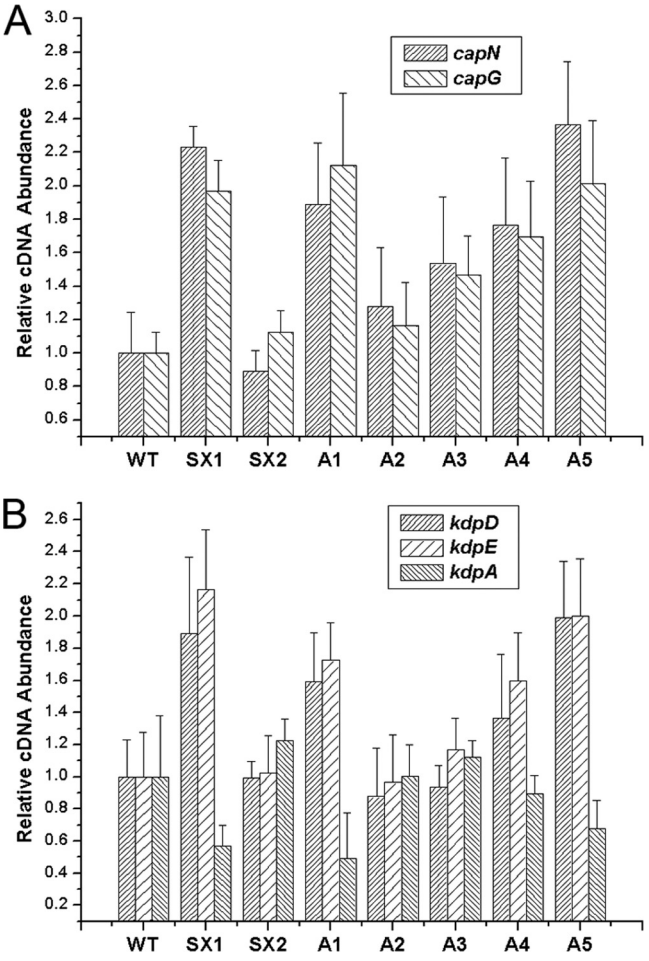

FIG. 3. Transcriptional regulation of the *cap* gene and *kdpDE* gene expression by LuxS/AI-2. (A) Relative transcript levels of the *cap* gene (represented by *capN* and *capG*). (B) Relative transcript levels of the *kdpD*, *kdpE*, and *kdpA* genes. The levels of transcription of these genes were measured by real-time RT-PCR in the *S. aureus* NCTC8325 wild type (WT), the *luxS* mutant (SX1), the *luxS* mutant with a plasmid containing *luxS* for genetic complementation (SX2), and the *luxS* mutant with 3.9 nM to 39 μM AI-2 for complementation (A1, 3.9 nM; A2, 39 nM; A3, 390 nM; A4, 3.9 μM; A5, 39 μM).

response, and reaching an appropriate AI-2 threshold level is important for the *luxS* mutant to restore the wild-type phenotype in *S. aureus*, which is consistent with the finding of other studies of AI-2 (1, 22, 48). On the other hand, *kdpA* transcription was inversely regulated by *kdpDE* by AI-2 quorum sensing (Fig. 3B), suggesting that KdpE may suppress KdpABC expression in *S. aureus* through the same pathway found in *E. coli*.

Inactivation of the two-component regulatory system gene *kdpDE* resulted in decreased transcript level of *cap*. The results presented above suggested that the KdpDE two-component system may mediate the AI-2 QS regulation of *cap* gene transcription. We then performed *kdpDE* mutational analysis to determine whether deletion of *kdpDE* would affect *cap* gene transcription. An allelic replacement mutant of *kdpDE* was constructed and found to have no obvious growth defects (data not shown). This strain was then tested for *cap* gene transcrip-

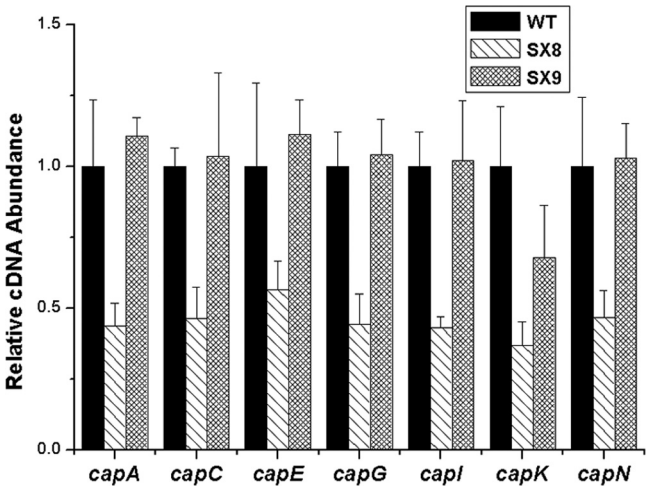

FIG. 4. Transcriptional regulation of CP synthesis by KdpDE. The relative transcript levels of CP synthesis-related genes (represented by *capA*, *capC*, *capE*, *capG*, *capI*, *capK*, and *capN*) were measured by real-time RT-PCR in the *S. aureus* NCTC8325 wild type (WT), the *kdpDE* mutant (SX8), and the *kdpDE* mutant with a plasmid containing *kdpDE* (SX9).

tion by real-time RT-PCR. The transcript levels of the *cap* genes were decreased in the *kdpDE* mutant compared to those in the wild type, and this decrease could be restored by genetic complementation (Fig. 4). These results suggest that the KdpDE system could affect *cap* gene transcription.

KdpDE-regulated *cap* transcription through the KdpE phosphorylation pathway. KdpD and KdpE are cotranscribed and belong to a family of sensor kinase and response regulator systems with no characterized function in *S. aureus*. In *E. coli*, they mediate the transcriptional activation of the *kdp* operon to control KdpFABC expression through phosphorylation of the signal transmission pathway. To further explore the mechanism of the involvement of KdpDE in *cap* gene transcription, we constructed the *kdpE* mutant strain and assessed the role of the phosphorylation site of KdpE in *cap* gene transcription. The results of real-time RT-PCR showed that deletion of *kdpE* resulted in the decreased transcription of *cap*, which could be restored by complementation with a plasmid with *kdpE*, whereas it could not be restored by the plasmid with *kdpE* whose phosphorylation site was defective (Fig. 5A), indicating that KdpE regulates the transcription of *cap* through a phosphorylation pathway. These observations showed for the first time that KdpDE functions as a two-component system in *S. aureus* and that KdpE relies on phosphorylation to function, just like other response regulators.

To further investigate whether KdpE regulates the transcription of *cap* directly, we performed a gel-shift assay. The purified His_6-tagged KdpE protein was used to bind the DIG-labeled fragment of the *cap* promoter. The purified KdpE protein bound to the *cap* promoter region in a dose-dependent fashion (Fig. 5B). The retarded protein-DNA complex was detected at KdpE concentrations of 0.5 to 4 μM. Unlabeled *cap* promoter DNA, added in excess (10-fold in a molar ratio) as a specific competitor, was found to reduce the formation of the protein-DNA complex. Meanwhile, retarded protein-DNA complex was not detected when the DIG-labeled random

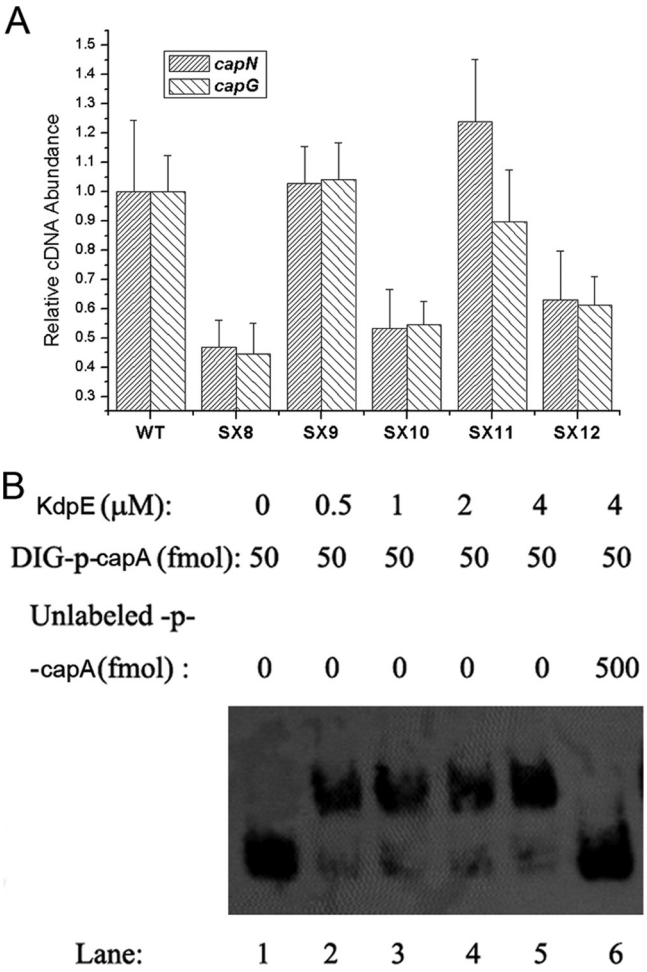

FIG. 5. Transcriptional regulation of *cap* gene expression by KdpE. (A) Relative transcript levels of the *cap* gene (represented by *capN* and *capG*) in the *S. aureus* NCTC8325 wild type (WT), the *kdpDE* mutant (SX8), the *kdpDE* mutant with a plasmid containing *kdpDE* (SX9), the *kdpE* mutant (SX10), the *kdpE* mutant with a plasmid containing *kdpE* (SX11), and the *kdpE* mutant with a plasmid encoding KdpE with a mutated Asp-phosphorylation site (SX12). (B) Binding of KdpE protein to the promoter of *cap*. The ability of KdpE to bind to the *cap* promoter was determined by gel-shift assay. Increasing amounts of KdpE were incubated with excess DIG-labeled probes. Lanes 1 to 6, KdpE concentrations of 0, 0.5, 1, 2, 4, and 4 μM, respectively; the amount of DIG-labeled probe in each case was 50 fmol (the concentration was 5 nM). In lane 6, besides the labeled probes, 500 fmol of unlabeled probes was incubated with the KdpE protein.

DNA sequence was used as a probe (data not shown). These results indicate that KdpE can specifically bind to the *cap* promoter region *in vitro*.

Absence of *luxS* increased *S. aureus* survival in human blood and human U937 monocytic cells, and absence of *kdpDE* attenuated *S. aureus* survival. Previous studies have shown that the expression of CP enhances bacterial virulence in a mouse bacteremia model or in macrophages owing to its antiphagocytic nature (42, 56) and that several two-component systems are upregulated by *S. aureus* during human neutrophil phagocytosis, including the KdpDE system (58). Therefore, we examined the effects of inactivation of *luxS*, the addition of AI-2

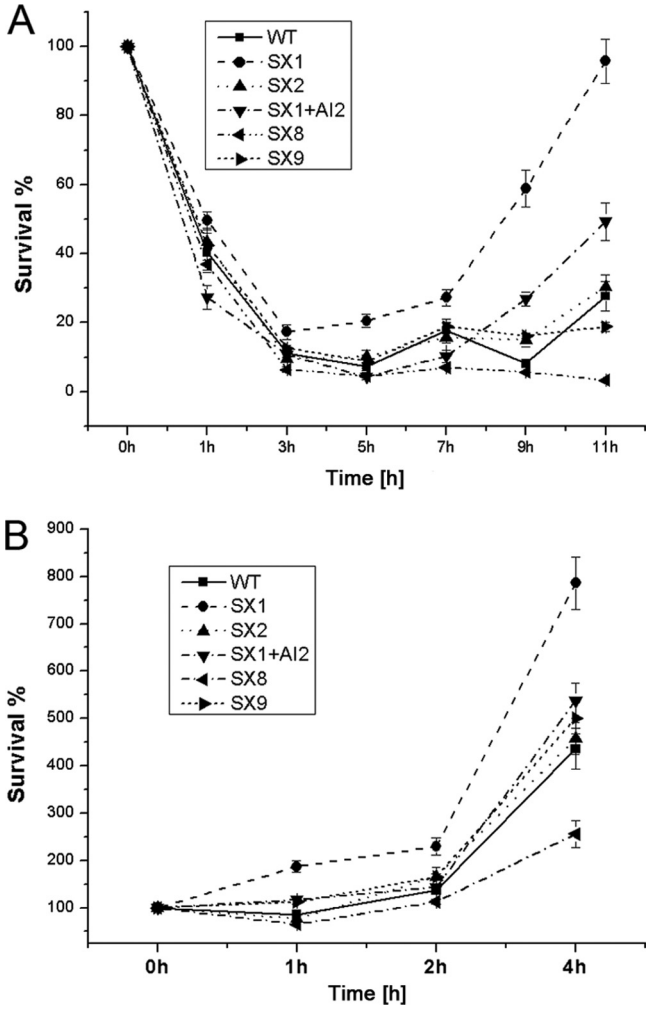

FIG. 6. Alteration of survival of *S. aureus* in human blood and in U937 monocytic cells due to absence of *luxS* or *kdpDE*. (A) Rates of survival for the *S. aureus* NCTC8325 wild type (WT), the *luxS* mutant (SX1), the *luxS* mutant with a plasmid containing *luxS* (SX2), the *luxS* mutant with 39 nM AI-2 (SX1 + AI-2), the *kdpDE* mutant (SX8), and the *kdpDE* mutant with a plasmid containing *kdpDE* (SX9). The strains were incubated in heparinized human blood, and the results are from five separate blood donors. (B) *S. aureus* survival when it was cultured with U937 monocytic cells. The percentage of *S. aureus* CFU that survived was determined as described in Materials and Methods.

to the *luxS* mutant, and the absence of *kdpDE* on the survival of *S. aureus* in human whole blood and human U937 monocytic cells. The survival and growth of the *luxS* mutant were higher when it was cultured in whole human blood (Fig. 6A), although there was no statistically significant difference in survival between the wild type, the *kdpDE* mutant, and several complemented strains during 5 h of incubation. After 7 h incubation, the rate of survival of the *luxS* mutant was significantly higher, whereas the rate of survival of the *kdpDE* mutant was reduced accordingly. After 11 h of incubation, the mean rate of survival ± standard deviation ($n = 4$) of the wild type was 27.6% ± 4.3%, that of the *luxS* mutant was 95.8% ± 6.4%, and that of the *kdpDE* mutant was 5.4% ± 3.3%. When these strains were cultured with human U937 monocytic cells, they demonstrated a tendency for survival similar to that in blood (Fig. 6B). Of importance, complementation of the *luxS* mutant with exogenous AI-2, the plasmid containing *luxS*, and the *kdpDE* strain restored the wild-type phenotype. These results indicated that the AI-2 signaling and the KdpDE system were involved in bacterial virulence in invasive *S. aureus* infection. The altered survival of *S. aureus* in human blood and monocytic cells correlated with the changes in the transcript levels of CP. The data further validated our hypothesis that *S. aureus* AI-2 quorum sensing regulates CP synthesis and virulence through the KdpDE two-component regulatory system.

DISCUSSION

AI-2 has been recognized to be a universal language for bacterial communication in recent years. However, knowledge of whether AI-2 serves as a signal molecule in Gram-positive bacteria, especially in *S. aureus*, remains elusive, mostly because no homologue of the known AI-2 receptor has been identified in the bacterium. A previous study has shown that the *luxS* gene in *S. aureus* was transcribed throughout growth and that AI-2 was produced in rich medium under aerobic and anaerobic conditions, peaking during the transition to the stationary phase (19). In addition, the *luxS* mutants exhibited a growth defect when they were grown in a limited medium, and this defect could be reduced only by transformation with the complementation vector rather than by using the AI-2 produced by this organism. No cross talk was displayed between the Agr and LuxS/AI-2 systems, and AI-2 did not exert a feedback effect on its own production in *S. aureus*. Furthermore, virulent phenotypic differences existed between the wild type and the *luxS* mutant. However, the parental phenotype could not be restored by the complementation vector or *in vitro*-synthesized AI-2, so they attributed this phenotype to second-site mutations. On the basis of these reasons, they drew the conclusion that LuxS played a role in metabolism but not QS. Undoubtedly, considering the metabolic role of LuxS in the activated methyl cycle, it was the complement vector but not AI-2 that restored the growth of *luxS* mutants in a sulfur-limited medium. However, it seems inaccurate to deny the QS function of AI-2 just because it cannot complement the virulent phenotypic alteration that is caused by second-site mutations. Our study demonstrated the involvement of LuxS in the regulation of CP production in *S. aureus* via a signaling process. Our data show that inactivation of *luxS* resulted in enhancement of the transcriptional level of the CP synthesis gene and resistance of this organism to uptake and killing by phagocytes. Of importance, exogenous AI-2 can complement these alterations, which indicated that the *luxS* mutation in *S. aureus* affected these phenotypes in an AI-2-dependent manner. These data lead us to conclude that the change in *cap* gene expression between the wild type and the *luxS* mutant could mainly be due to the role of AI-2 as a signal rather than due to the defect of methionine metabolism. Moreover, it is interesting to note that the complementation effect changed with the exogenous AI-2 concentrations, indicating that a concentration-sensing and -responding mechanism is involved, which is consistent with most QS processes.

Although it has been reported that the LuxS/AI-2 system plays important roles in cellular functions in a variety of bac-

teria, the detailed mechanism through which AI-2 functions has been investigated in only a few species. In *V. harveyi*, AI-2 detection and transduction rely on a two-component system. Borate–AI-2 is recognized by a soluble periplasmic protein that interacts with cytoplasmic response regulator proteins, thus triggering a sensor-kinase couple to finally control the expression of the luciferase structural operon (*luxCDABE*) (9, 31, 52). In *E. coli* and *S.* Typhimurium, AI-2 is first imported into the cell by the LsrR transporter, and then gene expression control is initiated (40, 55). In another Gram-positive bacterium, *Streptococcus mutans*, AI-2-regulated genes were identified, even though no homologue of the known AI-2 receptor has been recognized in that organism (37, 54). Our study demonstrated that LuxS/AI-2 can regulate cellular functions through a two-component system, KdpDE, whose role in *S. aureus* has not been well characterized. Inactivation of *luxS* results in a clear increase in the levels of transcription of *kdpD* and *kdpE*, which encode the sensor histidine kinase KdpD and the response regulator KdpE, respectively. Furthermore, gel-shift assays show that the regulatory protein KdpE can bind to the promoter region of the *cap* operon. These observations not only reveal that AI-2 regulates CP production through KdpDE but also provide new clues to the function of the KdpDE system in *S. aureus*. It is reported that a furanone could enhance staphylococcal biofilm formation by *luxS* repression (25), and we suppose that it may potentially involve the KdpDE two component system, although in that case the experimental evidence is missing. The exact mechanism by which LuxS/AI-2 interacts with KdpDE requires further study. Besides, knowledge of which forms of DPD are active in *S. aureus* remains elusive, since conversion between DPD derivatives is spontaneous and fast, making isolation or synthesis of each compound for biological activity testing almost impossible. Actually, explicit determination of the active form of DPD came only from biochemical and structural studies of various AI-2 receptors (16, 22, 40).

It is known that *S. aureus* normally produces surface proteins and polysaccharides during the exponential growth phase and secreted proteins during the stationary phase. In the invasive process, the surface proteins and polysaccharides are virulence determinants that are required to colonize host tissues and initiate the infection, and the secreted proteins are virulence factors that spread to adjacent tissues (46). Changes in the protein expression profile are controlled by a complicated regulatory network. In this study, we found that AI-2 has a negative effect on CP production in *S. aureus*, which leads us to propose that the LuxS/AI-2 QS system might participate in the downregulation of surface components after the colonization of host tissues.

As a major determinant of the virulence of *S. aureus*, it has been suggested that CP is regulated in a complicated way by a variety of factors in *S. aureus*, such as *spoVG*, *arlRS*, *agr*, *ccpA*, *mgr*, *sae*, and *sarA*. However, our results showed no significant differences in the transcriptional levels of these regulators between the *luxS* mutant and the wild type, indicating that the LuxS/AI-2 QS system does not merge with the characterized regulatory network of CP but might function in a novel and distinct pathway. It is known that the *agr* QS system regulates many virulence-associated traits in *S. aureus*. In contrast, according to our experimental data, the LuxS/AI-2 QS system exhibits clear involvement only in CP production (Fig. 7). We thus propose that the LuxS/AI-2 QS system might play a supplementary role in the overall QS process in *S. aureus*, although the details await further investigation.

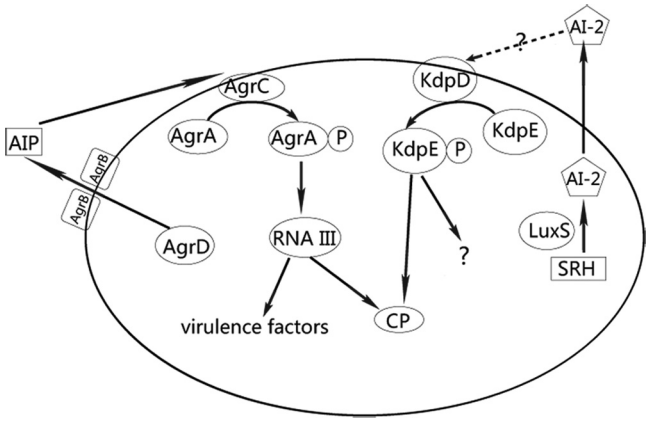

FIG. 7. The QS systems in *S. aureus*. The Agr QS system regulates many virulence-associated traits in *S. aureus*, and the LuxS/AI-2 QS system exhibits clear involvement in CP production.

ACKNOWLEDGMENTS

We thank our colleagues D. Hong, X. Zhang, R. Ma, and Y. You for their technical assistance. We thank the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) for providing the bacterial strains.

This work was supported by the National Natural Science Foundation of China (grants 30970118 and 30721002).

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Editor: A. Camilli