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Modulation of *ccrAB* Expression and SCC*mec* Excision by an Inverted Repeat Element and SarS in Methicillin-Resistant *Staphylococcus aureus*

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Methicillin-resistant *Staphylococcus aureus* (MRSA) is a notorious human pathogen that can cause a broad spectrum of infections. MRSA strains are resistant to almost the entire family of β-lactam antibiotics due to the acquisition of staphylococcal cassette chromosome *mec* (SCC*mec*). The chromosome cassette recombinases A and B, encoded by *ccrAB* genes located on SCC*mec*, play a key role in the excision of SCC*mec*. Studies have shown that *ccrAB* genes are expressed in only a minority of cells, suggesting the involvement of a subtle regulatory mechanism in *ccrAB* expression which has not been uncovered. Here, we found that an inverted repeat (IR) element, existing extensively and conservatively within the *ccrAB* promoter of different SCC*mec* types, played a repressive role in *ccrAB* expression and SCC*mec* excision in MRSA strain N315. Replacement of the IR sequence led to a significant increase in *ccrAB* expression and curing of SCC*mec* from strain N315 cells. In addition, we identified the transcriptional regulator SarS using DNA-affinity chromatography and further demonstrated that SarS can bind to the IR sequence and upregulate *ccrAB* expression and SCC*mec* excision. These findings reveal a molecular mechanism regulating *ccrAB* expression and SCC*mec* excision and may provide mechanic insights into the lateral transfer of SCC*mec* and spread of antibiotic resistance in *S. aureus*.

Staphylococcus aureus is a bacterial pathogen that can cause a variety of infectious diseases, ranging from superficial skin infections to life-threatening septicemia, osteomyelitis, and toxic shock syndrome (1). Worldwide emergence and spreading of antibiotic-resistant *S. aureus* pose serious challenges to chemotherapeutic treatment, which were emphasized for many years by methicillin-resistant *Staphylococcus aureus* (MRSA) strains. MRSA strains are resistant to almost the entire class of β-lactam antibiotics due to the acquisition of the staphylococcal cassette chromosome *mec* (SCC*mec*) (2–4). As a mobile genetic element, SCC*mec* can be excised from and integrated into the bacterial chromosome (5), which may subsequently lead to the transfer of methicillin resistance among staphylococcal strains. The phenotype of MRSA is exerted by an alternative penicillin-binding protein, PBP2a, encoded by the *mecA* gene (6), which exhibits a much lower affinity to β-lactam antibiotics than PBP2 (7). The *mecA* gene is located on SCC*mec* and is regulated by a three-component regulator system, *mecR1-mecI-mecR2* (8), which is also located on SCC*mec* within the 3′ end of the *orfX* gene coding for 23S rRNA methyltransferase (4, 9).

Another important gene cluster located on SCC*mec* is cassette chromosome recombinase genes *ccrAB* or *ccrC*, which code for large serine recombinases of the resolvase/invertase family (5, 10). CcrA, CcrB, and CcrC are three phylogenetically distinct proteins and play a key role in the site-specific excision of SCC*mec* (11). While *ccrAB* are always present together and exist in most SCC*mec* types, *ccrC* exists in only a minority of SCC*mec* types. Interestingly, CcrAB recombinases can excise most SCC*mec* types (12), whereas CcrC only plays a role in the excision of a certain type of SCC*mec* that encodes it (10). CcrB can bind to the specific sites *attS* and *attB* and can interact with CcrA (13), enabling the recognition of a recombination site without an inverted repeat and mediating SCC*mec* transfer (11, 14). According to the combinations of *mec* gene complex classes and *ccr* gene complex types, 11 types of SCC*mec* have been identified to date (15).

Spontaneous SCC*mec* excision has been observed on different occasions at low frequency (12, 16, 17). Although different studies have shown that treatment, such as heating or antibiotic stress, may have effect on *ccrAB* expression (18, 19), the regulatory mechanism controlling *ccrAB* expression remains largely unknown. The expression level of *ccrAB* remains low under normal growth conditions (18), and *ccrAB* is expressed in only a minority of cells (19), suggesting the existence of a subtle regulatory system. In *S. aureus*, recent years have seen the findings of complex regulatory networks and novel regulatory factors, such as two-component systems (20), regulatory RNAs (21), Sar protein families (22), and alternative sigma factors. Although it has been predicted that the expression of *ccrAB* was regulated by genetic elements outside SCC*mec* (19), transcriptional regulators controlling *ccrAB* expression have not been identified.

In this study, we identified an inverted repeat (IR) element that is located on almost the same site upstream of the *ccrA* translation

Received 30 April 2015 **Returned for modification** 13 June 2015
Accepted 18 July 2015

Accepted manuscript posted online 27 July 2015

Citation Zhang S, Ma R, Liu X, Zhang X, Sun B. 2015. Modulation of *ccrAB* expression and SCC*mec* excision by an inverted repeat element and SarS in methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 59:6223–6232. doi:10.1128/AAC.01041-15.

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/AAC.01041-15.

doi:10.1128/AAC.01041-15

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference[b]
S. aureus strains		
RN4220	8325-4, r^-, initial recipient for modification of plasmids which are introduced into *S. aureus* from *E. coli*	NARSA
N315	HA-MRSA, SCC*mec* type II	NARSA
N315Δ*sarS*	N315 strain deletion of *sarS*	This study
E. coli strains		
*Trans*1-T1	Clone host strain; F^- ϕ80(*lacZ*) ΔM15 Δ*lacX74 hsdR* (r_K^- m_K^+) Δ*recA*1398 *endA1 tonA*	TransGen
BL21	Expression strain; F^- *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm*(DE3)	TransGen
Plasmids		
pEASY blunt	Commercial TA cloning vector, Amp^r Kan^r	TransGen
pOS1	Shuttle vector, with *lacZ* ORF lacking first 6 amino acids, Amp^r Chl^r	23
pOS*ccrAB*	POS1 derivative, harboring 350-bp region of *ccrAB* promoter and 18 bp of *ccrA* coding sequence from strain N315, Amp^r Chl^r	This study
pOS*ccrAB*IR1	POS1 derivative, harboring 350-bp region of *ccrAB* promoter with upstream region of the IR replaced and 18 bp of *ccrA* coding sequence, Amp^r Chl^r	This study
pOS*ccrAB*IR2	POS1 derivative, harboring 350-bp region of *ccrAB* promoter with downstream region of the IR replaced and 18 bp of *ccrA* coding sequence, Amp^r Chl^r	This study
pBTs	Shuttle vector, temp sensitive, Amp^r Chl^r	27
pBTsΔIR1	pBTs derivative, for IR sequence substitution of *ccrAB* promoter in N315 chromosome, Amp^r Chl^r	This study
pBTsΔ*sarS*	pBTs derivative, for *sarS* deletion in strain N315, Amp^r Chl^r	This study
pET28a(+)	Expression vector with a hexahistidine tag, Kan^r	Novagen
pE*sarS*	pET28a(+) derivative, with ORF of *sarS* from strain N315, Kan^r	This study
pLI50	Shuttle vector, Amp^r Chl^r	26
pLI*ccrAB*	pLI50 derivative, harboring ORF of *ccrAB* and its promoter from strain N315, for *ccrAB* overexpression, Amp^r Chl^r	This study
pLI*ccrAB*-IR1	pLI50 derivative, harboring ORF of *ccrAB* and its promoter from strain N315 with partial substitution of the IR sequence, Amp^r Chl^r	This study
pLI*sarS*	pLI50 derivative, harboring ORF of *sarS* and its promoter from strain N315, for continuous *sarS* overexpression; Amp^r Chl^r	This study
pRMC2	Shuttle vector, anhydrotetracycline inducible, Amp^r Chl^r	25
pRMC*sarS*	pRMC2 derivative, with ORF of *sarS* from strain N315, Amp^r Chl^r	This study

[a] r^-, restriction system negative; Kan^r, kanamycin resistant; Amp^r, ampicillin resistant; Chl^r, chloramphenicol resistant.
[b] NARSA, Network on Antimicrobial Resistance in *Staphylococcus aureus*.

initiation site of different SCC*mec* types through multiple DNA sequence alignment of various *ccrAB* promoter sequences. Further study indicated that the IR sequence played a repressive role in *ccrAB* expression and SCC*mec* excision. Partial nucleotide substitution of the IR sequence resulted in a significant increase in *ccrAB* expression and curing of SCC*mec* from strain N315 cells. Furthermore, a *ccrAB* promoter-specific binding protein, SarS, was identified by DNA affinity chromatography, and an electrophoretic mobility shift assay (EMSA) further showed that SarS can bind to the IR sequence. *In vivo* experiments indicated that SarS upregulated *ccrAB* expression and enhanced SCC*mec* excision.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *S. aureus* strains were grown with aeration (220 rpm) in tryptic soy broth (TSB; Difco) or on tryptic soy agar plates (TSA; Difco) at 37°C. *Escherichia coli* was grown with aeration (220 rpm) in lysogeny broth medium (LB; Franklin Lakes) or on lysogeny broth agar (LA) at 37°C. When needed, 100 μg/ml ampicillin sodium salt or 50 μg/ml kanamycin sulfate for *E. coli* and 15 μg/ml chloromycetin for *S. aureus* strains were supplemented in cultivation.

Construction of LacZ reporter plasmids. To construct the reporter plasmid pOS*ccrAB* for detection of *ccrAB* expression, a fragment containing the 350-bp promoter of *ccrAB* and the first 18-bp region of the *ccrA*-coding sequence was amplified from strain N315 genomic DNA with primers p*ccrA*-f and p*ccrA*-r. The fragment was digested with BamHI/EcoRI and cloned into the upstream region of the reporter gene *lacZ* of the shuttle vector pOS1 (23). To construct the reporter plasmid pOS*ccrAB*IR1, in which GCCCAACATC was replaced with CGGGAAGATG in the upstream region of the IR sequence for stem-loop structure destruction, the upstream fragment was amplified with primers p*ccrA*-f and p*ccrA*-IR1s-r, and the downstream fragment was amplified with primers p*ccrA*-IR1s-f and p*ccrA*-r. The two fragments were ligated by SLiCE (24). Briefly, the two fragments were designed with 20-bp overlaps, and each fragment (50 to 200 ng) was mixed at a molar ratio of 1:1, and then 1 μl of 10× SLiCE buffer (500 mM Tris-HCl, 100 mM $MgCl_2$, 10 mM dithiothreitol [DTT], pH 7.5), 1 μl of SLiCE extract, 1 μl of 10 mM ATP, and double-distilled water (ddH_2O) were added to a final volume of 10 μl. The mixed solution was incubated at 37°C for 1 h and then amplified with primers p*ccrA*-f and p*ccrA*-r. The resultant fragment was digested with BamHI/EcoRI and cloned into pOS1. To construct the reporter plasmid pOS*ccrAB*IR2 in which GATGTTGGGC was replaced with CATCTTCCCG in the downstream region of the IR sequence, the upstream fragment was amplified with primers p*ccrA*-f and p*ccrA*-IR2s-r, and the downstream fragment was amplified with primers p*ccrA*-IR2s-f and p*ccrA*-r. The fragment ligated by SLiCE also was amplified with primers p*ccrA*-f and p*ccrA*-r and then digested with BamHI/EcoRI and cloned into pOS1. These reporter plasmids were first transformed into strain RN4220 and then strain N315 or its *sarS* deletion mutant strain for detection of *ccrAB* expression.

Construction of overexpression plasmids. To construct the inducible *sarS* overexpression plasmid, the fragment covering *sarS* and its ribo-

TABLE 2 Primers used in this study

Primer	Sequence[a] (5′-3′)	Application or reference
RT-*ccrA*-f	GCACAGTTATTAGAAGAAGATA	qRT-PCR
RT-*ccrA*-r	GCCATATTGATTGTTGACA	qRT-PCR
RT-*hu*-f	AAAAAGAAGCTGGTTCAGCAGTAG	qRT-PCR
RT-*hu*-r	TTTACGTGCAGCACGTTCAC	qRT-PCR
sarS-f	GCGgtaccTGTGTTATATTAAAATAAAGTGCATATACAAGGAGATAAAAC	pRMC*sarS*
sarS-r	GCGgtaccATGGTTTTAAGTGATATAGAAA	pRMC*sarS*
Up-*sarS*-f	CACTCATCGCAGTGCAGCGGAATTGAGCTTGAGAGTCATTAAGTT	pBTsΔ*sarS*
Up-*sarS*-r	AAGTCAAGCCTGAAGTCGAACAATCCACCATAAATACCC	pBTsΔ*sarS*
Down-*sarS*-f	CGACTTCAGGCTTGACTT	pBTsΔ*sarS*
Down-*sarS*-r	GACTCTAGAGGATCCCCCGGGTACCTGTTGTGCCGTTGTTAT	pBTsΔ*sarS*
oe-*sarS*-f	CGCggatccTTAGCTAAATGATTCAGTTA	pLI*sarS*
oe-*sarS*-r	CCCaagcttATGGTTTTAAGTGATATAGAAA	pLI*sarS*
p*ccrA*-r-biotin	ATCGGCTCCTCCTTTCACAG	Pulldown assay
p*ccrA*-fw	TTGTCTTTATCATACAACCG	Pulldown assay
gdpS-f	TAGTTCGAACATTTATATACAATA	Pulldown assay
gdps-r-biotin	GGATAGGGTAGACAGATAATAATAATGATACGATTGTCATTAAAACAGTGACATAAGCC	Pulldown assay
e-sarS-f	ATAAGAATgcggccgcAATGAAATATAATAACCATGACA	pE*sarS*
e-sarS-r	CCGctcgagTTATTCAAAAACAAGATGTA	pE*sarS*
oe-*ccrAB*-f	GCGgatccCCAAACAACTAAAAGAAGTG	pLI*ccrAB*
oe-*ccrAB*-r	GCGgatccCCTTCTGCTTCTTCGAATCT	pLI*ccrAB*
p*ccrA*-r	GCGgatccGGGCCTATGACTTGTTTCATAT	pOS*ccrAB*
p*ccrA*-f	GCGgaattcAAAGATAAGTCGTTAATTCA	pOS*ccrAB*
p*ccrA*-IR1s-f	AATAACATCTTCCCGGTTCATATTAGTCATTCATTTG	pOS*ccrAB*IR1, pBTsΔIR1, pLI*ccrAB*-IR1
p*ccrA*-IR1s-r	TGAACCGGGAAGATGTTATTGATGTTGGGCTTATATATCAATTCATCCATAAATATATA	pOS*ccrAB*IR1, pBTsΔIR1, pLI*ccrAB*-IR1
p*ccrA*-IR2s-f	AATAAGATGTTGGGCGTTCATATTAGTCATTCATTTG	pOS*ccrAB*IR2
p*ccrA*-IR2s-r	TGAACGCCCAACATCTTATTCATCTTCCCGTTATATATCAATTCATCCATAAATATATA	pOS*ccrAB*IR2
up-IR1-f	GCGgtaccGCGACCAATGCTGACAATAT	pBTsΔIR1
down-IR1-r	GCGgaattcACAGACGTAGTAACGTAATG	pBTsΔIR1
ccrA-IR sense	AATATGAACGCCCAACATCTTATTGATGTTGGGCTTATAT	EMSA
ccrA-IR antisense	ATATAAGCCCAACATCAATAAGATGTTGGGCGTTCATATT	EMSA
cL1	ATTTAATGTCCACCATTTAACA	5
cR1	AAGAATTGAACCAACGCATGA	5
mL1	GAATCTTCAGCATGTGATTTA	5
HVR1	ACTATTCCTCAGGCGTCC	SCC*mec* detection
HVR2	GGAGTTAATCTACGTCTCATC	SCC*mec* detection
*mecA*1	GTAGTTGTCGGGTTTGGT	*mecA* detection
*mecA*2	GGTATCATCTTGTACCCA	*mecA* detection
SPA1	ATCTGGTGGCGTAACACCTG	*spa* typing
SPA2	CGCTGCACCTAACGCTAATG	*spa* typing

[a] Lowercase letters indicate restriction sites. Underlined letters indicate overlap sequences used for SLiCE.

some binding site was amplified from strain N315 genomic DNA with primers *sarS*-f and *sarS*-r (Table 2). The fragment was digested with KpnI and cloned into the shuttle vector pRMC2 (25) to obtain pRMC*sarS*. To construct the *ccrAB* overexpression plasmid pLI*ccrAB*, a fragment covering *ccrAB* and the promoter was amplified from strain N315 genomic DNA with primers oe-*ccrAB*-f and oe-*ccrAB*-r. The fragment was digested with BamHI and cloned into the shuttle vector pLI50 (26). To create the *ccrAB* overexpression plasmid pLI*ccrAB*-IR1, in which the upstream region of the IR sequence was replaced with the same nucleotides as those of pOS*ccrAB*IR1, the upstream fragment was amplified with primers oe-*ccrAB*-f and p*ccrA*-IR1s-r, and the downstream fragment was amplified with primers p*ccrA*-IR1s-f and oe-*ccrAB*-r. The two fragments were ligated by SLiCE as described above, and the ligated fragment was amplified with primers oe-*ccrAB*-f and oe-*ccrAB*-r, and then the fragment was digested with BamHI and cloned into pLI50. To create the *sarS* overexpression plasmid pLI*sarS*, the fragment containing *sarS* and its promoter was amplified from strain N315 genomic DNA with primers oe-*sarS*-f and oe-*sarS*-r, digested with BamHI/HindIII, and cloned into pLI50.

Construction of recombinant strains. To create the *sarS* and IR sequence mutant strains without extra genes introduced, the plasmid pBTs was used as previously described (27). To construct the *sarS* mutant plasmid pBTsΔ*sarS*, the upstream and downstream regions of *sarS* were amplified from strain N315 genomic DNA with primer pairs up-*sarS*-f/up-*sarS*-r and down-*sarS*-f/down-*sarS*-r (Table 2). The two fragments were ligated by SLiCE to form an up-down fragment and then cloned into the KpnI/EcoRI-digested plasmid pBTs by SLiCE. To construct the IR mutant plasmid pBTsΔIR1, in which the upstream region of the IR sequence was replaced with the same nucleotides as those of pOS*ccrAB*IR1, the upstream and downstream regions were amplified from strain N315 genomic DNA with primer pairs up-IR1-f/p*ccrA*-IR1s-f and p*ccrA*-IR1s-r/down-IR1-r (Table 2). The two fragments were ligated by SLiCE, and the resultant fragment was digested with EcoRI/KpnI and then cloned into pBTs with T4 ligase. These plasmids were first introduced into strain RN4220 for modification and subsequently transformed into strain N315. The mutant strains were screened using a previously described method (28) and were further confirmed by PCR and sequencing.

β-Galactosidase assay. For LacZ activity assay with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) as the substrate, stationary-phase cultures of strain N315 containing different LacZ reporter plasmids were diluted 1:100 into TSB (2 ml) with chloromycetin (15 μg/ml) and X-Gal (40 μg/ml). The photos were taken after cultivated for 24 h, 36 h, 48 h, and 60 h, respectively.

For β-galactosidase assay with *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as the substrate, stationary-phase cultures of strain N315 containing different LacZ reporter plasmids were diluted 1:500 into TSB (10 ml) with chloromycetin (15 μg/ml), and cells were collected after incubation at 37°C for 6 h, 12 h, 18 h, and 24 h, respectively. First, cells were washed once with ABT buffer (60 mM K_2HPO_4, 40 mM KH_2PO_4, 100 mM NaCl, 0.1% Triton X-100) and then resuspended in 100 μl of ABT-LSA buffer (ABT buffer with 50 μg/ml lysostaphin). Cells were treated under shaking conditions at 37°C until thoroughly lysed. The galactosidase activity analysis was performed according to the method described previously (29).

Oxacillin susceptibility analysis. The oxacillin resistance of strain N315 and its IR mutant strains was analyzed with diffusion disks containing 1 mg of oxacillin at 37°C, and population analysis profiles were determined as previously described (30).

DNA pulldown assay. DNA pulldown assay was performed as previously described (31, 32), with minor modifications. Briefly, the biotin-labeled DNA fragment containing the 233-bp promoter region of *ccrAB* was amplified from strain N315 genomic DNA using primers p*ccrA*-r-biotin and p*ccrA*-fw. The control DNA fragment of *gdpS*-coding sequence was amplified with primers *gdps*-r-biotin and *gdpS*-f. Stationary-phase cultures of strain N315 were diluted 1:500 into TSB (20 ml). Cells were cultivated for 6 h and then collected and washed twice with lysis buffer (10 mM HEPES, 10 mM $MgCl_2$, 200 mM NaCl, 1 mM DTT, 1% Triton X-100, pH 7.0) and resuspended in lysis buffer (10 ml) with lysostaphin (10 mM HEPES, 10 mM $MgCl_2$, 200 mM NaCl, 1 mM DTT, 1% Triton X-100, 40 U/ml lysostaphin, 10 μg/ml DNase I, pH 7.0) under shaking conditions at 37°C for 15 min until thoroughly lysed. The lysate was centrifuged at 12,000 × *g* at 4°C for 40 min to remove insoluble debris, and the supernatant was concentrated to 2 ml with a Centrifuge Biomax-5 column (Millipore).

The prepared biotin-labeled DNA (25 μg) of the *ccrAB* promoter or control *gdpS* DNA was coupled to 0.9 ml of streptavidin-coated magnetic beads (Promega) with the same volume of 2× B/W buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 2 M NaCl) at room temperature. After incubation for 1 h, the beads were washed with lysis buffer 3 times to create an atmosphere suitable for protein binding. The supernatant (500 μl) with 10 μg/ml of poly(dI-dC) was added to the DNA-coated beads and incubated at 4°C for 1 h. The combination of supernatant and DNA-coated beads was repeated once to increase the amount proteins obtained. Beads were washed 5 times with lysis buffer (500 μl) containing 10 μg/ml of poly(dI-dC) and twice with lysis buffer (500 μl), and then it was supplemented with ddH_2O (70 μl) and incubated at 70°C for 10 min. Samples were segregated by SDS-PAGE and detected by silver staining. The specific bands different from the *gdpS* control were excised and in-gel digested with trypsin (0.6 mg). The tryptic peptides were subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis with an LTQ mass spectrometer (ProteomeX-LTQ; ThermoFisher Scientific). Sequence and peptide fingerprint data were analyzed using the NCBI database.

Expression and purification of SarS. The full-length open reading frame (ORF) of *sarS* was amplified from strain N315 genomic DNA using primers e-sarS-f and e-sarS-r (Table 2) and then cloned into the expression vector pET28a(+) (Novagen) to obtain pE*sarS*. The plasmid was transformed into *E. coli* BL21(DE3), and SarS was expressed with the induction of isopropyl-β-D-1-thiogalactopyranoside (1 mM) at 30°C for 3 h when the culture was grown to an optical density at 600 nm (OD_{600}) of 0.4 to 0.6. The cells then were harvested, resuspended in protein binding buffer (20 mM Tris-HCl, 200 mM NaCl, pH 8.0), and lysed by sonication on ice. SarS was purified using a nickel nitrilotriacetic acid agarose solution (Novagen) with its N-terminal His tag according to the manufacturer's instructions. The bound protein was eluted with elution buffer (20 mM Tris-HCl, 200 mM NaCl, 200 mM imidazole, pH 8.0). The imidazole in the eluent was removed using a Centrifuge Biomax-5 column (Millipore) with the incubation buffer (50 mM Tris-HCl, 200 mM NaCl, pH 8.0), and the protein was stored at −80°C until use. The protein purity was analyzed using SDS-PAGE, and the protein concentration was determined using the bicinchoninic acid (BCA) assay with bovine serum albumin as the standard. The final concentration of purified SarS was 53 μM, which was further used for EMSA.

EMSA. The biotin-labeled DNA fragment containing the promoter region of *ccrAB* was obtained in the same way as that used for the pulldown assay. The probe containing the IR sequence was obtained through primer fusion of *ccrA*-IR-sense and *ccrA*-IR-antisense (Table 2) and then labeled using a digoxigenin (DIG) gel shift kit (Roche). For EMSA, the biotin-labeled promoter of *ccrAB* (3.5 fmol) or the DIG-labeled IR sequence (10 fmol) was incubated at 25°C for 30 min with various amounts of SarS in 8 μl of incubation buffer (50 mM Tris-HCl, 200 mM NaCl, 0.1 mM EDTA, pH 8.0). After incubation, the mixtures were electrophoresed in a 5% or 8% native polyacrylamide gel in 1× Tris-borate-EDTA (TBE) buffer and then transferred to a nylon membrane in 0.5× TBE buffer. The band shifts were detected and analyzed according to the manufacturer's instructions. The images were obtained using an ImageQuant LAS 4000 mini (GE, Piscataway, NJ). The unlabeled fragment of the promoter region was used as a specific competitor at a ratio of approximately 100:1, 50:1, or 500:1 to the labeled fragment. The unlabeled *hu* fragment was added as a nonspecific competitor.

Total RNA extraction and real-time quantitative reverse transcription-PCR (qRT-PCR). For total RNA isolation from strain N315 with the *sarS* overexpression plasmid pRMC*sarS*, stationary-phase culture of strain N315 was diluted 1:100 into TSB (10 ml) with chloromycetin (15 μg/ml). Following cultivation for 3 h, cells were treated with anhydrotetracycline at a final concentration of 200 ng/ml for different periods of time and then collected. For total RNA isolation from wild-type strain N315 and the *sarS* mutant strain, stationary-phase cultures were diluted 1:500 into TSB (10 ml), and the cells grown to different growth phases were collected. The collected cells were immediately treated with 1 ml of RNAiso plus (TaKaRa) and lysed with 0.1-mm-diameter-silica beads in a FastPrep-24 automated system (MP Biomedicals). Total RNA was isolated by following the instructions of RNAiso plus, and residual DNA was removed with RNase-free DNase I (TaKaRa).

For reverse transcription, cDNA was synthesized using a PrimeScript first-strand cDNA synthesis kit (TaKaRa), and qRT-PCR was performed with SYBR *Ex Taq* premix (TaKaRa) using the StepOne real-time PCR system (Applied Biosystems). The cDNA quantity measured by real-time PCR was normalized to the abundance of *hu* cDNA (33). All qRT-PCR assays were repeated at least 3 times.

Determination of the SCC*mec* excision frequency and the existence of SCC*mec*. To determine the SCC*mec* excision frequency, strains carrying pLI*ccrAB* or pLI*ccrAB*-IR1 were cultivated in brain heart infusion (BHI) broth at 37°C for 24 h, cells were collected, and genomic DNA was extracted. The excision frequency was determined by PCR as described previously (34), and primers from two sides of *attB* (cL1 and cR1) were used to determine the ratio of the SCC*mec*-excised populations. Primers with one primer within SCC*mec* and the other primer in the strain N315 chromosome (mR1 and cL1) were used to determine the ratio of SCC*mec*-retained populations.

In order to determine the SCC*mec* excision frequency during consecutive cultivation, the strain transformed with pLI*sarS* was cultivated in BHI broth in consecutive passages at 37°C for 15 days. Cells were collected every 3 days and genomic DNA was extracted. The excision frequency was determined as described above, and strain N315 transformed with the empty shuttle vector pLI50 was used as a control.

To determine if SCC*mec* was cured from strain N315 cells, the following primer pairs were designed and used for PCR detection. Primer pair cL1 and cR1 was used to detect the *attB* fragment in SCC*mec*-excised chromosome, primer pair cL1 and mR1 was used to detect the *attL* fragment in SCC*mec*-integrated chromosome, primer pair HVR1 and HVR2 was used to detect the existence of SCC*mec* in the cells, primer pair *mecA*1

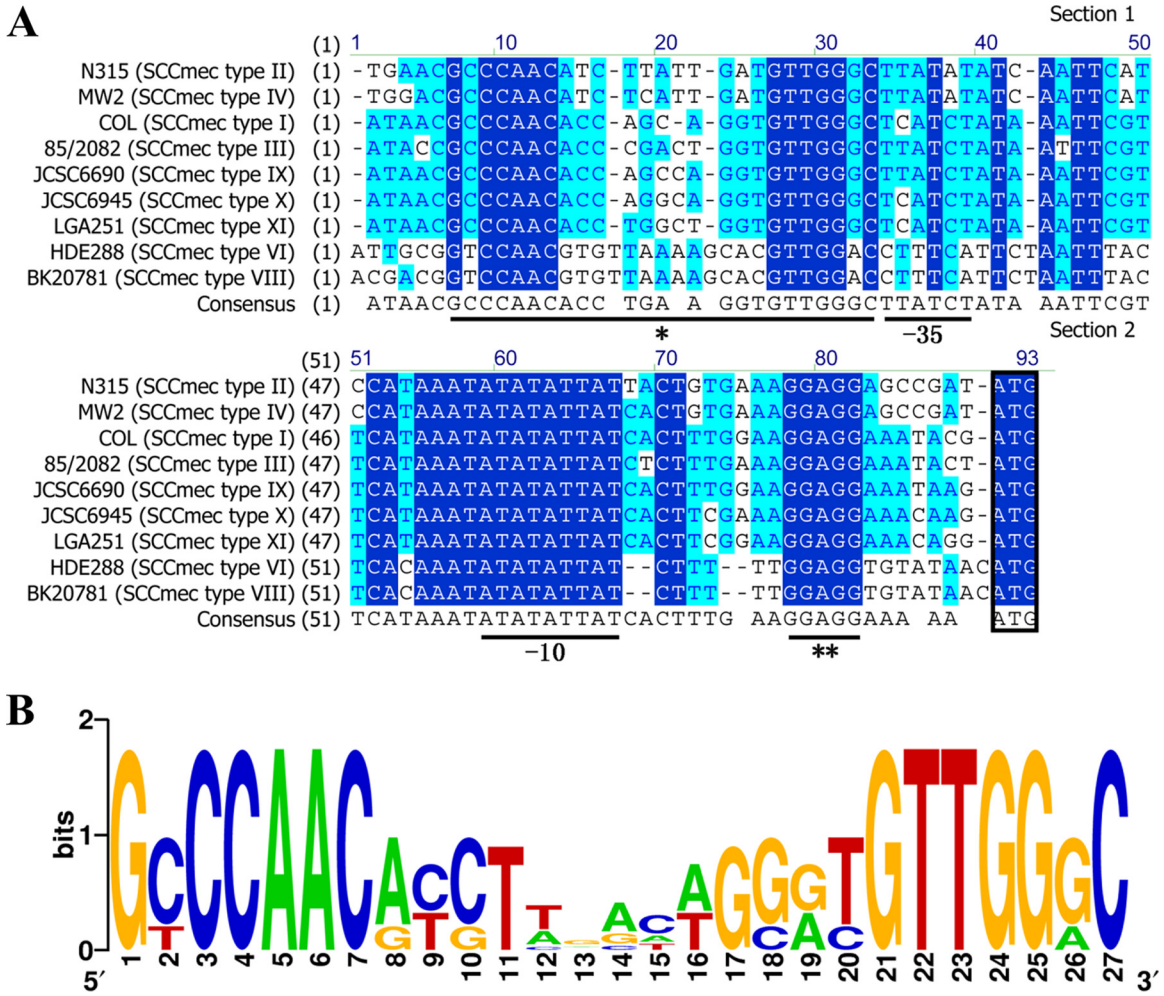

FIG 1 Identification of a highly conserved IR sequence within the *ccrAB* promoter. (A) Multiple-sequence alignment of the 5′-untranslated region of *ccrAB* among strains of different SCC*mec* types. A highly conserved IR sequence (*) and Shine-Dalgarno sequence (**) were identified within the *ccrAB* promoter region. The putative −35 and −10 regions are marked, and the start codon of the *ccrA* gene is boxed. Dark blue indicates identical bases, light blue indicates the conservative nucleotide, and white indicates the divergent bases. (B) A highly conserved region ($GNCCAACN_{13}GTTGGNC$) of the IR sequence was identified by consensus sequence analysis (42).

and *mecA*2 was used to detect the existence of the *mecA* gene in the cells, and primer pair SPA1 and SPA2 was used for *spa* typing.

RESULTS

Identification of a highly conserved IR sequence in *ccrAB* 5′-untranslated region. Upon analysis of the *ccrAB* promoter sequence from strain N315, we found a stem-loop structure of a 10-base inverted repeat with a 5-base loop (GCCCAACATCttatt GATGTTGGGC; the 5-base loop is in lowercase), which is located 55 bp upstream of the translation initiation site of *ccrA*. We compared the *ccrAB* promoter sequences from 9 different SCC*mec* types available from NCBI GenBank by multiple-sequence alignment in segments. From the results of the *in silico* analysis, we found that all of these SCC*mec* types harbor the IR sequence, which exhibits perfect base pairing, even though there are some base differentiations (Fig. 1A). Interestingly, an extremely conserved region comprises part of the IR sequence (Fig. 1B), and all of the IR sequences are located 55 bp (SCC*mec* types I to IV and IX to XI) or 53 bp (SCC*mec* types VI and VIII) upstream of the translation initiation site of *ccrA* (see Fig. S1 in the supplemental material).

The IR sequence played a repressive role in *ccrAB* expression and SCC*mec* excision. To determine the effect of the IR sequence on *ccrAB* expression, we created three different promoter-*lacZ* reporter gene plasmids. The plasmid pOS*ccrAB* was constructed with the original sequence of the *ccrAB* promoter, whereas the plasmids pOS*ccrAB*IR1 and pOS*ccrAB*IR2 were constructed with the base substitution of the upstream or the downstream region of the IR sequence (Fig. 2A). Analysis of these strains in TSB containing X-Gal indicated that the replacement of the IR sequence greatly increased the β-galactosidase activity, whereas the strain containing the original IR sequence exhibited no visible β-galactosidase activity even after cultivation for 60 h (Fig. 2B). Meanwhile, cells were collected at 6-h intervals to detect *ccrAB* expression with ONPG. The β-galactosidase activity changed dramatically in the strains harboring the substituted IR sequence compared with that of the original IR sequence (Fig. 2C), indicating that the IR sequence played a repressive role in *ccrAB* expression.

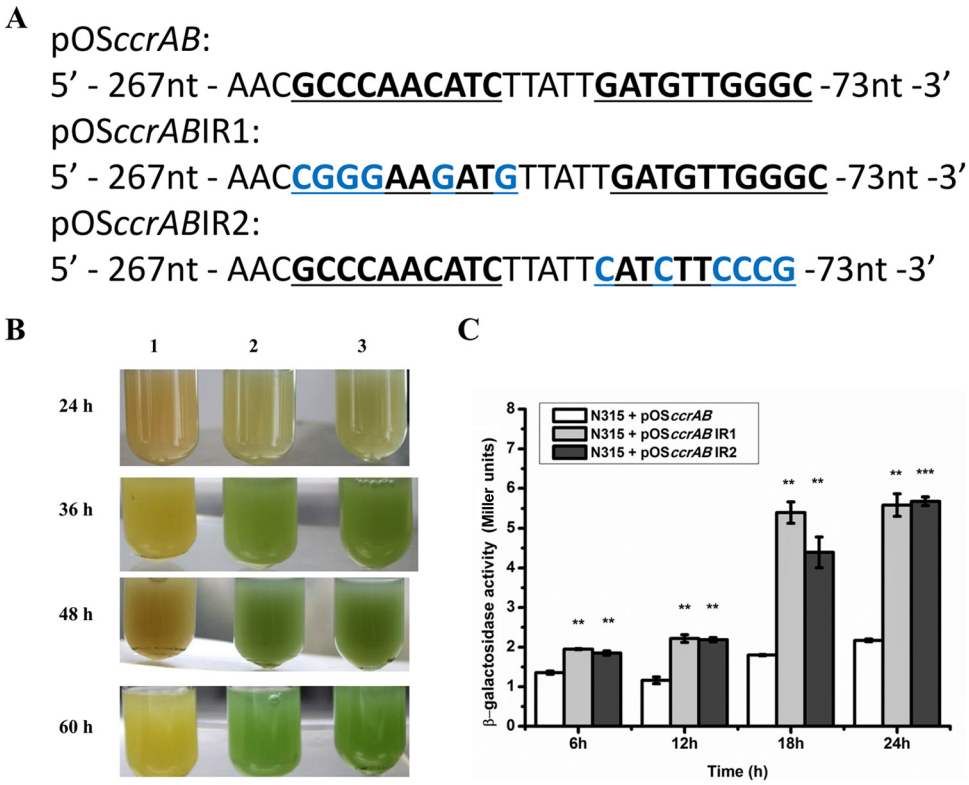

FIG 2 IR sequence represses *ccrAB* expression. (A) A schematic presents the promoter sequence involved in the construction of the reporter plasmids. The altered bases are marked in blue. (B) Replacement of the IR sequence greatly increases the β-galactosidase activity, whereas the strain containing the original IR sequence exhibited no visible β-galactosidase activity. Strains N315 carrying pOS*ccrAB*, pOS*ccrAB*IR1, or pOS*ccrAB*IR2 were cultivated in TSB containing 40 μg/ml X-Gal for different periods of time. Lane 1, strain N315 carrying pOS*ccrAB* (N315 + pOS*ccrAB*); lane 2, strain N315 carrying pOS*ccrAB*IR1 (N315 + pOS*ccrAB*IR1); lane 3, strain N315 carrying pOS*ccrAB*IR2 (N315 + pOS*ccrAB*IR2). (C) Partial nucleotide substitution of the IR sequence increases *ccrAB* expression. Cells were collected at 6-h intervals, and the β-galactosidase activity was detected with ONPG. Error bars represent standard deviations (SD) ($n = 3$). Statistically significant differences calculated by the unpaired two-tailed Student's *t* test are indicated: NS, not significant ($P > 0.05$); *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Since the substituted IR sequence enhanced *ccrAB* expression, it may subsequently affect SCC*mec* excision. To determine this, we tried to replace the upstream region of the IR sequence in the strain N315 chromosome by using the plasmid pBTsΔIR1, in which the substituted IR sequence was the same as that of pOS*ccrAB*IR1. Surprisingly, PCR analysis with primers spanning the IR sequence showed negative signals in all of these postulated mutant strains, and this phenomenon was further observed in other two separate screenings. We speculated that the replacement of the IR sequence probably had led to high *ccrAB* expression and, subsequently, SCC*mec* curing. Since methicillin resistance is an important characteristic of SCC*mec*, we first evaluated the oxacil-

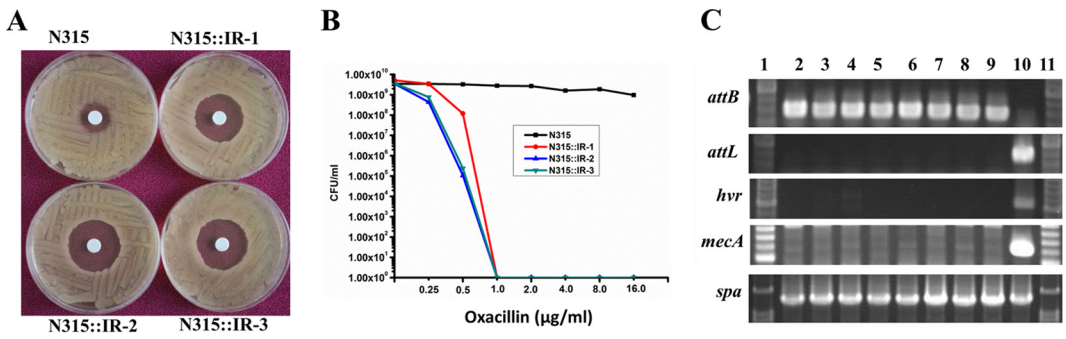

FIG 3 Substitution of the IR sequence causes SCC*mec* curing. (A) Evaluation of oxacillin resistance by diffusion disks containing oxacillin (1 mg) indicates that the putative IR mutant strains exhibit reduced resistance. N315::IR-1, N315::IR-2, and N315::IR-3 represent three randomly selected IR mutant strains. (B) Strain N315 is susceptible to oxacillin when the IR sequence in the chromosome was partially replaced. The oxacillin resistance levels were evaluated by population analysis profiles. N315::IR-1, N315::IR-2, and N315::IR-3 represent three randomly selected IR mutant strains. (C) PCR with primer pairs spanning *attB*, *attL*, *hvr*, and *mecA* indicates that SCC*mec* has been cured in the mutant strain. PCR results with primers spanning *spa* indicate that they are of the same *spa* type. Lanes 1 and 11, DNA marker; lanes 2 to 9, randomly selected IR mutant strains; lane 10, wild-type strain N315.

TABLE 3 SCC*mec* excision frequencies mediated by *ccrAB* overexpression with different promoter sequences

Strain	SCC*mec* excision frequency	SD
N315 + pLI50	3.5034×10^{-6}	2.6810×10^{-7}
N315 + pLI*ccrAB*	0.5213	1.4400×10^{-3}
N315 + pLI*ccrAB*-IR1	0.9986	1.6285×10^{-5}

[a] N315 + pLI50, N315 + pLI*ccrAB*, and N315 + pLI*ccrAB*-IR1 indicate strain N315 carrying pLI50, pLI*ccrAB*, and pLI*ccrAB*-IR1, respectively.

lin resistance of these strains. The results of diffusion disks indicated that the postulated IR mutant strains exhibited reduced resistance (Fig. 3A), and population analysis profiles revealed that the mutant strains had become susceptible to oxacillin (Fig. 3B). Further PCR analysis using genomic DNA extracted from the postulated mutant strains and the wild-type strain indicated that SCC*mec* was cured in all of the bacterial populations we collected (Fig. 3C).

To further determine if replacement of the IR sequence can lead to SCC*mec* curing, we evaluated the SCC*mec* excision frequency using plasmid-driven overexpression. We constructed the plasmid pLI*ccrAB* with the original *ccrAB* sequence and the plasmid pLI*ccrAB*-IR1 with the upstream region of the IR sequence altered the same way as that for pOS*ccrAB*IR1 and pBTsΔIR1. Our PCR data indicated that the strain carrying pLI*ccrAB* showed significantly higher SCC*mec* excision frequency than the wild-type strain N315 (Table 3). Interestingly, replacement of the IR sequence led to almost complete SCC*mec* curing (Table 3). These results allow us to conclude that the IR sequence plays a repressive role in *ccrAB* expression and SCC*mec* excision.

Identification of a *ccrAB* promoter binding protein, SarS. In order to identify proteins that are involved in the transcriptional regulation of *ccrAB*, we performed DNA affinity pulldown assays using a biotin-labeled fragment within *ccrAB* promoter as the probe. After washing extensively with the nonspecific competitor poly(dI-dC) at a low salt concentration, retained proteins were released by heating with purified water. The solution was analyzed by SDS-PAGE, and the regulatory protein SarS was identified by its mass spectrum (Fig. 4A and Table 4).

To confirm the binding ability and specificity, EMSA was performed with the *ccrAB* promoter labeled with biotin and SarS purified by a nickel nitrilotriacetic acid agarose solution (Fig. 4B). The results showed that SarS can retard the mobility of the *ccrAB* promoter in a dose-dependent manner. The protein-DNA complex can be disrupted with a 100-fold concentration of specific competitor, while the same amount of nonspecific competitor did

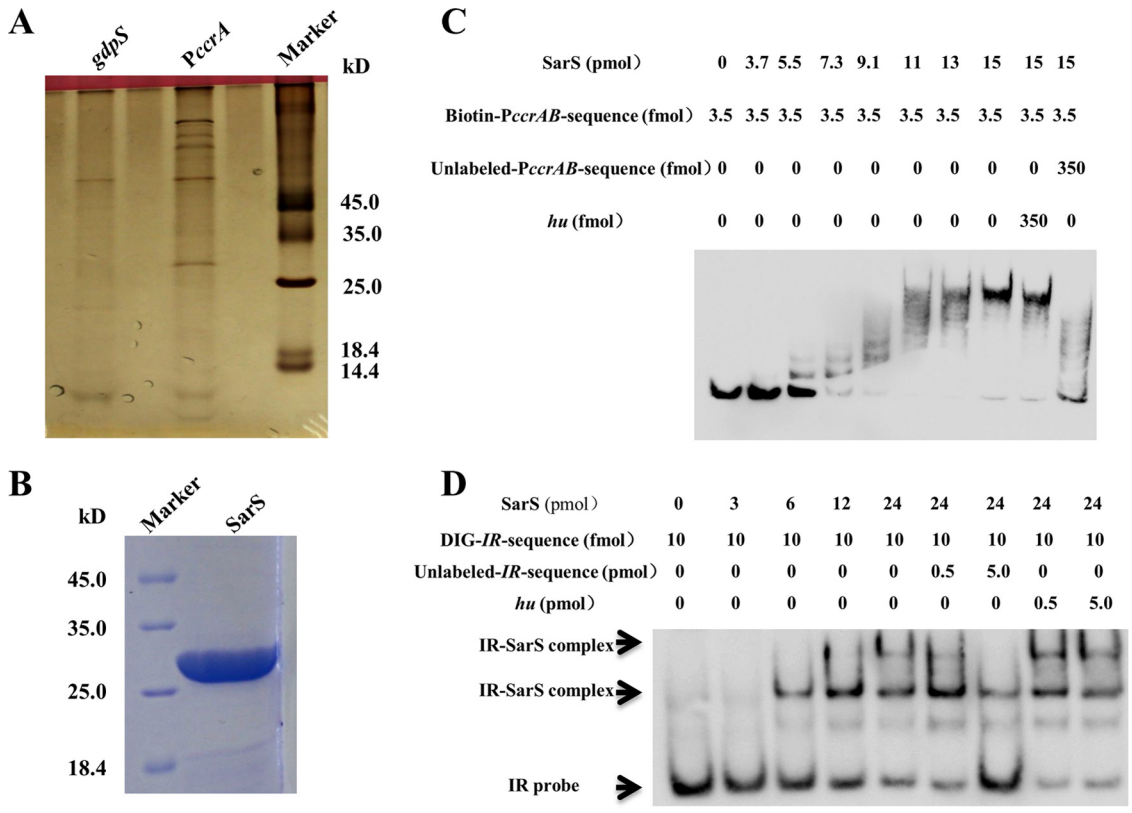

FIG 4 Identification of the *ccrAB* promoter-specific binding protein SarS. (A) SDS-PAGE analysis of proteins binding to *ccrAB* promoter region or *gdpS* coding sequence. The gel was treated with silver staining, and among the specific bands, protein SarS was identified by mass spectrometry. (B) SDS-PAGE analysis of SarS purified from the pET28a(+) expression vector. About 30 μg of the purified SarS protein was loaded onto the lane. The gel was stained with Coomassie blue. (C) EMSA of the purified SarS with a 233-bp *ccrAB* promoter fragment labeled with biotin. Increasing concentrations of purified SarS and 3.5 fmol of the biotin-labeled probe were used in the reactions. The specific competitor concentration was 100-fold higher that of the nonlabeled *ccrAB* promoter, and the concentration of the nonspecific competitor was 100-fold that of *hu*. (D) EMSA of purified SarS with the DIG-labeled IR sequence. Increasing concentrations of purified SarS and 10 fmol of the DIG-labeled probe were used in the reactions. The specific competitor was 50-fold or 500-fold of the nonlabeled IR sequence, and the nonspecific competitor was 50-fold or 500-fold of *hu*.

TABLE 4 Strain N315 proteins identified by mass spectrometry

ORF	Gene	Description
SA0108	*sarS*	Staphylococcal accessory regulator homolog
SA0500	*rpoB*	DNA-directed RNA polymerase beta chain, putative
SA0501	*rpoC*	DNA-directed RNA polymerase beta-prime chain, putative
SA1305	*hu*	DNA-binding protein HU, putative
SA1513	*polA*	DNA polymerase I
SA1720	*ligA*	DNA ligase, NAD dependent

not have the same effect (Fig. 4C). To further evaluate if SarS can bind to the IR sequence, we prepared a 40-bp DIG-labeled probe containing the whole IR sequence. Our EMSA results indicated that SarS was able to retard the mobility of the probe in a dose-dependent manner, and the binding can be disrupted with a 500-fold concentration of specific competitor (Fig. 4D), implying that the IR sequence is one acting site of SarS.

SarS upregulated *ccrAB* expression and enhanced SCC*mec* excision. We then created the *sarS* deletion strain (see Fig. S2 in the supplemental material) and evaluated the effect of SarS on *ccrAB* expression in the wild-type strain N315 and the *sarS* mutant strain by qRT-PCR analysis. Compared with the wild-type strain, the *sarS* mutant strain exhibited no significant difference in *ccrA* transcription at the exponential phase (Fig. 5A). In order to explore the effect of SarS on *ccrAB* expression at the stationary growth phase, we transformed the plasmid pOS*ccrAB* into the N315 and N315Δ*sarS* strains. The cells were collected at 6-h intervals, and the β-galactosidase activity was detected with ONPG as the substrate. The result showed that the β-galactosidase activity displayed no difference at 6 h, which was consistent with the qRT-PCR result. However, the β-galactosidase activity decreased at the stationary growth phase in the N315Δ*sarS* strain compared with that of the wild-type strain (Fig. 5B), suggesting that SarS is involved in the stationary-phase regulation of *ccrAB* expression. We then determined the effect of SarS on *ccrAB* transcription by creating the inducible *sarS* overexpression plasmid pRMC*sarS*. Cells were collected and total RNA was extracted after induction with anhydrotetracycline for different periods of time. The qRT-PCR assay showed that the transcriptional level of *ccrA* increased significantly with anhydrotetracycline induction (Fig. 5C).

To determine the effect of SarS on SCC*mec* excision, we constructed the *sarS* overexpression plasmid pLI*sarS* and transformed it into the N315Δ*sarS* strain. The transformed cells were cultivated in BHI broth and collected from consecutive cultures every 3 days. Genome DNA was extracted and PCR was performed to measure the SCC*mec* excision frequency. The results showed that SCC*mec* excision frequency increased significantly during consecutive cultivation (Fig. 6A). In contrast, the SCC*mec* excision frequency remained at almost the same level in the wild-type strain carrying pLI50 (Fig. 6B). These data indicated that SarS upregulated *ccrAB* expression and enhanced SCC*mec* excision.

DISCUSSION

SCC*mec* is a mobile genetic element and harbors the *mecA* gene, which codes for an alternative penicillin-binding protein, PBP2a. Acquisition of SCC*mec* not only gives *S. aureus* the ability to become resistant to almost the entire class of β-lactam antibiotics but also modulates the virulence (35) and *agr* quorum sensing (36) in some strains. SCC*mec* types II, III, and VIII and some irregular or truncated SCC*mec* elements harbor the toxin gene *psm-mec* (37), which codes for a regulatory RNA and modulates the virulence of MRSA through suppressing AgrA translation (38). As displayed before, SCC*mec* can be excised from or integrated into bacterial chromosomes mediated by CcrAB recombinases, which are encoded by *ccrAB* genes (5). It has been pointed out that CcrAB-mediated SCC*mec* excision may enhance the subsequent horizontal transfer of SCC*mec* and lead the donor strains to a disadvantageous state. Interestingly, *ccrAB* genes are expressed in only a minority of cells (19), displaying beneficial effect for both recipient strain and donor strain in genotype spreading and stability. The different expression levels of *ccrAB* in individual cells suggest that a subtle regulatory mechanism is involved in *ccrAB* expression and SCC*mec* excision.

The demonstration of regulatory details of *ccrAB* expression and SCC*mec* excision should provide insights into the dynamic equilibrium of mobile genetic elements and the emergence of antibiotic resistance in bacteria. In this work, we identified an IR sequence within the *ccrAB* promoter. Sequence alignment of different SCC*mec* types indicates that the IR sequence exists extensively among these SCC*mec* types and is highly conserved both in sequence and location, implying that the IR sequence is a regulator binding site in the *ccrAB* promoter. Partial replacement of the IR

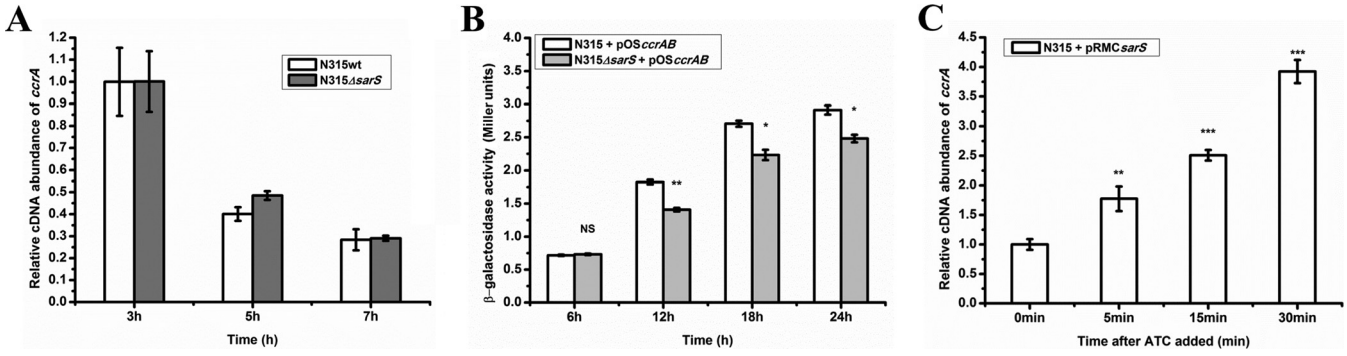

FIG 5 SarS upregulates *ccrAB* expression. (A) The transcriptional level of *ccrA* showed no difference in strain N315 and its *sarS* deletion mutant strain at the exponential phase. (B) Deletion of *sarS* from the strain N315 chromosome (N315Δ*sarS* + pOS*ccrAB*) decreases *ccrAB* expression at the stationary growth phase. Cells were collected at 6-h intervals, and the β-galactosidase activity was detected with ONPG. (C) qRT-PCR analysis of *ccrA* in strain N315 carrying pRMC*sarS* with the induction of anhydrotetracycline (ACT; 200 ng/ml). Cells were collected at 0, 5, 15, and 30 min. Error bars represent SD ($n = 3$). Statistically significant differences calculated by the unpaired two-tailed Student's *t* test are indicated: NS, not significant ($P > 0.05$); *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

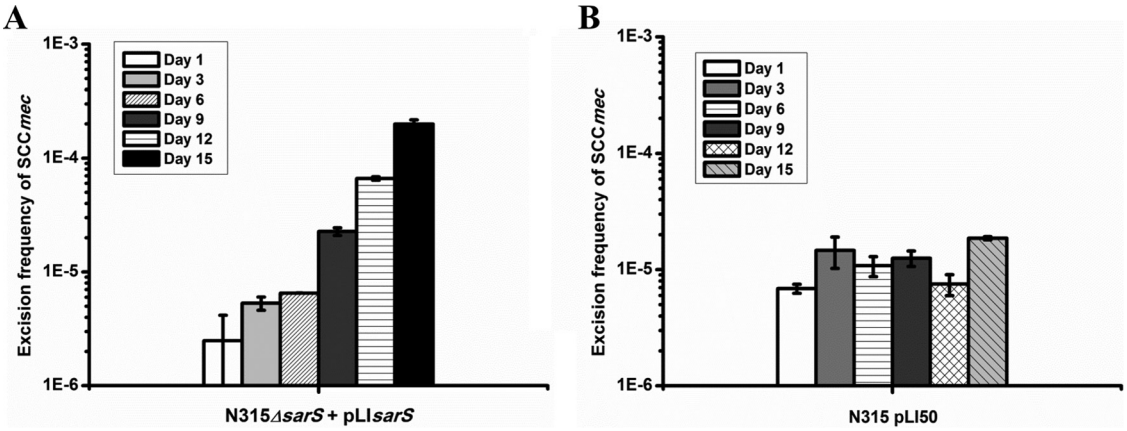

FIG 6 SarS enhances SCC*mec* excision. (A) SarS overexpression (N315Δ*sarS* + pLI*sarS*) enhances SCC*mec* excision during consecutive cultivation. Cells were cultivated for 1, 3, 6, 9, 12, and 15 days by passaging every 24 h in BHI broth at 37°C and then were collected. (B) The SCC*mec* excision frequency remained at almost the same level in the wild-type strain N315 carrying the empty shuttle vector pLI50 (N315 + pLI50). Cells were cultivated and collected in the same way as that described for the *sarS* overexpression strain.

sequence in reporter plasmids enhanced β-galactosidase activity, suggesting the repressive effect of this inverted repeat element in the regulation of *ccrAB* expression. Interestingly, partial substitution of the IR sequence in the chromosome can cause SCC*mec* curing, further pointing to the significance of the IR sequence in *ccrAB* expression and SCC*mec* stability. Notably, substitution of either the upstream region or the downstream region of the IR sequence exhibited the same effect on *ccrAB* expression, implying that the IR sequence functions through a special secondary structure. Studies have shown that IR elements are widely distributed in *S. aureus* and play multiple roles in gene regulation by acting as regulatory sites or transcription terminators. A highly conserved IR sequence is present upstream of the *int* gene in several staphylococcal prophages, fulfilling an important regulatory role (39). The IR sequence we identified also is located upstream of the putative promoter region, implying a role similar to that of the IR sequence in prophages.

Using DNA pulldown assay, we successfully identified a global transcriptional regulator, SarS, which is a member of the Sar protein family and a downregulator within the SarA/Agr global regulatory network (40, 41). A previous study has shown that SarS can bind to the promoters of *spa*, *hla*, and *ssp* and greatly promotes the expression of *spa* and *hla* (40). Our results indicated that SarS also can bind to the promoter of *ccrAB* in a laddering pattern similar to the EMSA results for SarS and the *spa* promoter (41). Notably, our further results indicated that SarS can bind to the IR sequence and enhance *ccrAB* expression and SCC*mec* excision, revealing the novel role that SarS plays in *S. aureus*. However, the regulation of *ccrAB* expression and SCC*mec* excision seems complicated. Even though we inferred that SarS binds to the IR sequence and affects its structural stability, it cannot be excluded that other regulatory proteins or small regulatory noncoding RNAs may be involved in the regulatory process.

Although *S. aureus* strain N315 carries intact *ccrAB* on its chromosome, the spontaneous excision of SCC*mec* occurs at an extremely low frequency with dynamic equilibrium (19). The transfer of SCC*mec* to methicillin-susceptible *Staphylococcus aureus* (MSSA) poses an advantage to the recipient strain under antibiotic stress, whereas curing of SCC*mec* poses a disadvantage to the survival of the donor strain. It would be easy to understand that genetic variability occurs in only a minority of cells, which can be precisely controlled through regulation of *ccrAB* expression. Although SCC*mec* excision occurs at a very low frequency, the selective pressures promoting the breakup of the dynamic equilibrium would induce a rapid and global expansion of methicillin resistance. Our findings that an inverted repeat DNA element and SarS are involved in the regulation of *ccrAB* expression and SCC*mec* excision should provide mechanistic insights into the lateral transfer of SCC*mec* and expansion of antibiotic resistance in staphylococci. Further identification of molecular regulatory systems and detailed mechanisms controlling *ccrAB* expression and SCC*mec* excision may help us understand SCC*mec* transfer in the environment and develop a novel therapeutic strategy in converting MRSA *in vivo* into MSSA.

ACKNOWLEDGMENTS

We thank the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) for providing the bacterial strains.

This study was supported by the National Natural Science Foundation of China (81371850).

REFERENCES

1. **Lowy FD.** 1998. *Staphylococcus aureus* infections. N Engl J Med **339:**520–532. http://dx.doi.org/10.1056/NEJM199808203390806.
2. **Chambers HF, Deleo FR.** 2009. Waves of resistance: *Staphylococcus aureus* in the antibiotic era. Nat Rev Microbiol **7:**629–641. http://dx.doi.org/10.1038/nrmicro2200.
3. **Malachowa N, DeLeo FR.** 2010. Mobile genetic elements of *Staphylococcus aureus*. Cell Mol Life Sci **67:**3057–3071. http://dx.doi.org/10.1007/s00018-010-0389-4.
4. **Ito T, Katayama Y, Hiramatsu K.** 1999. Cloning and nucleotide sequence determination of the entire *mec* DNA of pre-methicillin-resistant *Staphylococcus aureus* N315. Antimicrob Agents Chemother **43:**1449–1458.
5. **Katayama Y, Ito T, Hiramatsu K.** 2000. A new class of genetic element, staphylococcus cassette chromosome *mec*, encodes methicillin resistance in *Staphylococcus aureus*. Antimicrob Agents Chemother **44:**1549–1555. http://dx.doi.org/10.1128/AAC.44.6.1549-1555.2000.
6. **Song MD, Wachi M, Doi M, Ishino F, Matsuhashi M.** 1987. Evolution of an inducible penicillin-target protein in methicillin-resistant *Staphylococcus aureus* by gene fusion. FEBS Lett **221:**167–171. http://dx.doi.org/10.1016/0014-5793(87)80373-3.
7. **Hartman B, Tomasz A.** 1981. Altered penicillin-binding proteins in me-

thicillin-resistant strains of *Staphylococcus aureus*. Antimicrob Agents Chemother **19:**726–735. http://dx.doi.org/10.1128/AAC.19.5.726.
8. **Arede P, Milheirico C, de Lencastre H, Oliveira DC.** 2012. The antirepressor MecR2 promotes the proteolysis of the *mecA* repressor and enables optimal expression of β-lactam resistance in MRSA. PLoS Pathog **8:**e1002816. http://dx.doi.org/10.1371/journal.ppat.1002816.
9. **Boundy S, Safo MK, Wang L, Musayev FN, O'Farrell HC, Rife JP, Archer GL.** 2013. Characterization of the *Staphylococcus aureus* rRNA methyltransferase encoded by *orfX*, the gene containing the staphylococcal chromosome Cassette *mec* (SCC*mec*) insertion site. J Biol Chem **288:** 132–140. http://dx.doi.org/10.1074/jbc.M112.385138.
10. **Ito T, Ma XX, Takeuchi F, Okuma K, Yuzawa H, Hiramatsu K.** 2004. Novel type V staphylococcal cassette chromosome *mec* driven by a novel cassette chromosome recombinase, *ccrC*. Antimicrob Agents Chemother **48:**2637–2651. http://dx.doi.org/10.1128/AAC.48.7.2637-2651.2004.
11. **Misiura A, Pigli YZ, Boyle-Vavra S, Daum RS, Boocock MR, Rice PA.** 2013. Roles of two large serine recombinases in mobilizing the methicillin-resistance cassette SCC*mec*. Mol Microbiol **88:**1218–1229. http://dx.doi.org/10.1111/mmi.12253.
12. **Noto MJ, Archer GL.** 2006. A subset of *Staphylococcus aureus* strains harboring staphylococcal cassette chromosome *mec* (SCC*mec*) type IV is deficient in CcrAB-mediated SCC*mec* excision. Antimicrob Agents Chemother **50:**2782–2788. http://dx.doi.org/10.1128/AAC.00032-06.
13. **Wang L, Safo M, Archer GL.** 2012. Characterization of DNA sequences required for the CcrAB-mediated integration of staphylococcal cassette chromosome *mec*, a *Staphylococcus aureus* genomic island. J Bacteriol **194:** 486–498. http://dx.doi.org/10.1128/JB.05047-11.
14. **Wang L, Archer GL.** 2010. Roles of CcrA and CcrB in excision and integration of staphylococcal cassette chromosome *mec*, a *Staphylococcus aureus* genomic island. J Bacteriol **192:**3204–3212. http://dx.doi.org/10.1128/JB.01520-09.
15. **Shore AC, Coleman DC.** 2013. Staphylococcal cassette chromosome *mec*: recent advances and new insights. Int J Med Microbiol **303:**350–359. http://dx.doi.org/10.1016/j.ijmm.2013.02.002.
16. **Bloemendaal AL, Brouwer EC, Fluit AC.** 2010. Methicillin resistance transfer from *Staphylococcus epidermidis* to methicillin-susceptible *Staphylococcus aureus* in a patient during antibiotic therapy. PLoS One **5:**e11841. http://dx.doi.org/10.1371/journal.pone.0011841.
17. **Boundy S, Zhao Q, Fairbanks C, Folgosa L, Climo M, Archer GL.** 2012. Spontaneous staphylococcal cassette chromosome *mec* element excision in *Staphylococcus aureus* nasal carriers. J Clin Microbiol **50:**469–471. http://dx.doi.org/10.1128/JCM.01063-11.
18. **Higgins PG, Rosato AE, Seifert H, Archer GL, Wisplinghoff H.** 2009. Differential expression of *ccrA* in methicillin-resistant *Staphylococcus aureus* strains carrying staphylococcal cassette chromosome *mec* type II and IVa elements. Antimicrob Agents Chemother **53:**4556–4558. http://dx.doi.org/10.1128/AAC.00395-09.
19. **Stojanov M, Sakwinska O, Moreillon P.** 2013. Expression of SCC*mec* cassette chromosome recombinases in methicillin-resistant *Staphylococcus aureus* and *Staphylococcus epidermidis*. J Antimicrob Chemother **68:** 749–757. http://dx.doi.org/10.1093/jac/dks494.
20. **Laub MT, Goulian M.** 2007. Specificity in two-component signal transduction pathways. Annu Rev Genet **41:**121–145. http://dx.doi.org/10.1146/annurev.genet.41.042007.170548.
21. **Gripenland J, Netterling S, Loh E, Tiensuu T, Toledo-Arana A, Johansson J.** 2010. RNAs: regulators of bacterial virulence. Nat Rev Microbiol **8:**857–866. http://dx.doi.org/10.1038/nrmicro2457.
22. **Cheung AL, Nishina KA, Trotonda MP, Tamber S.** 2008. The SarA protein family of *Staphylococcus aureus*. Int J Biochem Cell Biol **40:**355–361. http://dx.doi.org/10.1016/j.biocel.2007.10.032.
23. **Liu Y, Mu C, Ying X, Li W, Wu N, Dong J, Gao Y, Shao N, Fan M, Yang G.** 2011. RNAIII activates map expression by forming an RNA-RNA complex in *Staphylococcus aureus*. FEBS Lett **585:**899–905. http://dx.doi.org/10.1016/j.febslet.2011.02.021.
24. **Zhang Y, Werling U, Edelmann W.** 2012. SLiCE: a novel bacterial cell extract-based DNA cloning method. Nucleic Acids Res **40:**e55. http://dx.doi.org/10.1093/nar/gkr1288.
25. **Corrigan RM, Foster TJ.** 2009. An improved tetracycline-inducible expression vector for *Staphylococcus aureus*. Plasmid **61:**126–129. http://dx.doi.org/10.1016/j.plasmid.2008.10.001.
26. **Lee CY, Buranen SL, Ye ZH.** 1991. Construction of single-copy integration vectors for *Staphylococcus aureus*. Gene **103:**101–105. http://dx.doi.org/10.1016/0378-1119(91)90399-V.
27. **Hu J, Zhang X, Liu X, Chen C, Sun B.** 2015. Mechanism of reduced vancomycin susceptibility conferred by walK mutation in community-acquired methicillin-resistant *Staphylococcus aureus* strain MW2. Antimicrob Agents Chemother **59:**1352–1355. http://dx.doi.org/10.1128/AAC.04290-14.
28. **Bae T, Schneewind O.** 2006. Allelic replacement in Staphylococcus aureus with inducible counter-selection. Plasmid **55:**58–63. http://dx.doi.org/10.1016/j.plasmid.2005.05.005.
29. **Xue T, Zhao L, Sun H, Zhou X, Sun B.** 2009. LsrR-binding site recognition and regulatory characteristics in *Escherichia coli* AI-2 quorum sensing. Cell Res **19:**1258–1268. http://dx.doi.org/10.1038/cr.2009.91.
30. **Jett BD, Hatter KL, Huycke MM, Gilmore MS.** 1997. Simplified agar plate method for quantifying viable bacteria. Biotechniques **23:**648–650.
31. **Iioka H, Loiselle D, Haystead TA, Macara IG.** 2011. Efficient detection of RNA-protein interactions using tethered RNAs. Nucleic Acids Res **39:** e53. http://dx.doi.org/10.1093/nar/gkq1316.
32. **Jutras BL, Verma A, Stevenson B.** 2012. Identification of novel DNA-binding proteins using DNA-affinity chromatography/pull down. Curr Protoc Microbiol **Chapter 1:**Unit 1F.1.
33. **Valihrach L, Demnerova K.** 2012. Impact of normalization method on experimental outcome using RT-qPCR in Staphylococcus aureus. J Microbiol Methods **90:**214–216. http://dx.doi.org/10.1016/j.mimet.2012.05.008.
34. **Tao L, Wu X, Sun B.** 2010. Alternative sigma factor sigmaH modulates prophage integration and excision in *Staphylococcus aureus*. PLoS Pathog **6:**e1000888. http://dx.doi.org/10.1371/journal.ppat.1000888.
35. **Collins J, Rudkin J, Recker M, Pozzi C, O'Gara JP, Massey RC.** 2010. Offsetting virulence and antibiotic resistance costs by MRSA. ISME J **4:**577–584. http://dx.doi.org/10.1038/ismej.2009.151.
36. **Rudkin JK, Edwards AM, Bowden MG, Brown EL, Pozzi C, Waters EM, Chan WC, Williams P, O'Gara JP, Massey RC.** 2012. Methicillin resistance reduces the virulence of healthcare-associated methicillin-resistant *Staphylococcus aureus* by interfering with the agr quorum sensing system. J Infect Dis **205:**798–806. http://dx.doi.org/10.1093/infdis/jir845.
37. **Monecke S, Engelmann I, Archambault M, Coleman DC, Coombs GW, Cortez de Jackel S, Pelletier-Jacques G, Schwarz S, Shore AC, Slickers P, Ehricht R.** 2012. Distribution of SCC*mec*-associated phenol-soluble modulin in staphylococci. Mol Cell Probes **26:**99–103. http://dx.doi.org/10.1016/j.mcp.2012.01.001.
38. **Kaito C, Saito Y, Ikuo M, Omae Y, Mao H, Nagano G, Fujiyuki T, Numata S, Han X, Obata K, Hasegawa S, Yamaguchi H, Inokuchi K, Ito T, Hiramatsu K, Sekimizu K.** 2013. Mobile genetic element SCC*mec*-encoded *psm-mec* RNA suppresses translation of *agrA* and attenuates MRSA virulence. PLoS Pathog **9:**e1003269. http://dx.doi.org/10.1371/journal.ppat.1003269.
39. **Iandolo JJ, Worrell V, Groicher KH, Qian Y, Tian R, Kenton S, Dorman A, Ji H, Lin S, Loh P, Qi S, Zhu H, Roe BA.** 2002. Comparative analysis of the genomes of the temperate bacteriophages phi11, phi12 and phi13 of *Staphylococcus aureus* 8325. Gene **289:**109–118. http://dx.doi.org/10.1016/S0378-1119(02)00481-X.
40. **Tegmark K, Karlsson A, Arvidson S.** 2000. Identification and characterization of SarH1, a new global regulator of virulence gene expression in *Staphylococcus aureus*. Mol Microbiol **37:**398–409. http://dx.doi.org/10.1046/j.1365-2958.2000.02003.x.
41. **Cheung AL, Schmidt K, Bateman B, Manna AC.** 2001. SarS, a SarA homolog repressible by agr, is an activator of protein A synthesis in *Staphylococcus aureus*. Infect Immun **69:**2448–2455. http://dx.doi.org/10.1128/IAI.69.4.2448-2455.2001.
42. **Crooks GE, Hon G, Chandonia JM, Brenner SE.** 2004. WebLogo: a sequence logo generator. Genome Res **14:**1188–1190. http://dx.doi.org/10.1101/gr.849004.