International Journal of Medical Microbiology 306 (2016) 406–414

Contents lists available at ScienceDirect

International Journal of Medical Microbiology

journal homepage: www.elsevier.com/locate/ijmm

# SigmaB regulates *ccrAB* expression and SCC*mec* excision in methicillin-resistant *Staphylococcus aureus*

Shijie Zhang [a], Xueqin Shu [a], Baolin Sun [a,b,*]

[a] CAS Key Laboratory of Innate Immunity and Chronic Disease, School of Life Sciences and Medical Center, University of Science and Technology of China, Hefei, Anhui 230027, China
[b] Hefei National Laboratory for Physical Sciences at Microscale, Hefei, Anhui 230027, China

ARTICLE INFO

*Article history:*
Received 26 January 2016
Received in revised form 11 April 2016
Accepted 12 May 2016

*Keywords:*
Methicillin-resistant *Staphylococcus aureus*
SigmaB
*CcrAB* gene organization
*CcrAB* expression
SCC*mec* excision

ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a worldwide pathogen that is resistant to practically the entire class of β-lactam antibiotics due to the presence of the *mecA* gene. The *mecA* gene is located on a large mobile genetic element referred to as staphylococcal cassette chromosome *mec* (SCC*mec*), and the excision and integration of SCC*mec* are mediated by the Ccr recombinase encoded by *ccrAB* or *ccrC*, which are also located on SCC*mec*. Previous studies have shown that the *ccrAB* genes are only expressed in a minority of cells and that their expression levels can be affected by certain environmental stimuli, but the molecular mechanisms controlling these phenotypes remain elusive. Here, we found that overexpression of SigB can dramatically enhance *ccrA* transcription and SCC*mec* excision in MRSA strain N315, revealing an important role for this alternative sigma factor in the lateral transfer of SCC*mec*. Further primer extension-blot analysis and 5′RACE (Rapid Amplification of cDNA Ends) indicated that an unrecognized SigB-dependent promoter region, which exists in certain SCC*mec* type II and IV strains, is responsible for the enhancement, and the *ccrAB* genes are in fact transcribed in a two-promoter pattern with a low activity of the SigB-dependent promoter under normal growth conditions.



## 1. Introduction

Acquisition of the mobile genetic element staphylococcal cassette chromosome *mec* (SCC*mec*) has prompted methicillin-resistant *Staphylococcus aureus* (MRSA) to become one of the most prevalent and threatening pathogens throughout the world (Ayliffe, 1997). The penicillin-binding protein PBP2a encoded by the *mecA* gene located on SCC*mec* provides MRSA strains with resistance to nearly all β-lactam antibiotics (Hartman and Tomasz, 1984; Ito et al., 1999), and a set of site-specific recombinases encoded by *ccrAB* or *ccrC* carried by SCC*mec* can excise SCC*mec* and make it transferable among staphylococcal strains (Ito et al., 2004; Katayama et al., 2000). CcrA, CcrB, and CcrC are three phylogenetically distinct proteins of the resolvase/invertase family (Katayama et al., 2000), and the genes *ccrA* and *ccrB* have been predicted to be co-transcribed with a presumptive promoter located immediately upstream of *ccrA* (Katayama et al., 2000). Because the *ccrAB* genes are only expressed in a minority of cells and their expression levels can be affected by some environmental stimuli (Higgins et al., 2009; Stojanov et al., 2013), it was suspected that *ccrAB* are strictly regulated by unknown regulatory networks. We previously demonstrated that a conserved inverted repeat element located upstream of *ccrA* and the Sar family protein SarS play roles in the modulation of *ccrAB* expression and SCC*mec* excision (Zhang et al., 2015). Since alternative sigma factor SigH is recruited by the staphylococcal temperate phage for the regulation of *int* gene transcription and bacteriophage transfer (Tao et al., 2010), the roles of alternative sigma factors in *ccrAB* transcription are of great interest.

In *S. aureus*, four types of sigma factors have been identified, which includes the housekeeping sigma factor SigA, and the alternative sigma factors SigB (Wu et al., 1996), SigH (Morikawa et al., 2003), and SigS (Shaw et al., 2008). SigB is a classic and well-characterized alternative sigma factor involved in a variety of cellular processes controlling the general stress response (Kullik and Giachino, 1997; Pane-Farre et al., 2006) and the resistance to β-lactam and glycopeptide antibiotics (Morikawa et al., 2001; Singh et al., 2003). Generally, SigB is kept inactive by the anti-sigma factor RsbW and can be turned on by the stimulus sensor RsbU through the anti-anti-sigma factor RsbV (Senn et al., 2005). As for SigH, in addition to the role it plays in prophage *int* gene transcription, which

* Corresponding author at: CAS Key Laboratory of Innate Immunity and Chronic Disease, School of Life Sciences and Medical Center, University of Science and Technology of China, Hefei, Anhui 230027, China.
*E-mail address:* sunb@ustc.edu.cn (B. Sun).

http://dx.doi.org/10.1016/j.ijmm.2016.05.008
1438-4221/

is in control of prophage integration and excision (Tao et al., 2010), it also participates in the regulation of competence genes that are responsible for natural DNA transformation (Morikawa et al., 2003; Morikawa et al., 2012). It has been recognized that SigH can be activated through chromosomal gene duplication rearrangement and post-transcriptional regulation with an inverted repeat sequence covering the ribosome binding site (Morikawa et al., 2012). SigS is a newly identified extracytoplasmic function sigma factor (Shaw et al., 2008), the role of which is still unclear.

In this study, we found that overexpression of SigB can significantly increase *ccrA* transcription and SCC*mec* excision in MRSA strain N315, and the effect on *ccrA* transcription greatly resembles that of the SigB-activity report gene *asp23* (Engelmann, 1999; Knobloch et al., 2004). Further *in vitro* analysis showed that overexpression of SigB induced the emergence of a specific transcript that was different from the transcript initiated by the promoter located immediately upstream of *ccrA* and this uncharacterized promoter sequence was revealed by 5′RACE (Rapid Amplification of cDNA Ends). However, the promoter-LacZ fusion assay showed that the activity of the SigB-dependent promoter is limited under normal growth conditions when compared to the SigA-dependent promoter.

## 2. Materials and methods

### 2.1. Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown in Luria-Bertani (LB; BD, Franklin Lakes, NJ, USA) medium with aeration (220 rpm) at 37 °C supplemented with appropriate antibiotics (ampicillin, 100 μg/ml; kanamycin, 50 μg/ml). *S. aureus* and its derivative strains were routinely grown in tryptic soy broth (TSB; BD) medium with aeration (220 rpm) at 37 °C and supplemented with chloromycetin (15 μg/ml) when necessary. If required, the media were solidified with 1.5% (w/v) agar.

### 2.2. DNA manipulations

Genomic DNA was extracted from *S. aureus* using the Wizard Genomic DNA Purification Kit (Promega, San Luis Obispo, CA, USA) according to the manufacturer's instructions, with the cells pre-treated with digestion buffer containing 40 U/ml lysostaphin, 10 mg/ml lysozyme and 10% (v/v) glycerol for 1 h. Plasmid DNA from *E. coli* was extracted using a Plasmid Purification Kit (Promega) according to the manufacturer's instructions. Plasmid DNA from *S. aureus* was extracted using the same kit, except that the cells were pre-incubated with digestion buffer containing 40 U/ml lysostaphin, 10 mg/ml lysozyme and 10% (v/v) glycerol for 30–60 min. Taq DNA polymerase obtained from TransGen was used for routine PCR and PrimerSTAR HS DNA polymerase obtained from TaKaRa was used for amplification of cloning inserts. The DNA fragments were purified with a Gel and PCR Clean-up System (Promega) according to the manufacturer's instructions. Restriction enzymes were used following the manufacturer's instructions (Fermentas, Maryland, USA). Dephosphorylation of vector arms was performed with Antarctica phosphatase (Biolabs, Beverly, MA, USA) and insert ligation was performed with T4 ligase (TransGen, Beijing, China) according to the manufacturers' recommendations.

### 2.3. Construction of overexpression plasmids

To construct the inducible sigma factor overexpression plasmids pRMC*sigB*, pRMC*sigH*, and pRMC*sigS*, the target genes were amplified with the primer pairs *sigB*-f/*sigB*-r, *sigH*-f/*sigH*-r, and *sigS*-f/*sigS*-r, and cloned into the shuttle vector pRMC2 (Corrigan and Foster, 2009). To create the SigB overexpression plasmid pLI*sigB*, a fragment containing *sigB* was amplified with the primer pair oe-*sigB*-f/oe-*sigB*-r, and the fragment covering the *spa* promoter was amplified with the primer pair p*spa*-f/p*spa*-r. The two fragments were ligated with T4 ligase and cloned into the shuttle vector pLI50 (Lee et al., 1991). The plasmids were first introduced into strain RN4220 for modification and subsequently transformed into the strain N315 by electroporation.

### 2.4. Primer extension-blot assay and 5′RACE

Primer extension analysis was performed with the primer r-inner. Briefly, total RNA (5 μg each) was prepared and reverse transcribed with Transcript II RT/RI MIX (TransGen) according to the manufacturer's instructions. The products were separated with a 6.0% denatured polyacrylamide–7 M urea gel in 1 × Tris-borate-EDTA (TBE) and transferred onto a nylon membrane in 0.5 × TBE. The products were then immobilized by UV cross-linking and blotted with the probe p*ccrA*-probe-biotin. DNA markers were prepared using the primer pairs p*ccrA*-r/PCR233 (271 bp), r-inner/PCR233 (242 bp), r-outer/PCR158 (190 bp), and r-inner/PCR158 (158 bp). Biotin was detected with a Chemiluminescent Nucleic Acid Detection Module (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. The images were obtained using ImageQuant LAS 4000 mini (GE, Piscataway, NJ, USA).

5′RACE was performed using the 5′-Full RACE Kit (TaKaRa, Kyoto, Japan) according to the manufacturer's instruction using primers r-outer and r-inner. The PCR products were segregated in a 1.5% DNA agarose gel and purified with a Gel and PCR Clean-up System. The cleanup fragments were cloned into pEASY-blunt (TransGen) and sequenced.

### 2.5. Construction of the LacZ reporter plasmids

To construct the SigA-recognition sequence deletion reporter plasmid pOS*ccrAB*Δ*sigA* of *ccrAB*, an upstream fragment of the 5′ untranslated region (5′UTR) spanning nucleotides −350 to −75 was amplified with the primer pair p*ccrA*-f/up-p*ccrA*-r using N315 genomic DNA as a template, the downstream fragment spanning nucleotides −24 to +201 was amplified with the primer pair down-p*ccrA*-f/*ccrA-r*, and the junction fragment designed with 20-bp overlap with the upstream and downstream fragment was acquired through primer fusion of jun-sense and jun-antisense. The three fragments were ligated by SLiCE as previously mentioned (Zhang et al., 2012). Briefly, the three fragments (50–200 ng) were mixed at a molar ratio of approximately 1:1:1, and then 1 μl of 10 × SLiCE buffer (500 mM Tris–HCl, 100 mM $MgCl_2$, 10 mM DTT, pH 7.5), 1 μl of SLiCE extract, 1 μl of 10 mM ATP, and double-distilled $H_2O$ were added to a final volume of 10 μl. The mixed solution was incubated at 37 °C for 1 h and then amplified with the primer pair p*ccrA*-f/p*ccrA*-r and cloned into plasmid pOS1 (Liu et al., 2011). To construct the SigB-recognition sequence deletion reporter plasmid pOS*ccrAB*Δ*sigB* of *ccrAB*, a previously reported PCR method (Xue et al., 2009) was used. Briefly, a fragment containing the entire length of the plasmid pOS*ccrAB* except for the SigB-recognition sequence was amplified with pOS*ccrAB* as the template using primer pair *sigB*-del-f/*sigB*-del-r. The result fragment was digested with DpnI and SalI and then ligated to generate pOS*ccrAB*Δ*sigB*. The constructed plasmids were first introduced into strain RN4220 for modification and subsequently transformed into strain N315.

### 2.6. β-galactosidase activity assays

For β-galactosidase activity assays, stationary-phase cultures of strain N315 transformed with different promoter-LacZ fusion

**Table 1**
Strains and plasmids used in this study.

| Strain or plasmid | Relevant characteristics | Source or reference |
|---|---|---|
| *S. aureus* | | |
| RN4220 | 8325-4, r⁻, initial recipient for modification of plasmids which are introduced into *S. aureus* from *E. coli* | NARSA[a] |
| N315 | HA-MRSA[b], SCC*mec* type II | NARSA |
| MRSA252 | HA-MRSA[b], SCC*mec* type II | NARSA |
| MW2 | CA-MRSA[b], SCC*mec* type IV | NARSA |
| N315ex | SCC*mec* cured strain of N315 | Zhang et al. (2015) |
| *E. coli* | | |
| *Trans*1-T1 | Clone host strain, F⁻ j80(*lacZ*) ΔM15 Δ*lacX*74 *hsdR* ($r_K^-$ $m_K^+$) Δ*recA*1398 *endA*1*tonA* | TransGen |
| Plasmids | | |
| pEASY blunt | Commercial TA cloning vector, $Amp^r$, $Kan^{rc}$ | TransGen |
| pRMC2 | Shuttle vector, anhydrotetracycline inducible, $Amp^r$, $Cm^r$ [c] | Corrigan and Foster (2009) |
| pRMC*sigB* | pRMC2 derivative, with the coding sequence of *sigB* from strain N315, $Amp^r$, $Cm^r$ | This study |
| pRMC*sigH* | pRMC2 derivative, with the coding sequence of *sigH* from strain N315, $Amp^r$, $Cm^r$ | This study |
| pRMC*sigS* | pRMC2 derivative, with the coding sequence of *sigS* from strain N315, $Amp^r$, $Cm^r$ | This study |
| pLI50 | Shuttle vector, $Amp^r$, $Cm^r$ | Lee et al. (1991) |
| pLI*sigB* | pLI50 derivative, harboring the coding sequence of *sigB* and the promoter sequence of *spa*, for *sigB* overexpression; $Amp^r$, $Cm^r$ | This study |
| pOS1 | Shuttle vector, with *lacZ* coding sequence lacking the first 6 amino acids, $Amp^r$, $Cm^r$ | Liu et al. (2011) |
| pOS*ccrAB* | pOS1 derivative, harboring a 350-bp region of the *ccrAB* promoter and 18 bp of the *ccrA* coding sequence from strain N315, $Amp^r$, $Cm^r$ | Zhang et al. (2015) |
| pOS*ccrABΔsigA* | pOS1 derivative, harboring the same sequence as pOSccrAB except that the putative SigA-recognition sequence was deleted, $Amp^r$, $Cm^r$ | This study |
| pOS*ccrABΔsigB* | pOS1 derivative, harboring the same sequence as pOSccrAB except that the putative SigB-recognition sequence was deleted, $Amp^r$, $Cm^r$ | This study |

[a] NARSA, Network on Antimicrobial Resistance in *Staphylococcus aureus*.
[b] MRSA, methicillin-resistant *Staphylococcus aureus*.
[c] $Kan^r$, kanamycin-resistant; $Amp^r$, ampicillin-resistant; $Cm^r$, chloramphenicol-resistant.

reporter plasmids were diluted 1:500 into TSB (10 ml) with chloromycetin (15 μg/ml), and the cells were collected after incubation at 37 °C for 3 h, 5 h, and 7 h. The cells were first washed once with 500 μl ABT buffer (60 mM $K_2HPO4$, 40 mM $KH_2PO4$, 100 mM NaCl, 0.1% Triton X-100) and then thoroughly lysed at 37 °C with 100 μl ABT LSA buffer (ABT buffer with 50 μg/ml lysostaphin). Then, 100 μl ABT buffer and 100 μl ONPG (o-nitrophenyl-β-D-galactopyranoside, 4 mg/ml) were added to initiate the reaction. The samples were incubated in a 37 °C water bath and 1 ml $Na_2CO_3$ (1 M) was added to stop the reaction when a yellow color became apparent in the samples. The absorbance of each sample at 420 nm was read using a spectrophotometer (DU 800, Beckman Coulter, Fullerton, California, USA) and β-galactosidase activity units were calculated as described previously (Xue et al., 2009).

### 2.7. Total RNA extraction, cDNA generation, and real-time quantitative reverse transcription-PCR (qRT-PCR)

For total RNA isolation from wild-type strain N315 for *ccrAB* transcription profile analysis, a stationary-phase culture of strain N315 was diluted 1:500 into TSB medium. Growth was monitored each hour by measuring the $OD_{600}$ using a spectrophotometer (DU 800, Beckman Coulter), and cells represent variable growth phases were collected. For total RNA isolation from the sigma factor-overexpressing strains containing plasmid pRMC2, pRMC*sigB*, pRMC*sigH*, or pRMC*sigS*, stationary-phase cultures of *S. aureus* were diluted 1:100 into TSB medium containing chloromycetin (15 μg/ml). Following cultivation for 3 h, cells were treated with anhydrotetracycline (ATC) at a final concentration of 50 or 200 ng/ml for different periods of time and were collected.

The collected cells were immediately pelleted with 1 ml RNAiso plus (TaKaRa) and lysed with 0.1-mm-diameter-silica beads in a FastPrep-24 Automated system (MP Biomedicals Solon, OH, USA). The total RNA was isolated following the instructions of RNAiso plus, and residual DNA was removed with RNase-free DNase I (TaKaRa).

Transcription analysis of *ccrAB* was performed by reverse transcription PCR using the Reverse Transcriptase M-MLV (TaKaRa) with primer RT-*ccrB*-r, and the PCR products were analyzed with the primer pairs *ccrB*-f/RT-*ccrB*-r and RT-*ccrA*-f/RT-*ccrB*-r. Reverse transcription for qRT-PCR analysis was performed using a PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa) with random primers. Real-time PCR was carried out with SYBR Premix Ex Taq (TaKaRa) using the StepOne Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). The quantity of cDNA measured by real-time PCR was normalized to the abundance of *16S* or *hu* cDNA (Valihrach and Demnerova, 2012). The primers used in this study are listed in Table 2 and the real-time PCR assays were repeated at least three times.

### 2.8. SCCmec excision and excision frequency determination

The precise excision of SCC*mec* was determined as previously mentioned (Katayama et al., 2000). The plasmids pLI50 and pLI*sigB* were transformed into strain N315. After cultivation in brain heart infusion (BHI) broth at 37 °C for 24 h or 48 h, the cells were collected, and the genomic DNA was extracted. Then, four sets of primers were used to detect precise excision and the closed circle form of SCC*mec*. Primers cL1 and cR1 were used to identify *attB* (274 bp), primers mL1 and mR8 were used to identify *attSCC* (456 bp), and primers cL1 and mL1 were used to detect *attL* (371 bp). Finally, primers mA1 and mA2 were used to detect a fragment of *mecA* gene (286 bp). All the fragments were collected and further confirmed by sequencing.

The precise excision frequency of SCC*mec* was determined using a previously described PCR method (Tao et al., 2010; Zhang et al., 2015). The plasmids pLI50 and pLI*sigB* were transformed into strain N315. After cultivation in BHI broth at 37 °C for 48 h, the cells were collected, and the genomic DNA was extracted. Then, the primer pair mL1/cL1 from two sides of *attL* was used to determine the ratio of the SCC*mec*-retained N315 populations, and the primer pair cL1/cR1 from two sides of *attB* was used for determination of the ratio of the SCC*mec*-excised N315 populations.

## 3. Results

### 3.1. Transcriptional analysis of ccrAB

In MRSA strain N315, the *ccrAB* genes are located between genes encoding two hypothetical proteins with uncharacterized functions (Fig. 1A). Upstream of *ccrA* is a superfamily II helicase-like protein that is present in SCC*mec* type I–IV with high similarity (Lina et al., 2006). A highly conserved AT-enriched sequence located immediately upstream of *ccrA* was postulated to be the promoter sequence of *ccrA* (Katayama et al., 2000; Zhang et al., 2015). While no candidates for promoter sequences were identified in the adjacent upstream region of the *ccrB* gene, *ccrA* and *ccrB* were believed to be co-transcribed (Higgins et al., 2009; Katayama et al., 2000; Stojanov et al., 2013). To determine if the *ccrAB* genes are co-transcribed, reverse-transcriptase PCR of *ccrB* was performed with RNA extracted from strain N315. Using primer pairs spanning fragments of *ccrB* and *ccrAB* (Fig. 1A), and compared to the results with template of genomic DNA or the same amount of RNA only, positive signals were detected (Fig. 1B), suggesting that *ccrA* and *ccrB* can indeed be co-transcribed from the *ccrA* promoter.

Besides, the transcription profiles of *ccrA* and *ccrB* in strain N315 was determined using qRT-PCR. Total RNA was extracted from strain N315 during different phases of growth in TSB. As shown in Fig. 1C, the transcription of *ccrA* and *ccrB* was growth-phase dependent with a higher transcription at the early-exponential phase and a lower transcription at the mid-exponential phase.

### 3.2. SigB overexpression enhances ccrAB expression and SCCmec excision

To determine if the transcription of *ccrAB* is modulated by alternative sigma factors, we created three inducible sigma factor overexpression plasmids, namely pRMC*sigB*, pRMC*sigS*, and pRMC*sigH* (Fig. 2A). The sigma factors were overexpressed under ATC induction, and the qRT-PCR result showed that overexpression of SigB significantly increased the transcriptional level of *ccrA* compared to the wild-type strain, whereas overexpression of SigS and SigH did not (Fig. 2B).

To examine the effect of SigB overexpression on SCC*mec* excision, genomic DNA extracted from the control strain N315ex and strain N315 transformed with plasmid pLI50 (N315 + pLI50) or pLI*sigB* (N315 + pLI*sigB*) was examined with different sets of primers that amplify fragments of *attB*, *attL*, *mecA*, or *attSCC* (Fig. 3A). As shown in Fig. 3B, a fragment of *attB*, which would theoretically be amplified in SCC*mec*-excised cells, was detected in strain N315 carrying plasmid pLI*sigB* following 48 h cultivation, as well as in the control strain N315ex, indicating that overexpression of *sigB* can induce the excision of SCC*mec*. A fragment of *attL* and *mecA*, which would be amplified in SCC*mec*-retained cells, was detected in all the strains except for the strain N315ex (Fig. 3B). However, a fragment of *attSCC*, which represents a circular and extrachromosomal form of SCC*mec*, was not detectable in strain N315 carrying plasmid pLI*sigB* following 48 h cultivation (Fig. 3B). Since the overexpression of *ccrAB* in strains SE5 and SE63 also displayed no *attSCC* bands (Noto and Archer, 2006), we believe that this may be a result of low content of circular SCC*mec* template.

Moreover, we also determined the SCC*mec* excision efficiency of strains carrying plasmids pLI50 and pLI*sigB* following 48 h cultivation. The fragment of *attL* was used to determine the SCC*mec*-retained populations and the fragment of *attB* was used to determine the SCC*mec*-excised populations. The results showed that the strain N315 transformed with plasmid pLI*sigB* showed a significant increase in SCC*mec* excision frequency compared to that of the wild-type strain N315 (N315 + pLI50) (Fig. 3C), revealing an important role of the SigB-dependent promoter in SCC*mec* excision and transfer.

### 3.3. SigB recognizes the ccrAB promoter and initiates ccrAB transcription

To determine if SigB modulates *ccrA* through a specific promoter or some intermediate regulators, total RNA from the SigB overexpression strain (N315 + pRMC*sigB*) after induction with ATC for different periods of time was analyzed using qRT-PCR. The results indicated that *ccrA* upregulated immediately after SigB was induced and overexpression of SigB displayed a similar effect on the transcription of *ccrA* and the SigB-dependent gene *asp23* (Fig. 4A), suggesting that SigB may be responsible for the transcription of *ccrA* through a SigB-dependent promoter. To confirm this, RNA extracted from strain N315 carrying plasmid pRMC2 or pRMC*sigB* was analyzed with a primer extension assay combined with nucleotide blotting. Reverse transcription of *ccrAB* with a primer located near the translation initiation site was performed and fragments of different sizes within the *ccrAB* promoter were used as markers. The bands were separated by electrophoresis, transferred to a charged nylon membrane, and blotted with a

**Table 2**
Primers used in this study.

| Primer | Sequence (5′–3′)[a] | Application or reference |
|---|---|---|
| RT-*ccrA*-f | GCACAGTTATTAGAAGAAGATA | qRT-PCR |
| RT-*ccrA*-r | GCCATATTGATTGTTGACA | qRT-PCR |
| RT-*ccrB*-f | CAATACCACGAATACACTT | qRT-PCR |
| RT-*ccrB*-r | CATCACATAATCTTCAATCAC | qRT-PCR |
| RT-*asp23*-f | CAAGCATACGACAATCAA | qRT-PCR |
| RT-*asp23*-r | TATCAGTTAAGCCACCTT | qRT-PCR |
| RT-*hu*-f | AAAAAGAAGCTGGTTCAGCAGTAG | qRT-PCR |
| RT-*hu*-r | TTTACGTGCAGCACGTTCAC | qRT-PCR |
| RT-*16S*-f | CGTGGAGGGTCATTGGA | qRT-PCR |
| RT-*16S*-r | CGTTTACGGCGTGGACTA | qRT-PCR |
| *ccrB*-f | CGACGGACAAATCAATCAAA | PCR analysis |
| *sigB*-f | GCGggtaccGGGAGGTTTTAAACATGGCGAAAGAGTCGAAATCAGCT | pRMC*sigB* |
| *sigB*-r | GCGgaattcATTGAAAATGACACACCATC | pRMC*sigB* |
| *sigH*-f | GCGggtaccGGGAGGCCGTTTCATGAAATATGATTTGAC | pRMC*sigH* |
| *sigH*-r | GCGgaattcCAAGCTTCACAATTTAAAGG | pRMC*sigH* |
| *sigS*-f | GCGggtaccGGGAGGCCGTTTCATGAAATTTAATGACGTATACA | pRMC*sigS* |
| *sigS-r* | GCGgaattcGGAACTTTAAGAATGTAATCGC | pRMC*sigS* |
| p*spa*-f. | GCGgaattcATAATGAACAACTTTCTA | pLI*sigB* |
| p*spa*-r | GCGtctagaTACCCCCTGTATGTATTTGT | pLI*sigB* |
| oe-*sigB*-f | TAtctagaATGGCGAAAGAGTCGAAATC | pLI*sigB* |
| oe-*sigB*-r | GCGaagcttCTATTTATGTGCTGCTTCTTGT | pLI*sigB* |
| cL1 | ATTTAATGTCCACCATTTAACA | Katayama et al. (2000) |
| cR1 | AAGAATTGAACCAACGCATGA | Katayama et al. (2000) |
| mL1 | GAATCTTCAGCATGTGATTTA | Katayama et al. (2000) |
| mR8 | ATGAAAGACTGCGGAGGCTAACT | Katayama et al. (2000) |
| mA1 | TGCTATCCACCCTCAAACAGG | Katayama et al. (2000) |
| mA2 | AACGTTGTAACCACCCCAAGA | Katayama et al. (2000) |
| r-outer | CTTTGACGTAAATAGCCTAT | 5′RACE |
| r-inner | ATCGGCTCCTCCTTTCACAG | 5′RACE |
| PCR158 | TAAGTTCATCCATGTTTTTC | Markers prepare |
| PCR233. | GCGgaattcTTGTCTTTATCATACAACCG | Markers prepare |
| p*ccrA*-probe-biotin | GTTGGGCTTATATATCAATTCATCCATAAATATATATTATTACTGTGAAAGGAGGAGCC | 5′cDNA blot |
| p*ccrA*-f | GCGgaattcAAAGATAAGTCGTTAATTCA | Zhang et al. (2015) |
| p*ccrA*-r | GCGggatccGGGCCTATGACTTGTTTCATAT | Zhang et al. (2015) |
| *ccrA-r* | GTCACATTGCCCTTGTTGAA | pOS*ccrAB*Δ*sigA* |
| up-p*ccrA*-r | TTGGGCGTTCATATTAGTCA | pOS*ccrAB*Δ*sigA* |
| down-p*ccrA*-f | ATTACTGTGAAAGGAGGAGC | pOS*ccrAB*Δ*sigA* |
| jun-sense | GCTCCTCCTTTCACAGTAATGCCCAACATCAATAAGATGTTGGGCGTTCATATTAGTCA | pOS*ccrAB*Δ*sigA* |
| jun-antisense | TGACTAATATGAACGCCCAACATCTTATTGATGTTGGGCATTACTGTGAAAGGAGGAGC | pOS*ccrAB* Δ*sigA* |
| *sigB*-del-f | ACGCgtcgacAGGCCCTTCAGGAGTTCACGAAGA | pOS*ccrAB* Δ*sigB* |
| *sigB-del-r* | ACGCgtcgacAGTTCTTTCGCAGCTTTGTTATCAT | pOS*ccrAB* Δ*sigB* |

[a] The sequences in lower-case letters refer to the restriction endonuclease recognition sites, and the sequences underlined represent overlap sequences that are suitable for SLiCE.

biotin-labeled probe. The results showed that SigB overexpression induced the appearance of a specific band compared to the wild type strain (Fig. 4B). To determine the transcription start site of the SigB-dependent promoter, we performed 5′RACE using total RNA extracted from the SigB overexpression strain (N315 + pRMC*sigB*). The results showed that the transcription start site was located 255-bp upstream of the translation initiation site of *ccrA*. The SigB-recognition sequence P2 (tTTTAA-12-GGGgAT) (Fig. 5A) that we identified displays great similarity to the consensus SigB-recognition sequence of *Bacillus subtilis* (GTTTAA-12/14-GGGTAT) (Petersohn et al., 1999).

### 3.4. CcrAB *operon is transcribed in a two-promoter pattern*

Together with the previously predicted promoter of *ccrA* (Katayama et al., 2000), two promoter regions were identified: promoter P1 with a sequence similar to the SigA-dependent promoter and P2 with a sequence resembling the consensus SigB-dependent promoter (Fig. 5A). To verify the function of the putative SigA and SigB promoter sequences, we constructed the following three promoter-LacZ fusion reporter plasmids: pOS*ccrAB* with the promoter region containing both the putative SigA- and SigB-dependent sequences, pOS*ccrAB*Δ*sigA* with the putative SigA-recognition sequence precisely deleted, and pOS*ccrABs*Δ*sigB* with the putative SigB-recognition sequence substituted with the 6-bp linker sequence (“GTCGAC”, *SalI* linker). Then, the β-galactosidase activity of those strains was detected and compared. As shown in Fig. 5B, the absence of the SigA-recognition sequence significantly decreased *ccrA* promoter activity and the absence of the SigB-recognition sequence only showed a slight decrease in *ccrA* promoter activity, revealing a lower activity of the SigB-dependent promoter at common condition.

### 3.5. *Sequence alignment and transcriptional analysis of the SigB-recognition region in SCC*mec *type II and IV MRSA strains*

To further determine if the SigB-dependent promoter exists in other SCC*mec* type II or IV strains, in which *ccrAB* genes belong to the same *ccr* alleles, we analyzed the potential SigB-recognition sequences in these strains. As shown in Fig. 6A, there exists a homologous sequence located on the same site in these strains. To determine if the homologous region can be recognized by SigB, we chose strains MRSA252 (SCC*mec* type II, tTTTAA-12-aGGTAT) and MW2 (SCC*mec* type IV, tTTTAA-12-aGGgAT) for further analysis. The qRT-PCR result indicated that overexpression of SigB can significantly increase the transcriptional levels of *ccrA* and *asp23* in strain MRSA252 (Fig. 6B). However, overexpression of SigB had no effect on *ccrA* transcription in strain MW2, despite that it can dramatically increase the transcriptional level of gene *asp23* (Fig. 6C).

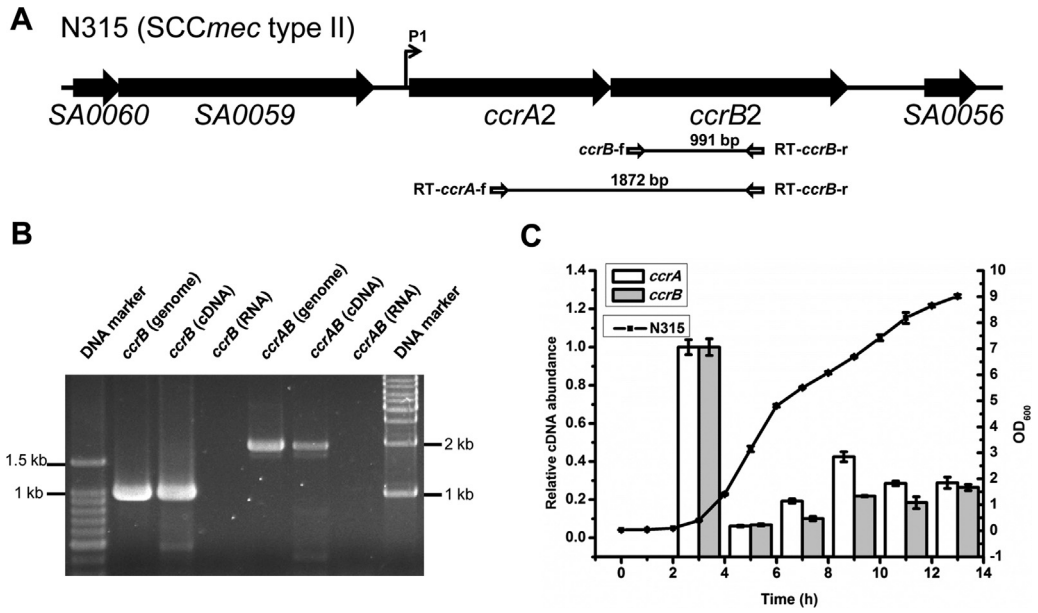


**Fig. 1.** Transcriptional analysis of *ccrAB*. (A) Genetic organization of the *ccrAB* gene complex and the location of primers used in the reverse-transcription PCR assay. Transcription start site of the putative promoter region is indicated by the bent arrow. (B) Gel electrophoresis analysis of PCR products amplified with primers spanning fragments of *ccrB* and *ccrAB*. Genomic DNA, reverse-transcription cDNA with primer RT-*ccrB*-r, and the same amount of total RNA from strain N315 were used as template. (C) Transcription of *ccrA* and *ccrB* is growth-phase dependent. The growth curve was monitored each hour by measuring the $OD_{600}$ using a spectrophotometer (DU 800, Beckman Coulter), and cells grown to different time points were collected. The profiles of *ccrA* and *ccrB* transcription were analyzed using qRT-PCR. Signals were normalized to the abundance of *hu* cDNA. Error bars represent standard deviations (SD) (n = 3).

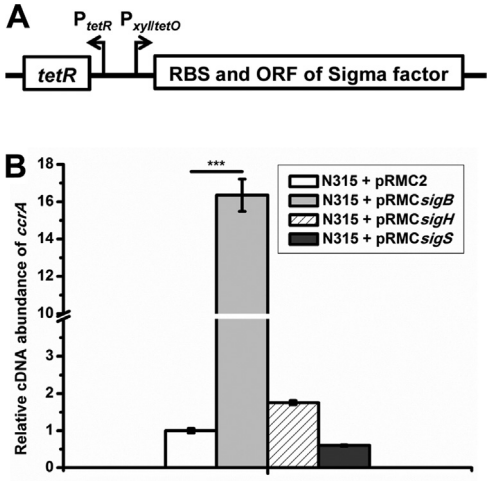


**Fig. 2.** SigB promotes the transcription of *ccrA*. (A) A diagram showing construction of the inducible sigma factors overexpression plasmids, consensus ribosomal binding sequence were inserted for proper translation. (B) qRT-PCR analysis of *ccrA* in strain N315 carrying plasmid pRMC2, pRMC*sigB*, pRMC*sigH*, or pRMC*sigS*. Cultures were induced with ATC (200 ng/ml) and cells were collected at 1 h. Signals were normalized to the abundance of *16S* cDNA. Error bars represent SD (n = 3). Statistically significant differences calculated by the unpaired two-tailed Student's *t*-test are indicated: NS, not significant, $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

## 4. Discussion

Mobile genetic elements (MGEs), which are important means in the transferring of genetic information, play a central role in the adaptation of bacteria to selective pressure. In *S. aureus*, many MGEs have been identified, including insertion sequences, transposons, bacteriophages, plasmids, pathogenicity islands, and staphylococcal cassette chromosomes (Malachowa and DeLeo, 2010). The CcrAB recombinases, which play a key role in the site-specific excision and integration of SCC*mec* (Katayama et al., 2000), belong to the large serine family of site-specific DNA recombinases that includes many bacteriophage integrases and transposases (Peacock and Paterson, 2015). Under some conditions, prophage genomes can be excised from bacterial chromosomes and reintegrated later by the *int-xis* system (Nash, 1981). In *S. aureus*, spontaneous SCC*mec* excision has also been observed at a low frequency (Ito et al., 1999). Besides, conserved inverted repeat sequences have been discovered in the promoter sequences of both *ccrAB* and *int* genes, and fulfill important regulatory roles (Iandolo JJ et al., 2002; Zhang et al., 2015). These findings indicated that the two different mobile genetic elements may be regulated by similar mechanisms.

Since SigH has been shown to modulate *int* gene expression and prophage integration and excision in *S. aureus* (Tao et al., 2010), alternative sigma factors may also be involved in the regulation of *ccrAB* expression and SCC*mec* excision. Through overexpression of alternative sigma factors that have been identified in *S. aureus*, we found that SigB is involved in the modulation of *ccrAB* transcription in MRSA strain N315. Then, SCC*mec* excision was

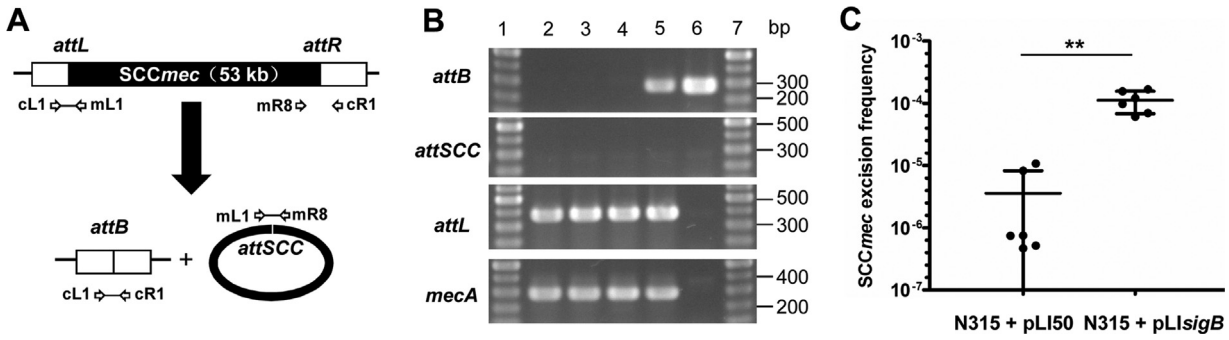


**Fig. 3.** Determination of the *sigB*-mediated SCC*mec* excision. (A) A schematic represent the SCC*mec* excision in strain N315. The primer pair cL1/cR1 from two sides of *attB* was used for determination of the ratio of the SCC*mec*-excised N315 genomic DNA, and the primer pair mL1/cL1 from two sides of *attL* was used to determine the ratio of the SCC*mec*-retained N315 genomic DNA. (B) PCR detection of SCC*mec* excision. Genomic DNA was extracted from N315 strains cultivated in BHI broth at 37 °C for 24 h or 48 h. Primer pairs cL1/cR1, mL1/mR8, cL1/mL1, and mA1/mA2 were used to detect *attB* (274 bp), *attSCC* (456 bp), *attL* (371 bp), and a part of the *mecA* gene (286 bp), respectively. Lanes 1 and 7: DNA marker; lane 2: strain N315 carrying plasmid pLI50 (24 h); lane 3: strain N315 carrying plasmid pLI*sigB* (24 h); lane 4: strain N315 carrying plasmid pLI50 (48 h); lane 5: strain N315 carrying plasmid pLI*sigB* (48 h); lane 6: strain N315ex. The molecular weight marker is a 100-bp DNA marker (TransGen) and some of the relevant bands are indicated. (C) SCC*mec* excision frequencies mediated by *sigB* overexpression. Genomic DNA was extracted from N315 strains cultivated in BHI broth at 37 °C for 48 h. Error bars represent SD (n = 6). 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.

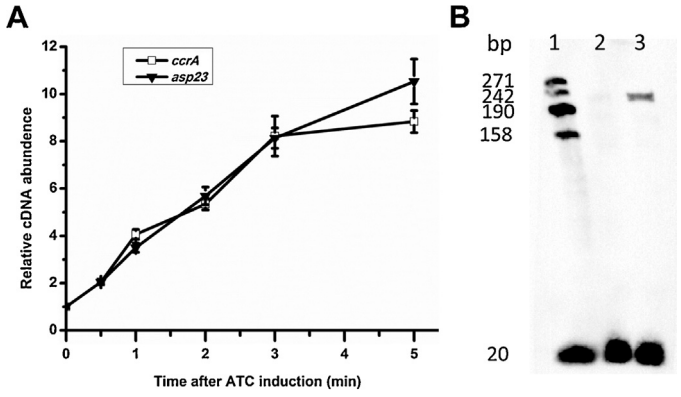


**Fig. 4.** SigB enhances *ccrA* transcription through an unreported SigB-dependent promoter. (A) Overexpression of SigB has a similar effect on the transcription of *ccrA* and the SigB-dependent gene *asp23*. Cultures were induced with ATC (50 ng/ml) and cells were collected at 0 min, 0.5 min, 1 min, 2 min, 3 min, and 5 min. Signals were normalized to the abundance of *hu* cDNA. Error bars represent SD (n = 3). (B) Primer extension analysis shows a specific band in strain N315 overexpressing SigB (N315 + pRMC*sigB*). Lane 1: DNA fragments containing different partial regions of the *ccrAB* promoter, which were used as molecular weight markers; lane 2: primer extension with total RNA extracted from the strain carrying pRMC2 (N315 + pRMC2) after induction with ATC (200 ng/ml) for 30 min; lane 3: primer extension with total RNA extracted from the strain carrying pRMC*sigB* (N315 + pRMC*sigB*) after induction with ATC (200 ng/ml) for 30 min.

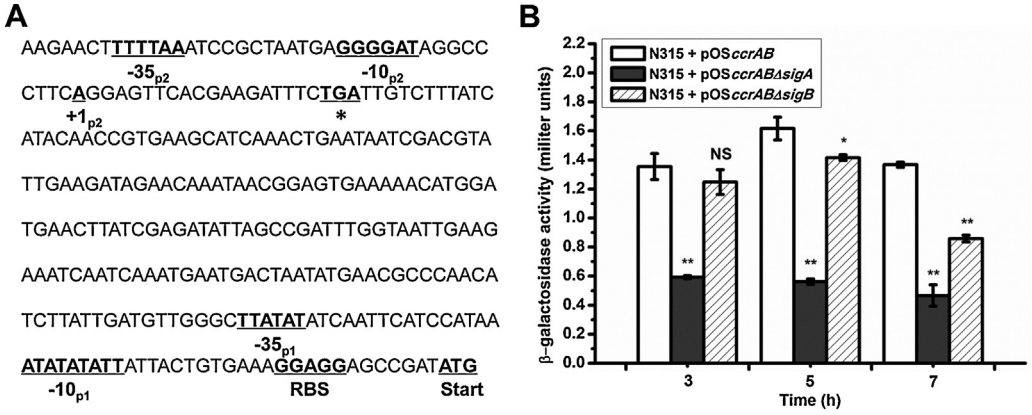


**Fig. 5.** *CcrAB* operon is transcribed in a two-promoter pattern. (A) Nucleotide sequence of the region upstream of *ccrA*. The putative SigA-dependent promoter P1 and the SigB-dependent promoter P2 are underlined and marked. The sequence of the P1 was predicted using the online software BPROM (http://linux1.softberry.com/berry.phtml) and the transcription start site (TSS) of the P2 was defined with 5′RACE. The putative ribosome-binding site is underlined and labeled with RBS, the start codon of *ccrA* is underlined and marked with Start, and the *SA0059* stop codon is indicated by an asterisk. (B) The β-galactosidase activities of plasmids pOS*ccrAB*, pOS*ccrABΔsigA*, and pOS*ccrABΔsigB* in strain N315. Cells were collected after 3, 5, and 7 h of growth in TSB containing chloromycetin (15 μg/ml) and the β-galactosidase activity was detected with ONPG (4 mg/ml). 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.

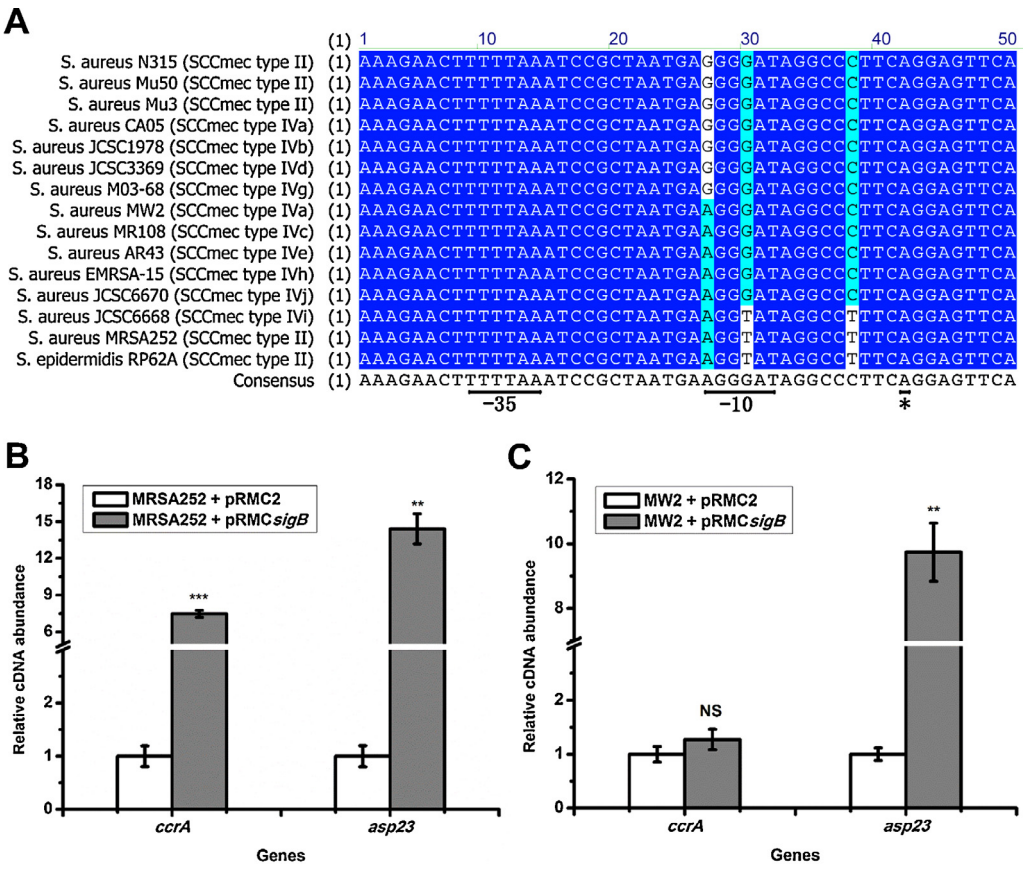


**Fig. 6.** Pervasive analysis of the SigB-recognition sequence in SCC*mec* type II and IV MRSA strains. (A) Multiple-sequence alignment of the potential SigB-recognition sequence. The putative −35 and −10 regions are marked, and the TSS (*) is indicated. Dark blue indicates identical bases, and light blue indicates the conservative nucleotide. (B) qRT-PCR analysis of *ccrA* and *asp23* in strain MRSA252 carrying plasmid pRMC2 or pRMC*sigB*. Cultures were induced with ATC (200 ng/ml) and cells were collected at 15 min. (C) qRT-PCR analysis of *ccrA* and *asp23* in strain MW2 carrying plasmid pRMC2 or pRMC*sigB*. Cultures were induced with ATC (200 ng/ml) and cells were collected at 15 min. Signals were normalized to the abundance of *hu* cDNA. 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. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

determined in the SigB overexpression strain, and a dramatic increase in SCC*mec* excision was exhibited. These results indicate that SigB plays an important role in *ccrAB* expression and SCC*mec* excision. The qRT-PCR results of *ccrA* and *asp23* revealed that SigB may be responsible for *ccrA* transcription directly, and further *in vitro* experiments revealed that a SigB-recognition promoter region of *ccrA* is located within the coding region of the upstream gene. The SigB-recognition sequence in *S. aureus* has been reported to resemble the SigB consensus promoter sequence of *B. subtilis* (Bischoff et al., 2004), and different SigB-recognition sequences have been identified with similar sequences in *S. aureus* (Homerova et al., 2004). The SigB-dependent promoter region that we identified was a heretofore unrecognized sequence, but it also shares high similarity with the conserved sequence of *B. subtilis*. Through multiple-sequence alignment of the potential SigB-recognition region in strains that carry type II or IV SCC*mec*, we found that a homologous region is located on the same site. Strain MRSA252, which also carries the type II SCC*mec*, displayed a significant upregulation in *ccrA* transcription when SigB was overexpressed. However, strain MW2, which carries the type IV SCC*mec*, exhibited no difference in *ccrA* transcription even though the transcriptional level of *asp23* was dramatically increased. Notably, there are plenty of SCC*mec* type II and IV strains share the same sequences to strain N315 or MRSA252, implying that the SigB-dependent *ccrA* transcription may exists in certain SCC*mec* type II and IV strians. However, a systematic analysis is needed to explain the difference and evolution of these strains.

Together with the previously postulated promoter (Katayama et al., 2000; Zhang et al., 2015), *ccrA* was identified to be transcribed in a two-promoter pattern. However, the SigB-dependent promoter that we identified was not included in the reporter plasmids designed by Higgins et al. (Higgins et al., 2009) and Stojanov et al. (Stojanov et al., 2013), and there is no sequence similar to the SigB-consensus promoter sequence within the sequence selected by the previous studies, so the environmental sensing identified in the previous studies may be the function of other regulators. Notably, the promoter sequences of *ccrA* that we uncovered shows high similarity to previous reports in which the genes *sarS* and *esxA* were identified as also harboring a remote SigB-recognition sequence in addition to the nearby SigA-dependent promoter, and were significantly regulated by the SigB-associated regulators (Schulthess et al., 2012; Tegmark et al., 2000). Moreover, the promoter-LacZ fusion assay showed that the activity of the SigB-dependent promoter is limited under normal growth conditions compared to the SigA-dependent promoter, which displays beneficial effects for the steady existence of SCC*mec* in MRSA strains. While SigB plays a significant role in sensing environmental stimuli and cell stresses, some environmental factors may induce a high activity of SigB and subsequent *ccrAB* expression, which should result in a high efficiency of SCC*mec* excision and transfer. Considering that the SigB-induced loss of SCC*mec* only happened in a low fraction of cells, the association of SigB expression with methicillin resistance in *Staphylococcus epidermidis* and *S. aureus* is perhaps modulated

by other regulatory mechanisms (Knobloch et al., 2005; Morikawa et al., 2001).

Antibiotic-resistant *S. aureus* emerged in a series of waves (Chambers and Deleo, 2009), and the emergence of MRSA strains posed serious challenges for the treatment of infections. Since coagulase-negative staphylococci are believed to be a potential reservoir of *mecA* for *S. aureus* (Hiramatsu et al., 2001) and SigH-associated competency provides *S. aureus* with the ability to acquire exogenous genes (Morikawa et al., 2012), the mechanisms by which MGEs are excised and transferred in environmental conditions have attracted much interest. In this study, we present a different understanding of *ccrAB* gene structure. Since SigB is a sensor of different environmental stimuli and cell stresses, we also provide a novel explanation regarding the relationship of environmental stimuli and SCC*mec* excision. Further investigation of the molecular mechanism of environmental stimuli associated with the effect of *sigB* and its downstream regulators on *ccrAB* expression, as well as subsequent SCC*mec* excision and transfer among different staphylococcal strains, should provide new targets and strategies for the treatment of MRSA infections.

## 5. Conclusions

In summary, this study revealed that an unrecognized SigB-dependent promoter is involved in the control of *ccrAB* transcription and SCC*mec* excision, and the *ccrAB* genes are in fact transcribed in a two-promoter pattern in MRSA strain N315.

## Acknowledgments

We thank Ma R and Liu X for technical assistance. This study was supported by the National Natural Science Foundation of China (81371850).

## References

Ayliffe, G.A., 1997. The progressive intercontinental spread of methicillin-resistant *Staphylococcus aureus*. Clin. Infect. Dis. 24 (Suppl 1), S74–79.
Bischoff, M., Dunman, P., Kormanec, J., Macapagal, D., Murphy, E., Mounts, W., Berger-Bachi, B., Projan, S., 2004. Microarray-based analysis of the *Staphylococcus aureus* sigmaB regulon. J. Bacteriol. 186, 4085–4099.
Chambers, H.F., Deleo, F.R., 2009. Waves of resistance: *Staphylococcus aureus* in the antibiotic era. Nat. Rev. Microbiol. 7, 629–641.
Corrigan, R.M., Foster, T.J., 2009. An improved tetracycline-inducible expression vector for *Staphylococcus aureus*. Plasmid 61, 126–129.
Engelmann, S.G.á.S., 1999. Regulation of sigmaB-dependent transcription of *sigB* and *asp23* in two different *Staphylococcus aureus* strains. Mol. Gen. Genet.
Hartman, B.J., Tomasz, A., 1984. Low-affinity penicillin-binding protein associated with beta-lactam resistance in *Staphylococcus aureus*. J. Bacteriol. 158, 513–516.
Higgins, P.G., Rosato, A.E., Seifert, H., Archer, G.L., 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.
Hiramatsu, K., Cui, L., Kuroda, M., Ito, T., 2001. The emergence and evolution of methicillin-resistant *Staphylococcus aureus*. Trends Microbiol. 9, 486–493.
Homerova, D., Bischoff, M., Dumolin, A., Kormanec, J., 2004. Optimization of a two-plasmid system for the identification of promoters recognized by RNA polymerase containing *Staphylococcus aureus* alternative sigma factor sigmaB. FEMS Microbiol. Lett. 232, 173–179.
Iandolo JJ, W.V., Groicher, K.H., Qian, Y., Tian, R., et al., 2002. Comparative analysis of the genomes of the temperate bacteriophages phi11, phi12 and phi13 of *Staphylococcus aureus* 8325. Gene 289, 109–118.
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.
Ito, T., Ma, X.X., 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.
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.
Knobloch, J.K., Jager, S., Horstkotte, M.A., Rohde, H., Mack, D., 2004. RsbU-dependent regulation of *Staphylococcus epidermidis* biofilm formation is mediated via the alternative sigma factor sigmaB by repression of the negative regulator gene *icaR*. Infect. Immun. 72, 3838–3848.
Knobloch, J.K., Jager, S., Huck, J., Horstkotte, M.A., Mack, D., 2005. *mecA* is not involved in the sigmaB-dependent switch of the expression phenotype of methicillin resistance in *Staphylococcus epidermidis*. Antimicrob. Agents Chemother. 49, 1216–1219.
Kullik, I., Giachino, P., 1997. The alternative sigma factor sigma(B) in *Staphylococcus aureus:* regulation of the *sigB* operon in response to growth phase and heat shock. Arch. Microbiol. 167, 151–159.
Lee, C.Y., Buranen, S.L., Ye, Z.H., 1991. Construction of single-copy integration vectors for *Staphylococcus aureus*. Gene 103, 101–105.
Lina, G., Durand, G., Berchich, C., Short, B., Meugnier, H., Vandenesch, F., Etienne, J., Enright, M.C., 2006. Staphylococcal chromosome cassette evolution in *Staphylococcus aureus* inferred from *ccr* gene complex sequence typing analysis. Clin. Microbiol. Infect. 12, 1175–1184.
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.
Malachowa, N., DeLeo, F.R., 2010. Mobile genetic elements of *Staphylococcus aureus*. Cell. Mol. Life Sci. 67, 3057–3071.
Morikawa, K., Maruyama, A., Inose, Y., Higashide, M., Hayashi, H., Ohta, T., 2001. Overexpression of sigma factor sigma(B), urges *Staphylococcus aureus* to thicken the cell wall and to resist beta-lactams. Biochem. Biophys. Res. Commun. 288, 385–389.
Morikawa, K., Inose, Y., Okamura, H., Maruyama, A., Hayashi, H., Takeyasu, K., Ohta, T., 2003. A new *staphylococcal sigma* factor in the conserved gene cassette: functional significance and implication for the evolutionary processes. Genes Cells 8, 699–712.
Morikawa, K., Takemura, A.J., Inose, Y., Tsai, M., Nguyen Thi le, T., Ohta, T., Msadek, T., 2012. Expression of a cryptic secondary sigma factor gene unveils natural competence for DNA transformation in *Staphylococcus aureus*. PLoS Pathog. 8, e1003003.
Nash, H.A., 1981. Integration and excision of bacteriophage lambda: the mechanism of conservation site specific recombination. Annu. Rev. Genet. 15, 143–167.
Noto, M.J., Archer, G.L., 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.
Pane-Farre, J., Jonas, B., Forstner, K., Engelmann, S., Hecker, M., 2006. The sigmaB regulon in *Staphylococcus aureus* and its regulation. Int. J. Med. Microbiol. 296, 237–258.
Peacock, S.J., Paterson, G.K., 2015. Mechanisms of methicillin resistance in *Staphylococcus aureus*. Annu. Rev. Biochem 84, 577–601.
Petersohn, A., Bernhardt, J., Gerth, U., Hoper, D., Koburger, T., Volker, U., Hecker, M., 1999. Identification of sigma(B)-dependent genes in *Bacillus subtilis* using a promoter consensus-directed search and oligonucleotide hybridization. J. Bacteriol. 181, 5718–5724.
Schulthess, B., Bloes, D.A., Berger-Bachi, B., 2012. Opposing roles of sigmaB and sigmaB-controlled SpoVG in the global regulation of *esx*A in *Staphylococcus aureus*. BMC Microbiol. 12, 17.
Senn, M.M., Giachino, P., Homerova, D., Steinhuber, A., Strassner, J., Kormanec, J., Fluckiger, U., Berger-Bachi, B., Bischoff, M., 2005. Molecular analysis and organization of the sigmaB operon in *Staphylococcus aureus*. J. Bacteriol. 187, 8006–8019.
Shaw, L.N., Lindholm, C., Prajsnar, T.K., Miller, H.K., Brown, M.C., Golonka, E., Stewart, G.C., Tarkowski, A., Potempa, J., 2008. Identification and characterization of $\sigma^S$, a novel component of the *Staphylococcus aureus* stress and virulence responses. PLoS One 3, e3844.
Singh, V.K., Schmidt, J.L., Jayaswal, R.K., Wilkinson, B.J., 2003. Impact of *sigB* mutation on *Staphylococcus aureus* oxacillin and vancomycin resistance varies with parental background and method of assessment. Int. J. Antimicrob. Agents 21, 256–261.
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.
Tao, L., Wu, X., Sun, B., 2010. Alternative sigma factor sigmaH modulates prophage integration and excision in *Staphylococcus aureus*. PLoS Pathog. 6, e1000888.
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.
Valihrach, L., Demnerova, K., 2012. Impact of normalization method on experimental outcome using RT-qPCR in *Staphylococcus aureus*. J. Microbiol. Methods 90, 214–216.
Wu, S., de Lencastre, H., Tomasz, A., 1996. Sigma-B, a putative operon encoding alternate sigma factor of *Staphylococcus aureus* RNA polymerase: molecular cloning and DNA sequencing. J. Bacteriol. 178, 6036–6042.
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.
Zhang, Y., Werling, U., Edelmann, W., 2012. SLiCE: a novel bacterial cell extract-based DNA cloning method. Nucleic Acids Res. 40, e55.
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.