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Antibiotics trigger initiation of SCC*mec* transfer by inducing SOS responses

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ABSTRACT

The rise of antimicrobial resistance limits therapeutic options for infections by methicillin-resistant staphylococci. The staphylococcal cassette chromosome *mec* (SCC*mec*) is a mobile genetic element as the only carrier of the methicillin-resistance determinants, the *mecA* or *mecC* gene. The use of antibiotics increases the spread of antibiotic resistance, but the mechanism by which antibiotics promote horizontal dissemination of SCC*mec* is largely unknown. In this study, we demonstrate that many antibiotics, including β-lactams, can induce the expression of *ccrC1* and SCC*mec* excision from the bacterial chromosome. In particular, three widely used antibiotics targeting DNA replication and repair (sulfamethoxazole, ciprofloxacin and trimethoprim) induced higher levels of *ccrC1* expression and higher rates of SCC*mec* excision even at low concentrations (1/8 × minimum inhibitory concentration). LexA was identified as a repressor of *ccrC1* and *ccrAB* by binding to the promoter regions of *ccrC1* and *ccrAB*. The activation of RecA after antibiotic induction alleviated the repression by LexA and increased the expression of *ccrC1* or *ccrAB*, consequently increasing the excision frequency of the SCC*mec* for SCC*mec* transfer. These findings lead us to propose a mechanism by which antimicrobial agents can promote horizontal gene transfer of the *mecA* gene and facilitate the spread of methicillin resistance.

INTRODUCTION

Methicillin-resistant staphylococci (MRS) are major causes of bacterial infections in humans and animals. The *mecA* or *mecC* gene responsible for methicillin resistance is located on a mobile genomic island, the staphylococcal cassette chromosome *mec*, which is a major gene acquisition machine and is responsible for the horizontal dissemination of many resistance genes (1).

Coagulase-negative staphylococci (CoNS) may serve as gene reservoirs facilitating the conversion of methicillin-susceptible *Staphylococcus aureus* (MSSA) to methicillin-resistant *S. aureus* (MRSA) (2). The transmission of staphylococcal cassette chromosome *mec* (SCC*mec*) from MR-CoNS to MSSA has been proposed based on the following: (i) the original source of the *mecA* gene appears to be *S. sciuri* or *S. fleurettii* (3,4); (ii) the IS*1272* element inserted in SCC*mec* is observed much more frequently in *Staphylococcus haemolyticus* than in *S. aureus* (5) and (iii) methicillin resistance is more frequent among CoNS than *S. aureus* (6,7). Although there is no evidence for SCC*mec* transfer from MRSA to CoNS, the restriction of the SCC*mec* element to the *Staphylococcus* genus and the homology of SCC*mec* DNA sequences between different staphylococcal species highly suggest that the transfer of SCC*mec* among different staphylococci is possible (8).

As a mobile genetic element, SCC*mec* integrates and excises from a unique location in the staphylococcal chromosome within the 3′ end of the highly conserved ribosomal methyltransferase gene *orfX*, which is also known as *rlmH* (9,10). For the movement, SCC*mec* carries specific gene complexes containing the cassette chromosome recombinase genes AB or C (*ccrAB or ccrC*), which encode proteins belonging to the large serine recombinase family and recognizing the specific recombination sites of SCC*mec* (11,12). The *ccr* genes with nucleotide identities of <50% are distinguished as distinct types. Within *ccrA* and *ccrB* types, allotypes *ccrA1–7* and *ccrB1–6* have been characterized based on their nucleotide identities (>85% within each allotype). In the *ccrC* type, only two allotypes have been identified: *ccrC1* and *ccrC2* (8,13). Eleven alleles of *ccrC1* have been assigned (14). The Ccr proteins mediate integration and excision of the SCC*mec* into and from the chromosome. The integration of SCC*mec* occurs via Ccr-mediated recombination between this unique sequence (designated *attB*) and a specific site in the circularized SCC*mec* element (designated *attSCC*). When SCC*mec* is inserted, a new pair of sites is generated, referred to as *attL* and *attR* flanking the

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element's left and right ends, respectively. During SCC*mec* excision the reverse happens-*attR* and *attL* sites are recombined to regenerate the original sites, *attB* and *attSCC* (9). β-lactam antibiotics have been recently shown to increase expression of *ccrAB* (15). However, the regulatory mechanisms by which antibiotics promote expression of *ccrAB* and *ccrC1* and initiate SCC*mec* transfer have not been elucidated. In the studies by Cuirolo *et al.* (16) and Plata *et al.* (17), oxacillin (OXA) induced the SOS response in MRSA and mitomycin C (MMC) induced *mecA* expression, showing that SOS was important for the resistant phenotype.

The SOS pathway is employed by bacteria to respond to various bactericidal agents, including β-lactam antibiotics (18,19). Governed by the products of *lexA* and *recA* genes, the SOS pathway coordinates a comprehensive response against DNA lesions (20). Under basal conditions, the transcription of dozens of genes involved in the SOS response is repressed (21). When chromosomes are damaged, persisting regions of single-stranded DNA allow the assembly of activated RecA nucleoprotein filaments called RecA*. The presence of RecA* activates the transcriptional upregulation of SOS genes by facilitating the cleavage of the LexA repressor (22). The SOS response is involved not only in cell division inhibition, nucleotide excision repair and recombination repair (23), but also in horizontal gene transfer (HGT) to neighboring cells (24), such as the transfer of pathogenicity island-encoded virulence factors in staphylococci (25,26), the transfer of integrating conjugative elements in *Vibrio cholerae* (27) and the recombination of chromosomal integrons in many Gram-negative pathogens (28–30). In this study, we provide evidence that antibiotics trigger the initiation of SCC*mec* transfer by inducing SOS responses.

MATERIALS AND METHODS

Bacterial strains, media and culture conditions

The bacterial strains and plasmids used in this study are listed in Supplementary Table S1. Strain NW19 is an OXA-susceptible, *mecA* positive *S. haemolyticus* with *ccrC1* allele 2 and 8 in type V(5C2&5) SCC*mec* (GenBank accession number, MRUY00000000). MRSA strain Mu50 has *ccrA2B2* genes in its type II SCC*mec*. *E.* coli DH5α was cultured in the Luria–Bertani medium supplemented with 100 mg/L ampicillin at 37°C. *S. aureus* and *S. haemolyticus* strains were grown in the trypticase soy broth (TSB) at 37°C with the appropriate antibiotic agents (chloramphenicol, 10 mg/L; Zeocin, 25 mg/L). MMC was used at a final concentration of 0.5 mg/L. The minimum inhibitory concentratidfons (MICs) of antibiotics were determined by the agar dilution method according to Clinical and Laboratory Standards Institute recommendations (31).

Transcript determinations

One colony of each sample was inoculated in 2 ml of the TSB medium and incubated at 37°C overnight. The overnight culture was diluted to $OD_{600} = 0.05$ and grown with constant aeration at 37°C until being treated with antibiotics at the mid-log phase of growth ($OD_{600} = 0.6$). After incubating for an additional 15 min, the cells were harvested by centrifugation at 4°C. Total RNA extraction was performed using a TaKaRa MiniBEST Universal RNA Extraction kit (Takara, Dalian, China). The cDNA was subsequently synthesized by a TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix kit (Takara, Dalian, China). SYBR Premix Ex Taq II (Takara, Dalian, China) was used for real-time polymerase chain reaction (RT-PCR) analyzes. The primers used for RT-PCR are listed in Supplementary Table S2. Each sample was analyzed in triplicate.

Western blot analysis

Total protein was prepared using a ProteoPrep® Total Extraction Sample kit (Sigma-Aldrich, Zwijndrecht, The Netherlands). To detect the amount of green fluorescent protein (GFP), 60 μg of total protein was resolved by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to 0.45 μm polyvinylidene fluoride membranes at 4°C for 1.5 h. A monoclonal anti-GFP primary antibody (GenScript Co., Nanjing, China) diluted by 1/5000 was used to probe the blots according to the manufacturer's protocol. The secondary antibody was horseradish peroxidase-conjugated goat anti-mouse IgG (GenScript Co., Nanjing, China). The immune complexes were detected using an ECL western blot detection system (Pierce, Thermo Fisher Scientific, Waltham, MA, USA).

Electrophoretic mobility shift assays

The *lexA* gene was amplified from the NW19 and Mu50 genomes using primers (LexAexpF/LexAexpR and Lex50expF/Lex50expR) and cloned into the pCold II vector. The corresponding LexA proteins were overexpressed and purified in *E. coli* (Supplementary Data Materials and Methods for detail). The wild-type *ccrC1* promoter was amplified using the primers (ccrCOPF and ccrCOPR) and cloned into the pEAZY-T vector (GenScript Co., Nanjing, China). Site-directed mutants of the *ccrC1* promoter were constructed using the primers (mut1F/R, mut2F/R and mut3F/R) by overlap extension PCR. Electrophoretic mobility shift assay (EMSA) probes were obtained by amplification using the oligonucleotides BIO-ccrCOPF and ccrCOPR. The *ccrA* promoter was amplified using the primers (BIO-ccrAOPF and ccrAOPR). The EMSA experiments were performed using the LightShift EMSA Optimization and Control Kit according to the manufacturer's instructions (Pierce, Thermo Fisher Scientific, Waltham, MA, USA). All samples were loaded in 6% non-denaturing Tris–glycine polyacrylamide gels. Biotin-labeled DNA–protein complexes were detected using the Chemiluminescent Nucleic Acid Detection Module Kit (Pierce, Thermo Fisher Scientific).

Construction of gene overexpressing and reducing reporter strains

Detailed protocols are described in SuppIementary Data Materials and Methods.

Excision frequency detection

Genomic DNA from staphylococci was extracted using a Column Bacterial DNAout kit according to the manufac-

turer's instructions (GenScript, Nanjing, China) and used as a template for the detection of excision, as previously described (32). Primer pairs from the two sides of SCC*mec* (Supplementary Table S2) were used to determine the ratio of the SCC-excised genome. One copy of the housekeeping gene *tpi* (triosephosphate isomerase gene) was used as a reference. Each sample was determined in triplicate. All PCR products were confirmed by nucleotide sequence determination.

Gene deletion and complementation in Mu50 strain

Deletions of the *recA* gene were obtained using the plasmid pKZΔrecA50, which was constructed using pKOR1 with some modifications; a standard protocol (33) allowed marker-less *recA* replacement in Mu50 and the construction of Mu50ΔrecA (for details, see Supplementary Data Materials and Methods).

UV treatment

Cells grown overnight were inoculated into the TSB medium and re-suspended in 0.9% NaCl when $OD_{600} = 0.6$. Cells were treated for 10 min with UV irradiation and kept in the dark before RNA and DNA preparation. Irradiation was conducted by placing the plate containing the suspension 30 cm below a UV germicidal lamp and the UV dose was ∼35 J/m^2.

RESULTS

DNA damage caused by antibiotics promotes the expression of *ccrC1*

Stress induction by antibiotics is expected to occur under concentrations close to the MIC. Therefore, a *ccrC1* promoter-*gfp* fusion was constructed to evaluate the activity of the *ccrC1* promoter under 1/8 × MIC, 1/4 × MIC, 1/2 × MIC and 1 × MIC concentrations. The MICs of each antibiotic under our experimental conditions for the strain *S. haemolyticus* NW19 were first determined (Supplementary Table S3). The activity of the *ccrC1* promoter tended to be higher upon exposure to DNA synthesis-targeting antibiotics exposure than those with other antibiotic treatments (data not shown). The effects of nine antibiotics on the expression of *ccrC1* at 1/8 × MIC concentrations were also investigated by qPCR. The targets of these antibiotics are cell wall synthesis (OXA, teicoplanin (TEC) and vancomycin (VAN)), protein synthesis (gentamicin (GEN), kanamycin (KAN) and tetracycline (TET)) and DNA replication or repair (sulfamethoxazole (SMX), ciprofloxacin (CIP) and trimethoprim (TMP)). No significant differences in *ccrC1* expression were observed for four antibiotics (TEC, VAN, KAN and TET) compared to the control (Figure 1). OXA and GEN showed a light induction of the promoter (Figure 1), although their effects were much lower than those observed with DNA-targeting antibiotics. Exposure to the three DNA synthesis-targeting antibiotics significantly increased the transcription of *ccrC1*.

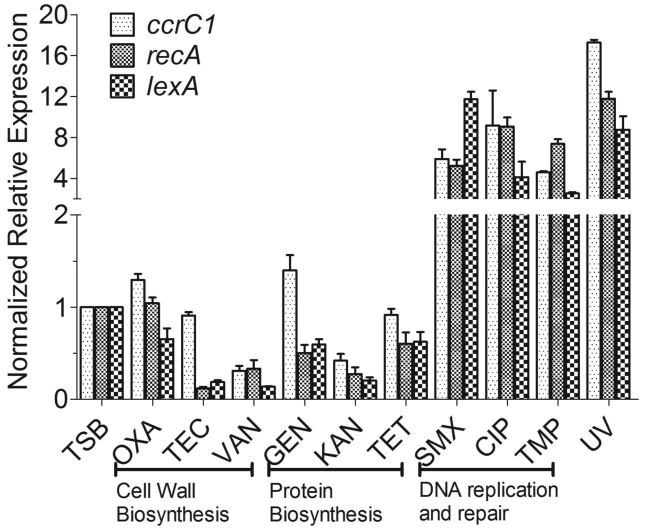

Figure 1. Antibiotics and Ultraviolet (UV) exposure promote *ccrC1* expression in *Staphylococcushaemolyticus* NW19. Transcriptional levels of *ccrC1, recA* and *lexA* in NW19 with different antibiotic induction (1/8 MIC) and UV exposure. Gene expression levels were normalized and are presented relative to the trypticase soy broth (TSB) cultured control.

Agents or Stimuli that cause DNA damage promote the excision of SCC*mec*

To identify the CcrC1-mediated excision of SCC*mec*, genomic DNA of NW19 was whole-genome sequenced (BGI-Shenzhen, Shenzhen, China). The SCC composite island in strain NW19 consists of five recombination sites, identical to the genome island in another strain (NW19A, GenBank accession no. KM369884) (34). The elements SCC*mec*, ΨSCC and ΨSCCcad/ars/cop are flanked by different direct repeats (DR1 and DR2, DR2 and DR3-4, DR3-4 and DR5, respectively). There are 83 base pairs between DR3 and DR4. The SCC*mec* element consists of the *ccrC1* allele 8 gene complex, the *mec* gene complex, the *ccrC1* allele 2 gene complex and the ydhk gene cluster (Supplementary Figure S2A). The recombinations between different DRs in the SCC-excised genome were identified by PCR. Three strong positive occurrences were identified (DR1 + DR2, DR1 + DR3 and DR1 + DR5; Supplementary Figure S2A). Detailed information on the different DR site sequences in the variant SCC-excised genome is shown in Supplementary Figure S2B. To assess the effects of antibiotics on the excision of SCC, RT-PCR was performed to evaluate the frequency of excision under 1/8 × MIC antibiotic exposure. The three SCC-excised genomes shown in Supplementary Figure S2A were detected, and the SCC*mec*-excised genome (DR1 + DR2 shown in Supplementary Figure S2B) exhibited the highest frequency. Therefore, the detection of the SCC*mec*-excised genome was chosen to evaluate the influence of different antibiotic treatments. The frequency of SCC*mec* excision in NW19 was $<10^{-11}$ without treatment (Figure 2). Treatment with SMX, CIP or TMP, three antibiotics targeting DNA replication and repair, led to significantly higher excision frequencies (3.4×10^{-5}, 2.3×10^{-6} and 1.1×10^{-5}, respectively) than the control. OXA and GEN showed an ∼100-fold increase in excision rates (8.8

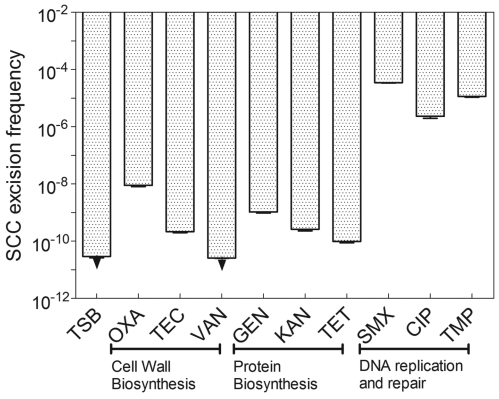

Figure 2. Antibiotics promote SCC excision via *ccrC1*. Primer pairs corresponding to the two sides of SCC*mec* (EGRT-F and EGRT-R) were used to determine the ratio of the SCC-excised genome. The arrowheads indicate values below the limit of detection ($<2.5 \times 10^{-11}$). One copy of the gene *tpi* (triosephosphate isomerase gene) was used as a reference. The bars represent the averages of three independent measurements in three different cultures, and the error bars represent the standard error of the mean. OXA, oxacillin; TEC, teicoplanin; VAN, vancomycin; GEN, gentamicin; KAN, kanamycin; TET, tetracycline; SMX, sulfamethoxazole; CIP, ciprofloxacin; TMP, trimethoprim.

$\times 10^{-9}$ and 1.0×10^{-9} respectively), which were much less than those observed with SMX, CIP or TMP, but still significant.

After showing that certain antibiotics initiated the expression of *ccrC1* and the excision of SCC*mec,* the effects of nine antibiotics on the expression of *recA* and *lexA*, two important and essential genes involved in DNA damage repair, were determined. Under antibiotic exposure at a concentration of 1/8 × MIC, only three antibiotics (SMX, CIP and TMP) induced a significant increase in the expression of *recA-lexA* compared to the control (Figure 1). In previous studies, it was shown that OXA could induce SOS response in MRSA (16,17,26). Thus, we further determined the expression of *recA* and *ccrC1* in strain NW19 with different concentrations of OXA exposure by RT-PCR. The results are showed in Supplementary Figure S1. Increased expression of gene *recA* and *ccrC1* was observed, when the concentrations of OXA at MIC and 2 MIC were used. The involvement of DNA damage in the effects of these antibiotics was confirmed by UV exposure. UV exposure promoted the expression of *recA-lexA* and SOS initiation. The transcription of *ccrC1* increased by ∼17-fold compared to the control (Figure 1). These results suggest that DNA damage is responsible for the expression of *ccrC1*.

An SOS box is highly conserved in the *ccrC1* promoter

In many bacterial species, DNA damage caused by DNA-targeting antibiotics triggers the SOS response, which involves induction of expressing SOS response genes, including *recA* and *lexA*. LexA is the repressor of the SOS response for all the genes of the chromosomal SOS regulon, except for some horizontally transferred genes such as phage lambda (35). Analysis and alignment of the promoter sequences of *ccrC1* and several other SOS genes in *S. aureus* (Table 1) revealed three putative overlapping LexA-binding motifs in the promoter of *ccrC1* in *S. haemolyticus* NW19. A search of *ccrC1* promoter sequences from seven different *ccrC1* allele types available in the NCBI GenBank indicated that all these allele types harbor the SOS box, which covers the partial −35 region and partial −10 region (Figure 3). All LexA-binding sequences are located 79 bp upstream of the translation initiation site of *ccrC1*. To further confirm the LexA binding sites, plasmids pCL::*ccr-gfp* and pCL::mut*ccr-gfp* (mutB, mutC and mutBC in Figure 4A), which contain mutations in the putative SOS box of the *ccrC1* promoter, were constructed. MutA site is in the −35 region of *ccrC1* promoter and mutation in this site affects binding of the RNA polymerase to the promoter and reduces the expression of GFP. Analysis of GFP levels indicated that the substitution of the putative SOS box greatly increased the expression of the GFP reporter (Figure 4B). The strain containing the original promoter also had a detectable level of GFP expression, which suggested a weak activity of the *ccrC1* promoter in the absence of environmental stress. These results indicate that mutations in the putative SOS box affect the transcription activity of the *ccrC1* promoter.

To confirm binding ability and specificity, purified LexA protein was used to examine the binding ability to the *ccrC1* promoter by an EMSA. The probes were titrated with increasing concentrations of purified LexA and subjected to gel-shift analysis. LexA specifically retarded the mobility of the *ccrC1* promoter in a dose-dependent manner (Figure 4C). The formation of a specific LexA–DNA complex was preceded by a detectable shift of the free probe but indistinct complex formation. This observation reflects the binding of the initial LexA monomer followed by the stable dimerization of LexA on DNA. Furthermore, the presence of the 100-fold specific competitor (unlabeled DNA of the *lexA* promoter) and mutations in key sites of the putative SOS box prevented the interaction between LexA and the wild-type promoter and mutant promoters, respectively (Figure 4A and D). These results indicate that the putative SOS box on the *ccrC1* promoter (5′-TGAAACGAAATTATAAATA-3′) is the LexA binding site.

Expression of *ccrC1* and excision of SCC*mec* are influenced by *recA* and *lexA in vivo*

To confirm the roles of RecA and LexA in the regulation of *ccrC1* expression *in vivo*, increased and reduced expression of *recA* and *lexA* were achieved by plasmid-mediated overexpression and antisense RNA as described previously (36). Cells were grown in TSB to $OD_{600} = 0.6$ and treated with MMC (an inducer of DNA damage) at a concentration of 0.5 mg/L for 30 min. In the absence of MMC, overexpression or reduction of *recA* had little or no effect on *ccrC1* expression (Figure 5A). Overexpression of *lexA* in strain 19lexUp caused reduced transcription of *ccrC1*, and antisense RNA-mediated reduction of *lexA* transcripts in strain 19lexDw led to upregulation of *ccrC1*. We also analyzed the expression of the GFP reporter in wild and mutant strains (Figure 5C), and the results were in agreement with the transcript detection of *ccrC1*. Compared to wild-type NW19, SCC excision frequency was much higher in strain 19lexDw

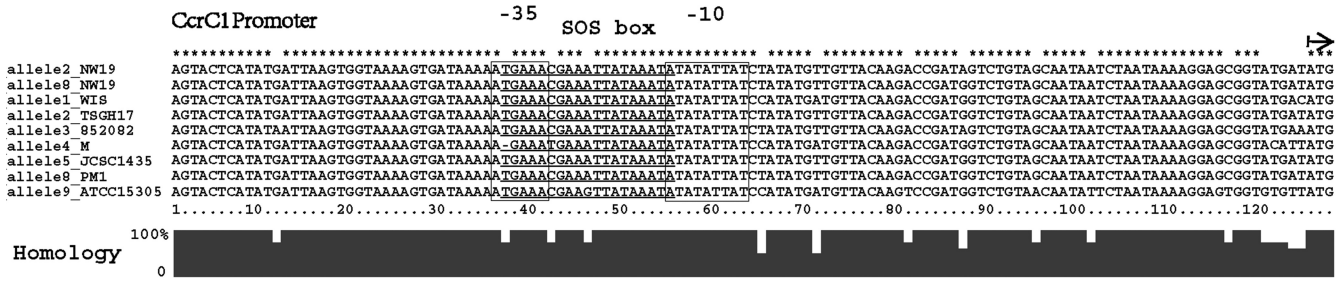

Figure 3. Alignment of the promoter regions of *ccrC1* genes from *Staphylococcus aureus* WIS (accession number AB121219), *S. aureus* TSGH17 (accession number AB512767), *S. aureus* 85/2082 (accession number AB037671), *S. aureus* M (U10927), *Staphylococcus haemolyticus* JCSC1435 (AP006716), *S. aureus* PM1 (AB462393) and *Staphylococcus saprophyticus* ATCC15305 (AP008934). Type V(5C2&5) SCC*mec* in *S. haemolyticus* NW19 contains two *ccrC*-carrying gene complexes on either side of *mec* gene complex. The *ccrC1* allele 8 is located in the region between *orfX* and *mec* gene complex, while *ccrC1* allele 2 is located between *mec* gene complex and *ydhK* complex (as showed in Supplementary Figure S2A). Two allele genes were 92.5% identical. The putative LexA-binding sequences are underlined. Promoter elements (−35 and −10) are framed. The start codon of *ccrC1* gene is indicated by a black arrow.

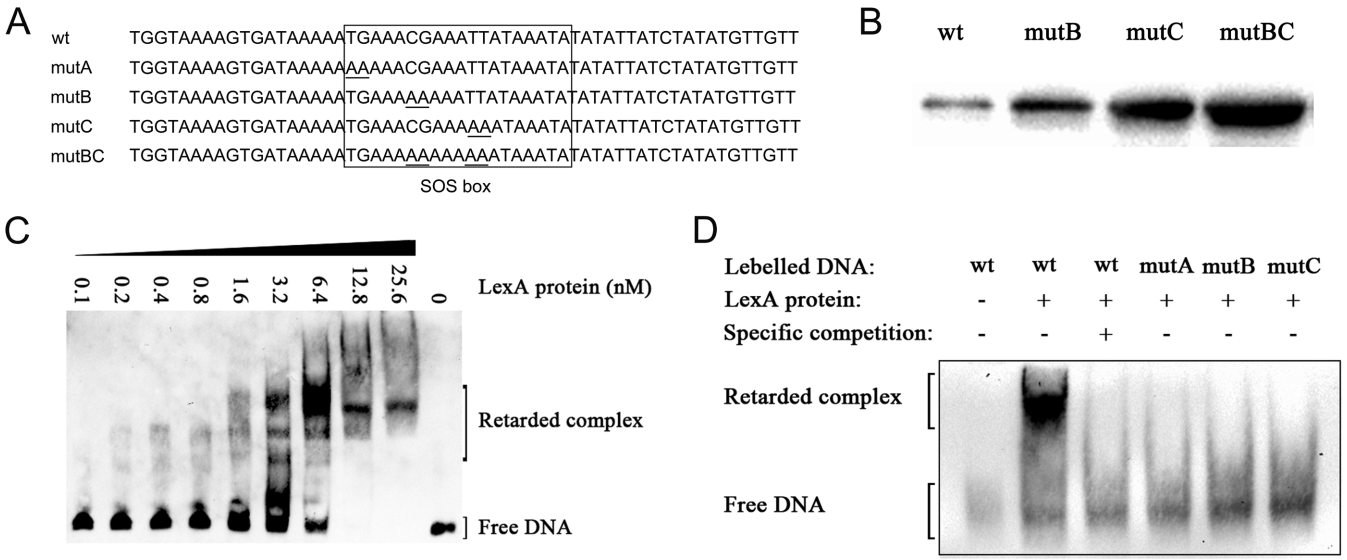

Figure 4. (**A**) Partial sequences of the *ccrC1* promoter region (wt) and SOS box mutants (mutA, mutB, mutC and mutBC) used in the electrophoretic mobility shift assays (EMSAs). (**B**) Western blotting detection of green fluorescent protein (GFP) under the wild-type and mutant promoters of *ccrC1*. The plasmids pCL::*ccr-gfp* and pCL::mut*ccr-gfp*, which encode the wild-type and mutant promoters of *ccrC1*, respectively, followed by *gfp*, were transformed into NW19. Cells were grown until $OD_{600} = 0.6$ and total bacterial protein was prepared. (**C**) EMSA experiments performed with the LexA protein and wild-type *ccrC1* promoter. (**D**) EMSA of the wild-type or mutant *ccrC1* promoter and LexA protein.

Table 1. Comparison of LexA binding sites in *S. aureus* and our isolate

Strain and Gene	SOS box				Reference
S. aureus RA1 *uvrA*	CG	AAA	GATTT AG	AT	(37)
S. aureus RA1 *uvrC*	CG	AAG	ATGTT GA	TT	(37)
S. aureus COL *dmpI*	CG	AAC	ACGTG TT	CT	(38)
S. aureus COL *ssb2*	CG	AAC	ATATG TT	CT	(38)
S. aureus COL *recA*	CG	AAC	AAATA TT	CG	(38)
S. aureus COL *lexA*	CG	AAC	AAATG TT	TG	(38)
S. aureus COL *2162*	GA	AAC	ATATT TT	CG	(38)
S. heamolyticus NW19 *ccrC1*[a]	GA	AAC	GAAAT TA	TA	This study
S. heamolyticus NW19 *ccrC1*[a]	TG	AAA	CGAAA TT	AT	This study
S. heamolyticus NW19 *ccrC1*[a]	CG	AAA	TTATA AA	TA	This study
S. aureus Mu50 *ccrA2B2*[b]	TG	AAA	GGAGGAG	CC	This study

[a]Three putative LexA-binding sequences on the promoter of *ccrC1* are overlapped and cover 19 bp in total (5′-TGAAACGAAATTATAAATA-3′).
[b]A putative LexA-binding sequences on the promoter of *ccrA2B2* in Mu50 (5′-TGAAAGGAGGAGCC-3′).

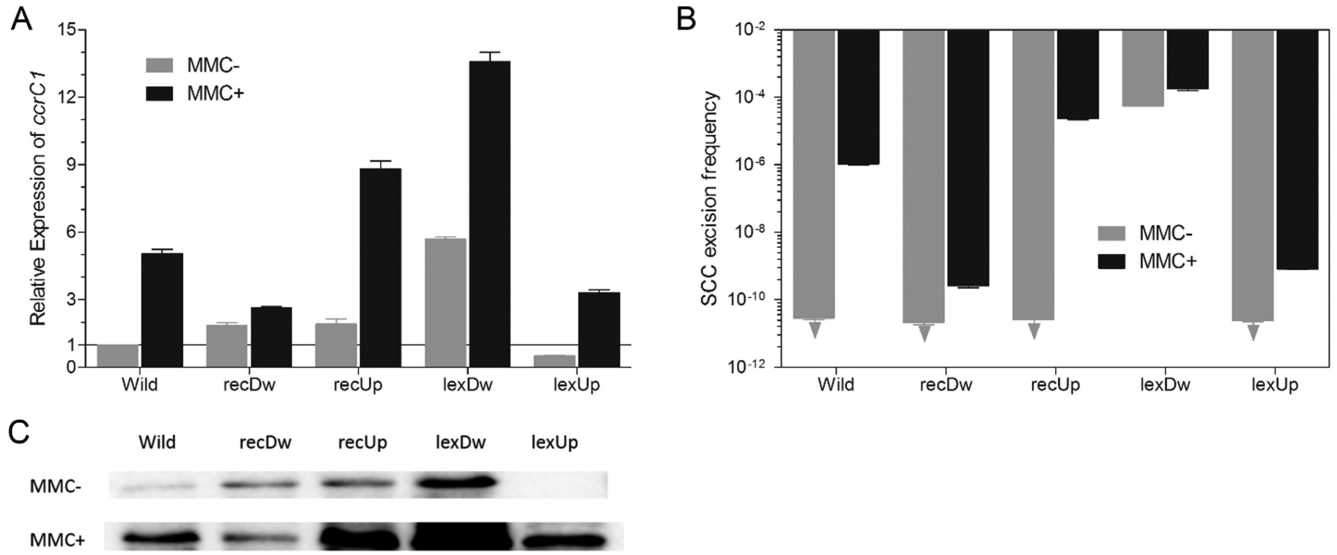

Figure 5. Effects of *recA* and *lexA* on the expression of *ccrC1* and SCC excision frequency in NW19. (**A**) Relative expression of *ccrC1* in different strains compared to the control (wild-type without mitomycin C (MMC)). (**B**) SCC excision frequency detection in different strains with and without MMC treatment. (**C**) Detection of GFP using ccr-gfp reporter strategy. Wild, wild-type NW19; recDw, NW19 with reduced expression of *recA*; recUp, NW19 with increased expression of *recA*; lexDw, NW19 with reduced *lexA* expression; lexUp, *lexA* transcript increased NW19; MMC−, without MMC exposure; MMC+, with MMC exposure. Arrowhead, below the limit of detection ($<2.5 \times 10^{-11}$). The relative expression of *ccrC1* and the SCC excision frequency were detected with and without MMC exposure (0.5 mg/L). Each sample was determined in triplicate. The data represent the mean ± Standard Error Mean (SEM).

(Figure 5B). By contrast, in strains NW19, 19recDw, 19recUp and 19lexUp, detection of SCC excision by the RT-PCR method was limited and frequencies of $<2.5 \times 10^{-11}$ were not detectable. In the presence of MMC, the expression level of *ccrC1* was upregulated to varying degrees in all five strains (Figure 5A). Compared to wild-type NW19, *recA* overexpression and *lexA* reduction caused a higher induction of *ccrC1* expression. Reduction of *recA* and overexpression of lexA led to lower induction of *ccrC1* expression. Compared to wild-type NW19, the SCC excision frequency was higher in strains 19recUp and 19lexDw and lower in strains 19recDw and 19lexUp (Figure 5B). These results suggest that LexA is the transcriptional repressor of *ccrC1* and that SCC transfer is initiated by the activated SOS response.

Expression of *ccrAB* and excision of SCC*mec* in Mu50 are dependent on the SOS pathway

The excision of the type II SCC*mec* in Mu50 is mediated by CcrA2B2 recombinases; CcrA2 allotype and CcrB2 allotype are encoded in the same operon. To further determine whether the expression of *ccrAB* is also controlled by the RecA-LexA system, different allotypes of the *ccrAB* promoter sequences were aligned. The putative LexA binding site (5′-TGAAAGGAGGAGCC-3′) overlapped with the transcription start sites (Figure 6A). *In vitro* EMSA experiments revealed specific retardation of the *ccrA* promoter (Figure 6B). The expression of *ccrA* was ∼3-fold higher in the *recA* deletion mutant than in the wild-type of Mu50 (Figure 6C). Expression of *lexA* is regulated by LexA itself. In the *recA* deletion mutant, repressed LexA led to a lower basal expression of *lexA* in Mu50ΔrecA strain (0.05-fold). Exposure to MMC upregulated the expression of *ccrA*, *recA* and *lexA* in the wild-type Mu50 and Mu50ΔrecA::precA strains. Little effect of MMC on the expression of *ccrA* was observed in the in *recA* deletion strain. The excision frequency of SCC*mec* was 6.8×10^{-5} and increased to 4.7×10^{-4} upon MMC treatment (Figure 6D). No significant difference was observed in the *recA*-deleted Mu50 with or without MMC exposure. These results show that the expression of *ccrAB* and excision of SCC*mec* in Mu50 is dependent on the RecA-LexA mediated SOS pathway.

DISCUSSION

In this study, we propose a model of the regulatory pathway by which antibiotics promote SCC*mec* transfer (Figure 7). Bactericidal drugs inducing DNA and protein damage are usually involved in triggering the SOS response (20,35). LexA is initially bound to its binding sites (SOS box) on the promoters of *ccrC1* and *ccrAB*, thus hindering the transcription of *ccrC1* and *ccrAB* by blocking RNA polymerase activity. The RecA protein becomes activated (RecA*) during the SOS response, which in turn facilitates LexA autocleavage. Inactivation of LexA alleviates the repression of CcrC1 and CcrAB expression. The increased levels of Ccr proteins, which can bind recombination sites (*attL* and *attR*) on both ends of SCC*mec*, lead to the integration and excision of SCC*mec* on the chromosome. HGT of the SCC*mec* cyclic intermediate may lead to transfer and spread of β-lactam resistance among staphylococci.

The frequency of SCC*mec* excision was dependent on the type and the concentration of antibiotics in this study. Low levels of the β-lactam antibiotic (OXA at 1/8 MIC and

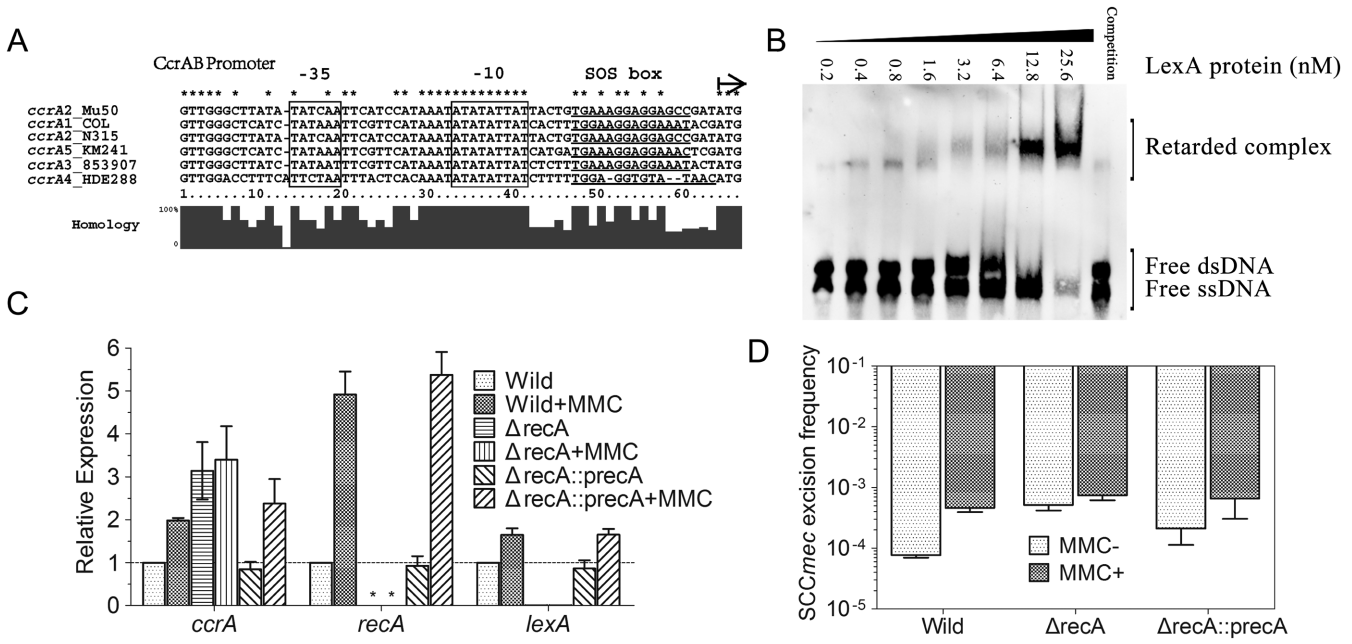

Figure 6. The SOS response promotes the expression of *ccrA* and SCC*mec* excision in Mu50. (**A**) Alignment of the promoter regions of *ccrA* genes from *Staphylococcus aureus* Mu50 (accession number BA000017), *S. aureus* COL (CP000046), *S. aureus* N315 (BA000018), *Staphylococcus pseudintermedius* KM241 (AM904731), *S. aureus* 85/3907 (AB047089) and *S. aureus* HDE288 (AF411935). The putative LexA-binding sequences are underlined. Promoter elements (−35 and −10) are boxed. The 5′ ATG of *ccrA* gene is indicated by a black arrow. (**B**) EMSA experiments performed with the LexA protein and *ccrA* promoter. (**C**) Relative expression of *ccrA*, *recA* and *lexA* in wild-type Mu50, the *recA*-deleted mutant (ΔrecA) and *recA*-complemented mutant (ΔrecA::precA) compared to the control (wild-type without MMC). *, not detectable. (**D**) SCC*mec* excision frequency detection in different strains with and without MMC treatment (0.5 mg/l). Each sample was determined in triplicate. Data are presented as the average ± SEM.

1/4 MIC) slightly affected expression of SOS proteins in *S. haemolyticus* NW19 (Figure 1 and Supplementary Figure S1), while OXA at the concentration of 1/100 MIC induced the SOS response in various Gram negative bacteria (39–41). The difference between *S. haemolyticus* NW19 and Gram-negative bacteria could be due to their different responses to OXA. *S. haemolyticus* NW19 became highly resistant to OXA (OXA MIC > 256 μg/ml) with OXA induction (36), due to increased expression of *mecA* (Supplementary Figure S1). In *E. coli*, cell wall stress induced by β-lactams induces the SOS response through a sensor protein DpiB and a replication site binding protein DpiA (18). Putative proteins (Figure 7) responsible for the cell wall stress response by β-lactams in staphylococcus remain to be identified. In our study, Ccr expression and SCC*mec* excision were also observed in non-stressed staphylococci, as reported in a previous study (42). This observation can be explained by the basal expression of *ccr* genes in the absence of SOS induction. In Stojanov's study, the *ccr* promoter activity was observed in the absence of antibiotic exposure, even though only in a small percentage of cell populations (∼3 and 1% among logarithmic and stationary growth phases respectively).

SCC*mec* is only part of the composite island in strain NW19; this island encodes not only methicillin resistance but also heavy metal resistance for cadmium, arsenic and copper. Many other composite islands in staphylococci have been reviewed by Shore and Coleman (43). Some SCCs that do not contain the *mec* gene complex are considered non-*mec* SCC. Ten non-*mec* SCC types have been identified, and four ($SCC_{ATCC12228}$, SCC_{pbp4}, SCC*Hg* and SCC476) carry additional antibiotic resistance or virulence genes (43). Because both the SCC composite island and non-*mec* SCC have *ccr* gene complexes, our results suggest that the excision and transfer of such SCCs could also be induced by antibiotics through the SOS response. Furthermore, we observed that the SCC*mec* excision frequency mediated by *ccrC1* was much lower than that mediated by *ccrAB*, even upon induction by MMC, which might explain why more SCCs carry the *ccrAB* gene complex than *ccrC1*.

The overuse and improper use of antibiotics have contributed to the rise of multidrug-resistant MRSA. In our study, β-lactams and antibiotics targeting DNA were observed to promote the SCC*mec* excision. Antibiotics were also reported to be responsible for maintaining antibiotic resistance in bacteria (44,45). The findings from this study and other studies may explain why MRS is widely distributed worldwide. An individual *mecA* positive staphylococcal bacterium with the SCC*mec* excised would become methicillin-susceptible and be destroyed under lactam pressure. It has been suggested that these few individuals commit this suicide, thus sacrificing themselves to transfer SCC*mec* into new recipient strains (42). However, we favor an alternative model in which the excision of SCC*mec* is an active process and is initiated when MRS is exposed to damage or environmental stress (Figure 7). Excision allows SCC*mec* to hitchhike from a feeble individual or species to a vigorous one. This 'hitchhike model' ensures that SCC*mec* is not eliminated in the long-term process of evolution.

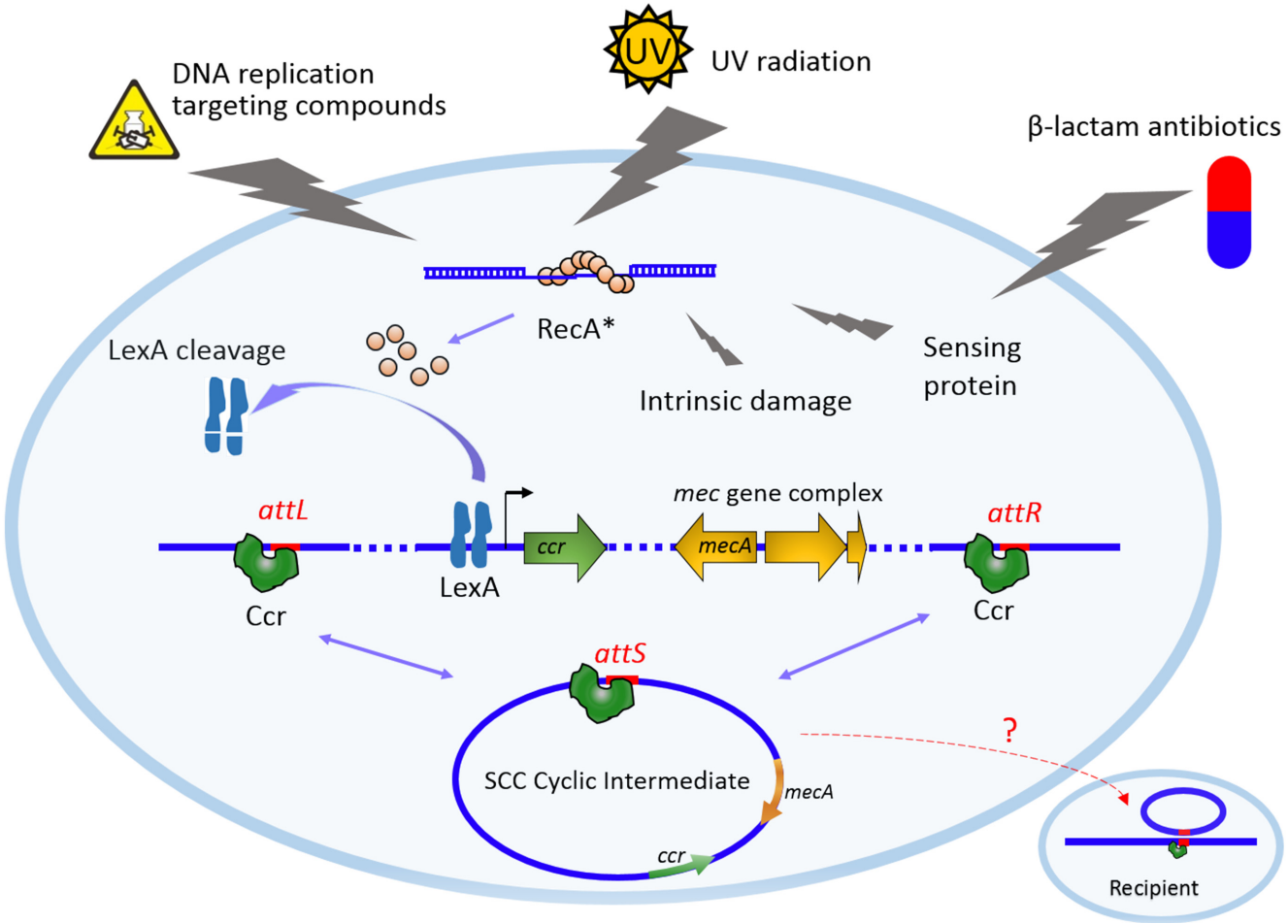

Figure 7. Proposed model of the regulatory pathway by which the SOS response promotes SCC*mec* transfer. Intermediary molecules involved in SOS induction are shown. The mechanism by which SCC*mec* transfers among staphylococci remains elusive.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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REFERENCES

1. Hanssen,A.M. and Ericson,S.J. (2006) SCC*mec* in staphylococci: genes on the move. *FEMS Immunol. Med. Microbiol.*, **46**, 8–20.
2. Otto,M. (2013) Coagulase-negative staphylococci as reservoirs of genes facilitating MRSA infection: Staphylococcal commensal species such as *Staphylococcus epidermidis* are being recognized as important sources of genes promoting MRSA colonization and virulence. *Bioessays*, **35**, 4–11.
3. Wu,S., Piscitelli,C., de Lencastre,H. and Tomasz,A. (1996) Tracking the evolutionary origin of the methicillin resistance gene: cloning and sequencing of a homologue of *mecA* from a methicillin susceptible strain of *Staphylococcus sciuri*. *Microb. Drug. Resist.*, **2**, 435–441.
4. Tsubakishita,S., Kuwahara-Arai,K., Sasaki,T. and Hiramatsu,K. (2010) Origin and molecular evolution of the determinant of methicillin resistance in staphylococci. *Antimicrob. Agents Chemother.*, **54**, 4352–4359.
5. Archer,G.L., Thanassi,J.A., Niemeyer,D.M. and Pucci,M.J. (1996) Characterization of IS1272, an insertion sequence-like element from *Staphylococcus haemolyticus*. *Antimicrob. Agents Chemother.*, **40**, 924–929.
6. Jones,R.N., Barry,A.L., Gardiner,R.V. and Packer,R.R. (1989) The prevalence of staphylococcal resistance to penicillinase-resistant penicillins. A retrospective and prospective national surveillance trial of isolates from 40 medical centers. *Diagn. Microbiol. Infect. Dis.*, **12**, 385–394.
7. Schmitz,F.J., Verhoef,J. and Fluit,A.C. (1999) Prevalence of resistance to MLS antibiotics in 20 European university hospitals participating in the european SENTRY surveillance programme. sentry participants group. *J. Antimicrob. Chemother.*, **43**, 783–792.
8. Ito,T., Kuwahara-Arai,K., Katayama,Y., Uehara,Y., Han,X., Kondo,Y. and Hiramatsu,K. (2014) Staphylococcal cassette chromosome *mec* (SCC*mec*) analysis of MRSA. *Methods Mol. Biol.*, **1085**, 131–148.
9. Misiura,A., Pigli,Y.Z., Boyle-Vavra,S., Daum,R.S., Boocock,M.R. and Rice,P.A. (2013) Roles of two large serine recombinases in mobilizing the methicillin-resistance cassette SCC*mec*. *Mol. Microbiol.*, **88**, 1218–1229.
10. Boundy,S., Safo,M.K., Wang,L., Musayev,F.N., O'Farrell,H.C., Rife,J.P. and Archer,G.L. (2013) Characterization of the *Staphylococcusaureus* rRNA methyltransferase encoded by *orfX*, the

gene containing the staphylococcal chromosome Cassette *mec* (SCC*mec*) insertion site. *J. Biol. Chem.*, **288**, 132–140.
11. Ito,T., Ma,X.X., Takeuchi,F., Okuma,K., Yuzawa,H. and Hiramatsu,K. (2004) Novel type V staphylococcal cassette chromosome mec driven by a novel cassette chromosome recombinase, *ccrC*. *Antimicrob. Agents Chemother.*, **48**, 2637–2651.
12. Katayama,Y., Ito,T. and 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.
13. Wu,Z., Li,F., Liu,D., Xue,H. and Zhao,X. (2015) Novel type XII Staphylococcal cassette chromosome *mec* harboring a new cassette chromosome recombinase, CcrC2. *Antimicrob. Agents Chemother.*, **59**, 7597–7601.
14. Kinnevey,P.M., Shore,A.C., Brennan,G.I., Sullivan,D.J., Ehricht,R., Monecke,S., Slickers,P. and Coleman,D.C. (2013) Emergence of sequence type 779 methicillin-resistant *Staphylococcusaureus* harboring a novel pseudo staphylococcal cassette chromosome *mec* (SCC*mec*)-SCC-SCC$_{CRISPR}$ composite element in Irish hospitals. *Antimicrob. Agents Chemother.*, **57**, 524–531.
15. Higgins,P.G., Rosato,A.E., Seifert,H., Archer,G.L. and 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.
16. Cuirolo,A., Plata,K. and Rosato,A.E. (2009) Development of homogeneous expression of resistance in methicillin-resistant *Staphylococcus aureus* clinical strains is functionally associated with a beta-lactam-mediated SOS response. *J. Antimicrob. Chemother.*, **64**, 37–45.
17. Plata,K.B., Riosa,S., Singh,C.R., Rosato,R.R. and Rosato,A.E. (2013) Targeting of PBP1 by beta-lactams determines *recA*/SOS response activation in heterogeneous MRSA clinical strains. *PLoS One*, **8**, e61083.
18. Miller,C., Thomsen,L.E., Gaggero,C., Mosseri,R., Ingmer,H. and Cohen,S.N. (2004) SOS response induction by beta-lactams and bacterial defense against antibiotic lethality. *Science*, **305**, 1629–1631.
19. Kohanski,M.A., Dwyer,D.J., Hayete,B., Lawrence,C.A. and Collins,J.J. (2007) A common mechanism of cellular death induced by bactericidal antibiotics. *Cell*, **130**, 797–810.
20. Erill,I., Campoy,S. and Barbe,J. (2007) Aeons of distress: an evolutionary perspective on the bacterial SOS response. *FEMS Microbiol. Rev.*, **31**, 637–656.
21. Al,M.A., Lombardo,M.J., Shee,C., Lisewski,A.M., Gonzalez,C., Lin,D., Nehring,R.B., Saint-Ruf,C., Gibson,J.L., Frisch,R.L. *et al.* (2012) Identity and function of a large gene network underlying mutagenic repair of DNA breaks. *Science*, **338**, 1344–1348.
22. Schlacher,K. and Goodman,M.F. (2007) Lessons from 50 years of SOS DNA-damage-induced mutagenesis. *Nat. Rev. Mol. Cell Biol.*, **8**, 587–594.
23. Janion,C. (2001) Some aspects of the SOS response system–a critical survey. *Acta Biochim. Pol.*, **48**, 599–610.
24. Fornelos,N., Browning,D.F. and Butala,M. (2016) The use and abuse of LexA by mobile genetic elements. *Trends Microbiol.*, **24**, 391–401.
25. Ubeda,C., Maiques,E., Knecht,E., Lasa,I., Novick,R.P. and Penades,J.R. (2005) Antibiotic-induced SOS response promotes horizontal dissemination of pathogenicity island-encoded virulence factors in staphylococci. *Mol. Microbiol.*, **56**, 836–844.
26. Maiques,E., Ubeda,C., Campoy,S., Salvador,N., Lasa,I., Novick,R.P., Barbe,J. and Penades,J.R. (2006) Beta-lactam antibiotics induce the SOS response and horizontal transfer of virulence factors in *Staphylococcus aureus*. *J. Bacteriol.*, **188**, 2726–2729.
27. Beaber,J.W., Hochhut,B. and Waldor,M.K. (2004) SOS response promotes horizontal dissemination of antibiotic resistance genes. *Nature*, **427**, 72–74.
28. Guerin,E., Cambray,G., Sanchez-Alberola,N., Campoy,S., Erill,I., Da,R.S., Gonzalez-Zorn,B., Barbe,J., Ploy,M.C. and Mazel,D. (2009) The SOS response controls integron recombination. *Science*, **324**, 1034.
29. Cambray,G., Sanchez-Alberola,N., Campoy,S., Guerin,E., Da,R.S., Gonzalez-Zorn,B., Ploy,M.C., Barbe,J., Mazel,D. and Erill,I. (2011) Prevalence of SOS-mediated control of integron integrase expression as an adaptive trait of chromosomal and mobile integrons. *Mob. DNA*, **2**,6–20.
30. Hocquet,D., Llanes,C., Thouverez,M., Kulasekara,H.D., Bertrand,X., Plesiat,P., Mazel,D. and Miller,S.I. (2012) Evidence for induction of integron-based antibiotic resistance by the SOS response in a clinical setting. *PLoS Pathog.*, **8**, e1002778.
31. Clinical and Laboratory Standards Institute (2014) *Performance Standards for Antimicrobial Susceptibility Testing: Twenty-fourth Informational Supplement M100-S24*. CLSI, Wayne.
32. Noto,M.J. and 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.
33. Bae,T. and Schneewind,O. (2006) Allelic replacement in *Staphylococcus aureus* with inducible counter-selection. *Plasmid*, **55**, 58–63.
34. Xue,H., Wu,Z., Li,L., Li,F., Wang,Y. and Zhao,X. (2015) Coexistence of heavy metal and antibiotic resistance within a novel composite staphylococcal cassette chromosome in a Staphylococcus haemolyticus isolate from bovine mastitis milk. *Antimicrob. Agents Chemother.*, **59**, 5788–5792.
35. Baharoglu,Z. and Mazel,D. (2014) SOS, the formidable strategy of bacteria against aggressions. *FEMS Microbiol. Rev.*, **38**, 1126–1145.
36. Liu,P., Xue,H., Wu,Z., Ma,J. and Zhao,X. (2016) Effect of *bla* regulators on the susceptible phenotype and phenotypic conversion for oxacillin-susceptible *mecA*-positive staphylococcal isolates. *J. Antimicrob. Chemother.*, **71**, 2105–2112.
37. Bisognano,C., Kelley,W.L., Estoppey,T., Francois,P., Schrenzel,J., Li,D., Lew,D.P., Hooper,D.C., Cheung,A.L. and Vaudaux,P. (2004) A RecA-LexA-dependent pathway mediates ciprofloxacin-induced fibronectin binding in *Staphylococcus aureus*. *J. Biol. Chem.*, **279**, 9064–9071.
38. Cirz,R.T., Jones,M.B., Gingles,N.A., Minogue,T.D., Jarrahi,B, Peterson,S.N. and Romesberg,F.E. (2007) Complete and SOS-mediated response of *Staphylococcus aureus*to the antibioticciprofloxacin. *J. Bacteriol.*, **189**, 531–539.
39. Gutierrez,A., Laureti,L., Crussard,S., Abida,H., Rodriguez-Rojas,A., Blazquez,J., Baharoglu,Z., Mazel,D., Darfeuille,F., Vogel,J. *et al.* (2013) β-lactam antibiotics promote bacterial mutagenesis via an RpoS-mediated reductionin replication fidelity. *Nat. Commun.*, **4**, 1610–1618.
40. Baharoglu,Z., Krin,E. and Mazel,D. (2013) RpoS plays a central role in the SOS induction by sub-lethal aminoglycoside concentrations in *Vibrio cholerae*. *PLoS Genet.*, **9**, e1003421.
41. Baharoglu,Z. and Mazel,D. (2011) *Vibrio cholerae* triggers SOS and mutagenesis in response to a wide range of antibiotics: a route towards multiresistance. *Antimicrob. Agents Chemother.*, **55**, 2438–2441.
42. Stojanov,M., Sakwinska,O. and Moreillon,P. (2013) Expression of SCC*mec* cassette chromosome recombinases in methicillin-resistant *Staphylococcus aureus* and *Staphylococcus epidermidis*. *J. Antimicrob. Chemother.*, **68**, 749–757.
43. Shore,A.C. and Coleman,D.C. (2013) Staphylococcal cassette chromosome *mec*: recent advances and new insights. *Int. J. Med. Microbiol.*, **303**, 350–359.
44. Bottery,M.J., Wood,A.J. and Brockhurst,M.A. (2016) Selective conditions for a multidrug resistance plasmid depend on the sociality of antibiotic resistance. *Antimicrob. Agents Chemother.*, **60**, 2524–2527.
45. Zhu,Y.G., Johnson,T.A., Su,J.Q., Qiao,M., Guo,G.X., Stedtfeld,R.D., Hashsham,S.A. and Tiedje,J.M. (2013) Diverse and abundant antibiotic resistance genes in chinese swine farms. *Proc. Natl. Acad. Sci. U.S.A.*, **110**, 3435–3440.