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Expression of SCC*mec* cassette chromosome recombinases in methicillin-resistant *Staphylococcus aureus* and *Staphylococcus epidermidis*

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Objectives: Methicillin resistance in staphylococci is mediated by the *mecA* gene, which is carried on the staphylococcal cassette chromosome *mec* (SCC*mec*). SCC*mec* is responsible for vertical and horizontal transfer of methicillin resistance. Horizontal transfer implies first SCC*mec* excision from the chromosome. Site-specific excision is catalysed by the Ccr recombinases, which are encoded by *ccrAB* genes located on the cassette. The aim of this study is to determine the promoter activity of *ccrAB* genes in individual cells of methicillin-resistant *Staphylococcus aureus* (N315, COL and MW2) and *Staphylococcus epidermidis* (RP62A). One mutant cured of its SCC*mec* (N315EX) was also used. Exposure to various stresses was included in the study.

Methods: For each strain, translational promoter-green fluorescent protein (*gfp*) fusions were used to assess the levels of *ccr* promoter activity in individual cells. Analyses were performed using epifluorescence microscopy and flow cytometry.

Results: *ccr* promoter activity was observed in only a small percentage of cell populations. This 'bistable' phenotype was strain dependent (GFP was expressed in N315 and RP62A, but not in COL and MW2) and growth dependent (GFP-expressing cells decreased from approximately 3% to 1% between logarithmic and stationary growth phases). The *ccr* promoter of strain N315 displayed normal promoter activity when expressed in SCC*mec*-negative N315EX. Likewise, the *ccr* promoter of strain COL (which was inactive in COL) showed normal N315-like activity when transformed into N315 and N315EX.

Conclusions: SCC*mec* excision operates through bistability, favouring a small fraction of cells to 'sacrifice' their genomic islands for transfer, while the rest of the population remains intact. Determinants responsible for the activity of the *ccr* promoter were not located on SCC*mec*, but were elsewhere on the genome. Thus, the staphylococcal chromosome plays a key role in determining SCC*mec* stability and transferability.

Keywords: excision, promoter, staphylococci, *gfp*

Introduction

In *Staphylococcus* spp. resistance to methicillin and to virtually all β-lactam drugs is mediated by the expression of the *mecA* gene, which encodes low-affinity penicillin-binding protein A, or PBP2A.[1] The *mecA* gene is carried by a genomic island named staphylococcal cassette chromosome *mec* (SCC*mec*), which is responsible for both vertical and horizontal transfer of methicillin resistance. It was originally believed that worldwide spread of methicillin-resistant *Staphylococcus aureus* (MRSA) was solely due to clonal expansion of a few successful strains, implying that *de novo* acquisition of SCC*mec* was extremely rare.[2] However, more recent studies indicate that lateral spread of SCC*mec* is more frequent than expected.[3,4] This is substantiated both by the fact that almost identical cassettes are present in unrelated staphylococcal strains—and even in different staphylococcal species—and by the presence of different types of SCC*mec* in closely related strains.[5–7]

To be transferred, SCC*mec* must first be excised from the chromosome of the donor strain. Site-specific excision of SCC*mec* is catalysed by the Ccr recombinases, which are large serine recombinases of the resolvase/invertase family and are encoded on the SCC*mec* cassette.[8] Three phylogenetically distinct *ccr* genes have been found so far on different cassettes. Certain SCC*mec* cassettes carry the *ccrA* and *ccrB* genes (*ccrAB*), which are part of the same transcription unit, whereas

other cassettes carry the *ccrC* gene, which shares less than 50% sequence similarity with *ccrAB*. Several allotypes (sequence similarity below 85%) have been described for *ccrA* and *ccrB* and are used for classification of their cognate SCC*mec* cassettes. In contrast, only one allotype was found for the *ccrC* gene.[9]

Site-specific excision/integration of SCC*mec* takes place at the 5′ end of *orfX*, a highly conserved gene located near the origin of replication in *S. aureus*. Integration involves a 15 bp core sequence (*attB*), which recombines with the cognate 15 bp core sequence located on the SCC*mec* (*attS*) resulting, upon integration, in two direct repeats (*attR* and *attL*) flanking the element.[8] In addition, accessory sequences located near the *attS* sites, which form imperfect inverted repeats of various sizes, seem also to play a role in site-specific integration/excision in many SCC*mec*.[10,11] Interestingly, CcrAB recombinases do not display cassette specificity, as all the allotypes are able to excise SCC*mec* of different types.[12] Conversely, CcrC recombinases are specific to their own SCC*mec*.[11]

Spontaneous SCC*mec* excision has been observed on several occasions both *in vivo* and *in vitro*.[12–15] For instance, overexpression of plasmid-located *ccrAB* genes generally leads to SCC*mec* excision and results in conversion of MRSA into methicillin-susceptible *S. aureus* (MSSA).[8] Moreover, β-galactosidase assays and quantitative RT–PCR studies showed that β-lactams and vancomycin increased transcription from *ccrAB* promoters of strains MW2 and N315.[16] This could favour the propagation of SCC*mec* via a two-step scenario. First, β-lactam drugs in the environment would select for methicillin-resistant staphylococci, thus promoting the expansion of the SCC*mec* reservoir. Second, transfer of SCC*mec* from this reservoir into new staphylococci would generate new methicillin-resistant strains capable of further amplifying the pool of SCC*mec*. In such a scenario, the optimal setting implies that not all individuals of the donor population do excise their cassette simultaneously, because they would become methicillin susceptible and thus be destroyed by the drug, but that only a few individuals commit this suicide, sacrificing themselves to transfer SCC*mec* into new recipient strains.

Here we tested this hypothesis by using promoter-green fluorescent protein (*gfp*) fusions to measure the expression of *ccrAB* genes in individual cells of various strains of MRSA carrying different types of SCC*mec* cassettes. Moreover, we also examined the methicillin-resistant *Staphylococcus epidermidis* strain RP62A, as more and more evidence suggests that coagulase-negative staphylococci are the reservoir of SCC*mec* for *S. aureus*.[13,17,18]

Materials and methods

Bacterial strains, media and culture conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strain DH5α, which was routinely used for plasmid propagation and cloning experiments, was cultivated on Luria-Bertani (LB) medium (Becton Dickinson, Sparks, MD, USA) supplemented with 100 mg/L ampicillin (AppliChem, Darmstadt, Germany) at 37°C. *S. aureus* strains were grown with aeration in Trypticase soy broth (TSB) (Difco Laboratories, Detroit, MI, USA) in a rotating incubator (at 180 rpm) at 37°C. For all experiments, bacterial cultures were inoculated with a 1/100 dilution of an overnight culture. If required, tetracycline and erythromycin (AppliChem) were added at a final concentration of 10 mg/L for plasmid propagation and 5 mg/L for flow cytometry analysis. Oxacillin was commercially purchased and used at the sub-MIC concentration

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference
Strain		
E. coli DH5α	host for DNA cloning	laboratory collection
S. aureus		
RN4220	restriction-deficient derivative of RN450; intermediate cloning host	19
N315	MRSA carrying type II SCC*mec*	20
N315EX	isogenic MSSA derivative of N315	this study
COL	MRSA carrying type I SCC*mec*	21
MW2	MRSA carrying type VI SCC*mec*	22
S. epidermidis RP62A	methicillin-resistant *S. epidermidis* carrying type II SCC*mec*	21
Plasmid		
pCN36	*E. coli*–*S. aureus* shuttle vector; Tc^R; ±22 copies/cell	23
pCN68	*E. coli*–*S. aureus* shuttle vector; source of *gfpmut2* gene	23
pSR3-1	thermosensitive-replicon plasmid carrying the *ccrAB* genes of strain N315 (used for SCC*mec* excision in N315)	8
pPGFP-N315	P_{ccrAB}N315-*gfpmut2* fusion cloned in pCN36; *tet*(M)	this study
pPGFP-MW2	P_{ccrAB}MW2-*gfpmut2* fusion cloned in pCN36; *erm*(C)	this study
pPGFP-COL	P_{ccrAB}COL-*gfpmut2* fusion cloned in pCN36; *erm*(C)	this study
pPGFP-COL-Tc	P_{ccrAB}COL-*gfpmut2* fusion cloned in pCN36; *tet*(M)	this study
pPGFP-RP62A	P_{ccrAB}RP62A-*gfpmut2* fusion cloned in pCN36; *tet*(M)	this study
pNEG-tet(M)	P_{NEG}-*gfpmut2* fusion cloned in pCN36; used as negative control	this study
pNEG-ermC	P_{NEG}-*gfpmut2* fusion cloned in pCN36; *tet*(M) replaced with *erm*(C); used as negative control	this study
pGFPS10-tet(M)	P_{PS10}-*gfpmut2* fusion cloned in pCN36; used as positive control	this study
pGFPS10-ermC	P_{PS10}-*gfpmut2* fusion cloned in pCN36; *tet*(M) replaced with *erm*(C); used as positive control	this study

of 4 mg/L for methicillin-resistant staphylococci. Mitomycin C (Sigma–Aldrich Chemie GmbH, Steinheim, Germany) was used at a final concentration of 0.5 mg/L.

DNA manipulations

For *S. aureus* and *S. epidermidis*, genomic DNA was extracted using a protocol adapted from Bae *et al.*[24] Briefly, 3 mL of an overnight culture was harvested and resuspended in 50 μL of Tris–EDTA buffer (10 mM Tris–Cl, pH 7.5, and 1 mM EDTA) supplemented with lysostaphin (final concentration 0.5 μg/mL). After 30 min of incubation at 37°C, 300 μL of 'Nuclei lysis solution' (Promega Corp., Madison, WI, USA) was added and the cell suspensions were heated at 80°C for 10 min. The samples were then treated with RNase and addition of 100 μL of 'Protein precipitation solution' (Promega Corp.) was followed by incubation for 5 min on ice. After centrifugation (4°C, 15 600 **g**), supernatants were collected and 300 μL of isopropanol was used to precipitate the DNA, which was subsequently washed with 70% ethanol, pelleted by centrifugation, air-dried and re-diluted at 4°C overnight in 20 μL of elution buffer (Qiagen Inc., Hilden, Germany).

Plasmids were isolated using the QIAprep Spin Miniprep Kit (Qiagen Inc.). For *S. aureus*, an additional step consisting of lysostaphin treatment (final concentration 0.5 μg/mL) was performed before the lysis step.

Digestions with restriction enzymes (Promega Corp.) were carried out according to the manufacturer's specifications. PCR fragments were purified using the QIAquick PCR Purification Kit (Qiagen Inc.) and gel-bands were purified using the QIAquick Gel Extraction Kit (Qiagen Inc.) according to the manufacturer's protocols. Ligations were performed using 1 μL of T4 ligase (Promega Corp.) according to the manufacturer's specifications.

PCR

GoTaq DNA polymerase (Promega Corp.) was routinely used for colony PCR screening analysis. DNA fragments required for cloning were amplified with KAPA HiFi DNA Polymerase (KAPA Biosystems, Cape Town, South Africa). All reactions were carried out according to the manufacturers' specifications. The primers used in this study are listed in Table 2.

Construction of translational fusion reporter plasmids

All the promoter-*gfp* fusion reporters were constructed as follows: specific primer pairs were used to PCR amplify the N315 *ccrAB* promoter and the *gfpmut2* gene. After enzymatic digestion, the two fragments were cloned by three-point ligation in the pCN36 plasmid. All the other plasmid reporters used in the study were made by substituting the N315 *ccrAB* promoter with their respective promoter (Table 1). To calibrate the levels of activity of the *ccrAB* promoters, a negative control plasmid (pNEG) was constructed by replacing the *ccrAB* promoter region with a 140 bp DNA fragment of the *secA* gene (SA2442 in strain N315), and a positive control plasmid (pGFPS10) was constructed by replacing the same region with the constitutive promoter of the *rpsJ* gene (SA2048 in strain N315).

Microscopy

Epifluorescence microscopy was performed using an Axioskop2 epifluorescence microscope (Zeiss, Germany), with a ×100 objective (Plan-NEOFLUAR ×100/1.30 oil, Zeiss). Bacteria from 3 mL aliquots from cultures in the exponential growth phase as described previously[25] were harvested by centrifugation and resuspended in 50 μL of PBS, of which 10 μL was deposited on glass slides and analysed.

Picture files (16-Bit) were scaled with Metamorph software (Visitron Systems, Germany) to visualize the fluorescence signal. GFP fluorescence pictures were digitally coloured using Photoshop 4.0 (Adobe Systems Europe Ltd, Edinburgh, UK) and superimposed on the corresponding phase contrast pictures, in order to visualize cells with an active *ccrAB* promoter.

Flow cytometry

Flow cytometry was performed with a FACS-Calibur (BD Biosciences, Erembodegem, Belgium), equipped with an air-cooled argon laser (488 nm). GFP fluorescence was recorded in the FL1 (525 ± 15 nm channel). Samples were removed from cultures in the exponential or stationary phase of growth, diluted in PBS in order to not exceed 800 events per second, and the fluorescence of 20 000 events was recorded for each sample. Analysis of flow cytometry data was performed using WinMDI software (version 2.8, Salk Institute, http://facs.scripps.edu/software.html).

Artificial SCCmec excision

SCC*mec* was cured from *S. aureus* N315 by using a method from Katayama *et al.*[8] Briefly, strain N315 was electroporated with the thermosensitive plasmid pSR3-1 (containing *ccrAB* genes) and transformants were cultured for 24 h at 30°C in TSB supplemented with tetracycline, serially diluted and plated on Trypticase soy agar (TSA) supplemented with tetracycline. Single colonies were picked, grown for 24 h at 42°C in TSB to

Table 2. Primers used in the study

Primer	Sequence[a]	Description
Prom fw	TTTTTTGGATCCTTGTCTTTATCATACAACTGTG	amplification of the promoter fragment in strains N315, MW2 and RP62A
Prom rev	TTTTTTACTAGTATCGGCTCCTCCTTTCACAGT	
PromCOL fw	TTTTTTGGATCCTAACTTAAAGATGAAATCGTACAGG	amplification of the promoter fragment in strain COL
PromCOL rev	TTTTTTACTAGTCGTATTTCCTCCTTCCAAAGT	
neg fw	TTTTTTGGATCCCCTTGTCTACCAGAACGACCACG	amplification of the fragment used as a negative control (*secA* gene—SA2442 in N315)
neg rev	TTTTTTACTAGTATGGCAGGTCGAGGCACAG	
PS10 fw	TTTTTTGCATGCCATTCACCACCGTTCTTATGAC	amplification of the promoter region of the *rpsJ* gene (30S ribosomal protein S10—SA2048 in N315)
PS10 rev	TTTTTTCTGCAGTCCCTCCTTATTCGTCTACATTT	
GFP fw	TTTTTTACTAGTATGAGTAAAGGAGAAGAACTT	amplification of the *gfpmut2* gene from pCN68
GFP rev	TTTTTTGAATTCTATTTGTATAGTTCATCCATG	
Excision fw	CGCAGTAACTACGCACTATCATTCAGC	amplification of the chromosomal junction in *S. aureus* N315EX
Excision rev	TGAATGAACGTGGATTTAATGTCCACC	

[a]Restriction sites are underlined.

promote curing of thermosensitive pSR3-1, and dilutions were plated on plain TSA to screen for colonies susceptible to oxacillin and tetracycline. One double-susceptible colony was purified and the absence of SCC*mec* in it was confirmed by PCR amplification of the chromosomal junction formed upon excision using the primer pair Excision fw and rev (Table 2). This isolate was named N315EX and used in further experiments.

Results

Allotype 1 and 2 ccrAB promoters

The expression of *ccrAB* genes was analysed using *gfp* translational fusions as depicted in Figure 1(a). This required first the characterization of the *ccr* gene promoter regions. Thus, the complete intergenic regions upstream of *ccrA*, which contain the putative promoter of the *ccrAB* transcriptional unit, were analysed for all the strains examined in this study (Table 3). Figure 1(b and c) depicts the homologies between these intergenic regions in different strains. The 233 bp promoter regions of strains N315, MW2 and RP62A, which all carry the *ccrAB* allotype 2, were well conserved, with a sequence similarity of 97% (Figure 1b). In contrast, the promoter region of strain COL was shorter (i.e. 187 bp), missing the 5′ part, and showing a sequence similarity of 77% when compared with N315 (Figure 1c).

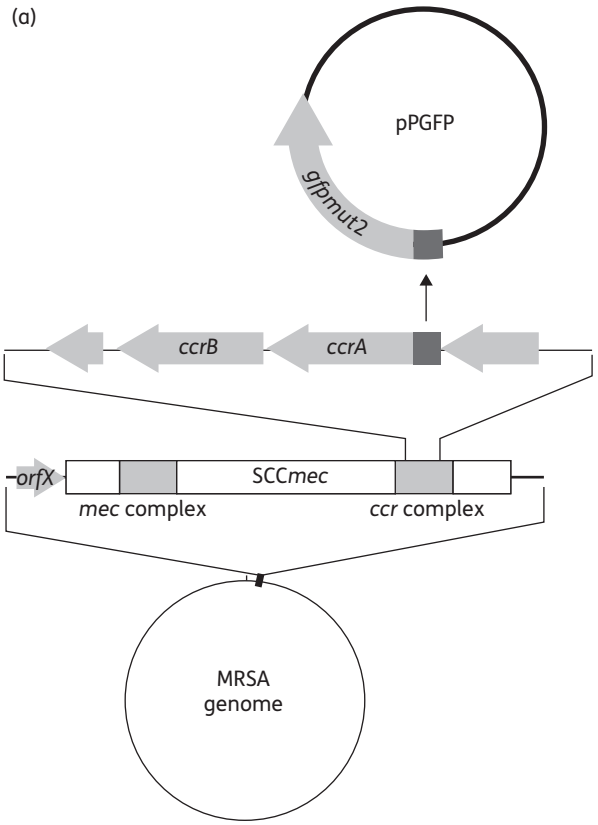
To test whether the activity of the promoters could be modulated in *trans* by genes from the core chromosome, rather than in *cis* or in *trans* by elements from the SCC*mec* cassette, we also studied the expression of the *ccrAB* promoters in *S. aureus* N315EX, from which SCC*mec* had been deleted.

Microscopy analysis

The activity of the *ccrAB* promoters was first evaluated by epifluorescence microscopy during the exponential phase of growth (i.e. samples taken after 3 h of inoculation). Figure 2 exemplifies such results with strains N315 and RP62A. It can be seen that both strains expressed GFP uniformly from the positive control vector pGFPS10, whereas cells carrying the negative control pNEG were devoid of fluorescence. In sharp contrast, only

Table 3. *ccrAB* allotype and SCC*mec* type of the strains used in the study

Strain	*ccrAB* allotype	SCC*mec* type
S. aureus COL	1	I
S. aureus N315	2	II
S. aureus N315EX	—	—
S. aureus MW2	2	IV
S. epidermidis RP62A	2	II



(b)

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MW2     TTGTCTTTATCATACAACTGTGAAGCATCAAACTGAATAATCGACGTATTGAAGATAGAA 60
RP62A   TTGTCTTTATCATACAACTGTGAAGCATCAAACTGAATAATCGACGTATTGAAGATAGAA 60
N315    TTGTCTTTATCATACAACCGTGAAGCATCAAACTGAATAATCGACGTATTGAAGATAGAA 60
        ****************** ****************************************

MW2     CAAATAACGGAGTGAAAAACATGGATGAACTTATCGAGATATTAGCCGATTTGGTAATTG 120
RP62A   CAAATAACGGAGTGAAAAACATGGATGAACTTATCGAGATATTAGCCGATTTGGTAATTG 120
N315    CAAATAACGGAGTGAAAAACATGGATGAACTTATCGAGATATTAGCCGATTTGGTAATTG 120
        ************************************************************

MW2     AAGAAATCAATCAAACAAATGACTAATATGGACGCCCAACATCTCATTGATGTTGGGCTT 180
RP62A   AAGAAATCAATCAAACGAATGACTAATATGGACGCCCAACATCTCATTGATGTTGGGCTT 180
N315    AAGAAATCAATCAAATGAATGACTAATATGAACGCCCAACATCTTATTGATGTTGGGCTT 180
        ***************  ************* ************* ***************

MW2     ATATATCAATTCATCCATAAATATATATTATCACTGTGAAAGGAGGAGCCGATATG 236
RP62A   ATATATCAATTCATCCATAAATATATATTATCACTGTGAAAGGAGGAGCCGATATG 236
N315    ATATATCAATTCATCCATAAATATATATTATTACTGTGAAAGGAGGAGCCGATATG 236
        ******************************* ********************
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(c)

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N315    TTGTCTTTATCATACAACCGTGAAGCATCAAACTGAATAATCGACGTATTGAAGATAGAA 60
COL     ------------------------------------TAAC-------TTAAAGATGAAA 16
                                             ***        ** *****  **

N315    CAAATAACGGAGTGAAAAACATGGATGAACTTATCGAGATATTAGCCGATTTGGTAATTG 120
COL     TCG-TACAGGAGTGAATAAAATGGATGAATTGATTGAATTACTAGCTGATTTAGTTTTTG 75
          **  ******** ** ********** * ** **  ** **** ***** **  ***

N315    AAGAAATCAATCAAATGAATGACTAATATGAACGCCCAACATCTTATTGATGTTGGGCTT 180
COL     AAGAAATTAATCATGCTGATGACTAATAATAACGCCCAACA-CCAGCAGGTGTTGGGCTC 134
        ******* *****     **********  *********** *      * *********

N315    ATATATCAATTCATCCATAAATATATATTATTACTGTGAAAGGAGGAGCCGATATG 236
COL     ATCTATAAATTCGTTCATAAATATATATTATCACTTTGGAAGGAGGAAATACGATG 190
        ** *** ***** * *************** *** ** ********
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Figure 1. (a) Construction scheme of reporter plasmids (pPGFP). The dark grey box represents the intergenic region containing the cloned *ccrAB* promoter regions. (b and c) Alignments of the intergenic regions containing the *ccrAB* promoter regions of the strains analysed in the study. The start codon of the *ccrA* gene is underlined.

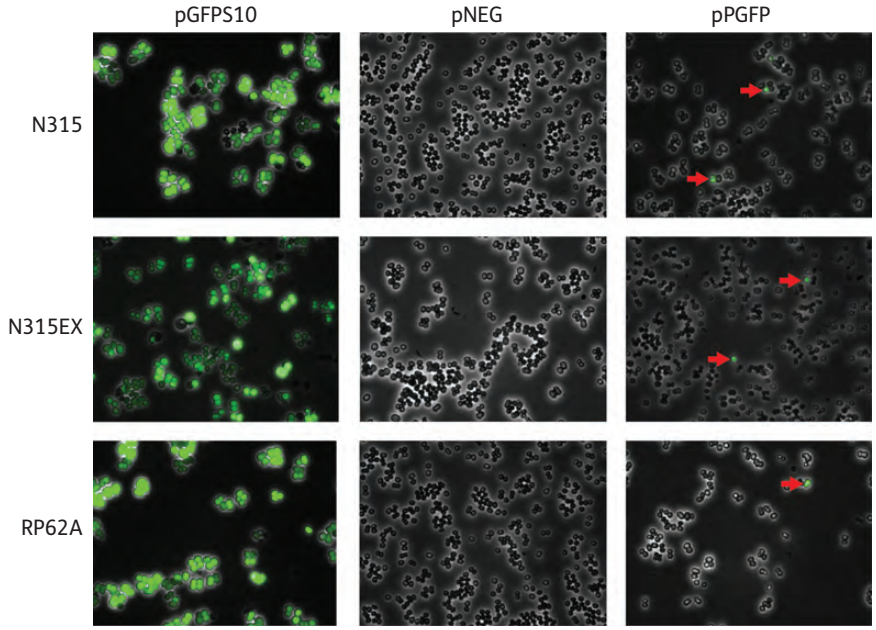

Figure 2. GFP expression. Digital overlay of GFP fluorescence image with the respective phase contrast image in cells carrying pPGFP, pNEG (negative control) and pGFPS10 (positive control). Red arrows indicate cells carrying pPGFP in which the *ccrAB* promoter is active.

a small proportion of the two cell populations showed GFP expression when they carried their specific pPGFP-N315 or pPGFP-RP62A. Most interestingly, roughly similar proportions of cells expressing GFP were observed by microscopy when pPGFP was transformed into the SCC*mec*-deleted mutant N315EX, indicating that the SCC*mec* of strain N315 did not contain determinants affecting the activity of the promoter. On the other hand, fluorescence signals were not observed by microscopy when pPGFP-MW2 and pPGFP-COL were introduced into strains MW2 and COL, respectively (data not shown). However, because microscopy results are qualitative and may be dependent on the observer, the experiments described above were repeated using flow cytometry analysis as described below. These, for instance, confirmed the absence of fluorescence in strains MW2 and COL (see Figure 4).

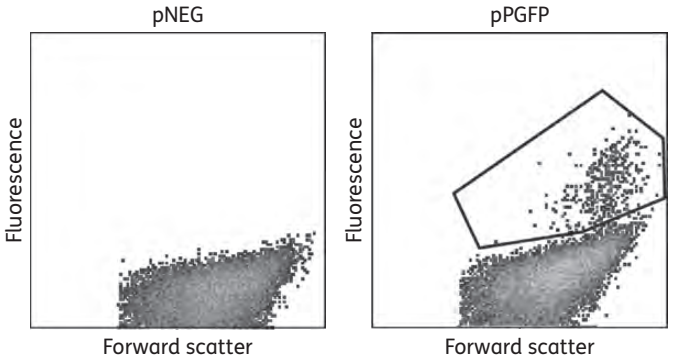

Figure 3. Quantification of N315 cells expressing GFP from the *ccrAB* promoter. Fluorescence expression profiles of cells carrying pNEG and pPGFP. Gated cells show the subpopulation expressing GFP. For each measurement, gates were arbitrarily determined with respect to the negative control.

Quantification of fluorescent cells by flow cytometry and influence of stress conditions on ccrAB expression

Flow cytometry was used to quantitatively assess the proportion of cells expressing the *ccrAB* promoter under different growth conditions. Figure 3 depicts prototype fluorescence profiles for N315 cells carrying the negative control pNEG (left panel) as well as the same strain expressing pPGFP (right panel). As observed using epifluorescence microscopy, only a minor proportion of the cell population expressed GFP. In order to determine the proportion of GFP-positive cells, we delineated a threshold using the GFP-negative control, as shown in Figure 3.

The experiment was repeated with each of the strains described in Table 3, tested in either the exponential or stationary phases of growth, and exposed to various stress conditions including growth at 42°C and exposure to oxacillin or mitomycin C. Figure 4 presents the details of these results. As a general feature, strains N315, N315EX and RP62A expressed GFP at a sizable level under all test conditions, whereas COL (which had a shorter promoter region of 187 bp versus 233 bp in the other strains) and MW2 (which had an N315-like promoter region) remained essentially below the limit of detection (Figure 4).

In the exponential growth phase strains N315, N315EX and RP62A exhibited subpopulations expressing GFP at rates between 2% and 3%, which is compatible with epifluorescence microscopy (Figure 2). Similar results were obtained under stress conditions, except for oxacillin treatment, which significantly reduced the GFP expression to 1.5% in strain N315. In contrast, in the stationary growth phase GFP expression was significantly reduced under most conditions, with some exceptions, i.e. for N315 at 42°C and RP62A with mitomycin C.

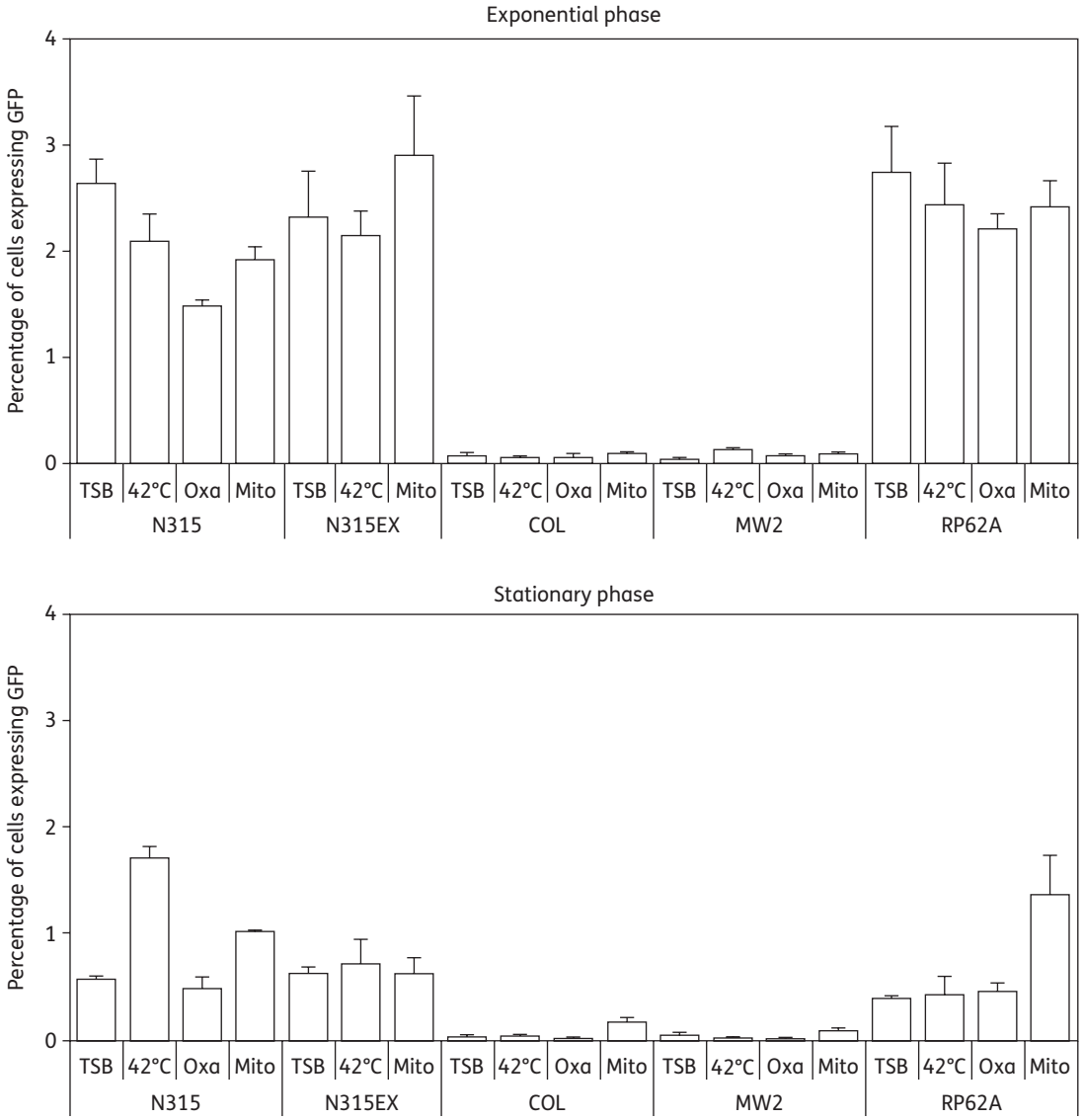

Figure 4. Flow cytometry analysis of the effects of various stresses on *ccrAB* promoter activity. Promoter constructions were transformed and expressed in their original strain. Cultures were grown either in plain TSB at 37°C (TSB) or in TSB submitted to various stress conditions from the very beginning of growth [i.e. 42°C, 4 mg/L oxacillin (Oxa) or 0.5 mg/L mitomycin C (Mito)]. Samples were removed from the cultures after 3 h (exponential phase) or 24 h (stationary phase) of growth, diluted in PBS in order not to exceed 800 events per second during flow cytometry, and the fluorescence of 20000 events was recorded for each sample. The bars represent the averages of three independent measurements in three different cultures and error bars represent the standard deviations.

It is noteworthy that the results were very similar when plasmid pPGFP-N315 was expressed in parent N315 or in its SCC*mec*-negative mutant N315EX, thus confirming the results obtained by epifluorescence microscopy and the fact that *ccrAB* genes were at least partially affected by non-SCC*mec* genetic determinants.

Finally, to test whether the shorter promoter region of strain COL was responsible for the absence of promoter activity, we transferred its specific expression plasmid (pPGFP-COL-Tc; Table 1) into the N315 background and repeated the expression experiment. Figure 5 shows that transfer of COL-specific pPGFP-COL-Tc into N315 or N315EX completely restored promoter activity. Thus, promoter activity was highly dependent on the bacterial background and the shorter COL promoter region was not responsible for its inactivity in the COL background.

Discussion

The present experiments employed a GFP expression system cloned into a low copy number plasmid in order to test the promoter activity of type 1 and 2 *ccrAB* allotypes in different staphylococcal backgrounds. The results yielded several findings that may help better understand the excision and transmission

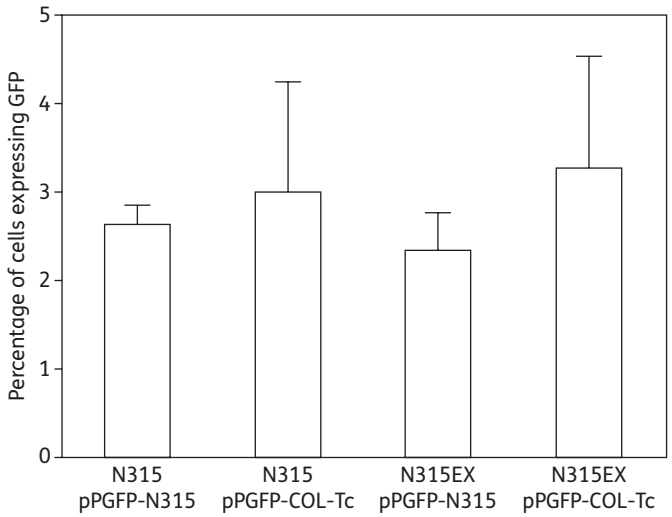

Figure 5. Flow cytometry analysis of N315 and N315EX transformed with either pPGFP-N315 or pPGFP-COL-Tc. The experimental protocol was as described in Figure 4. The bars represent the averages of three independent measurements in three different cultures and error bars represent the standard deviations.

processes of SCC*mec*. First, as predicted by the two-step transmission hypothesis discussed in the Introduction section, *ccrAB* promoter activity was not present simultaneously in all the cells of a culture, but only in a minority of them that represented a small fraction of the whole population. This is logical economics in a relatively primitive transfer system where the donor cell must lose its (presumably) beneficial genomic island in order to transfer it further. The results show that only a few cells in the population were activated for this purpose at a given time, while the remaining bacteria kept their advantageous genotype.

This stochastic gene expression system has been referred to as 'bistability' and was described in the transfer of other genomic islands,[26,27] including the ICE*clc* in *Pseudomonas knackmussi*.[28] ICE*clc* confers the capability to use aromatic compounds (e.g. chlorobenzoates and aminophenols) as carbon sources. Conjugative transfer of this island begins with its excision from the chromosome, which is driven by the activity of the IntB13 integrase promoter. Upstream of that, IntB13 promoter activity is controlled by the InrR protein, which is encoded on the island. Stochastic activation of IntB13 was observed by GFP expression in proportions of cells that increased from 0.1% to 3% between 24 h and 96 h of incubation. This suggested a dependency on cell concentration, and perhaps also on nutrient availability.

The present observations are very reminiscent of the bistability of ICE*clc*-related transfer in terms of frequency and dependency on growth phase. However, in contrast to ICE*clc*, the activity of the *ccrAB* promoters tested herein appeared to be influenced by determinants that were not encoded on the SCC*mec* cassette, but rather elsewhere on the genome. This was clear from the fact that the type 2 *ccrAB* promoter region of MRSA N315 was expressed similarly when transformed either into its N315 parent strain or into the isogenic N315EX mutant missing the whole SCC*mec* cassette. Thus, deletion of SCC*mec* did not affect promoter activity, which was therefore regulated by other genetic determinants. Moreover, the type 1 *ccrAB* promoter region of MRSA COL, which was totally inactive in its parental strain, regained full N315-like activity when transformed into MRSA N315 and MSSA N315EX. Thus, factors driving *ccrAB* gene expression were not only located in the remaining genetic background, but could vary between different strains. Eventually, the fact that the promoter region of the COL *ccrAB* was functional in N315 restricted critical parts of this region to the 3′ end of $\leq$187 bp.

Bose *et al.*[29] described the regulatory excision system of the conjugative island ICE*Bs1* of *Bacillus subtilis*. The excisionase gene appeared regulated by an original repressor/antirepressor (*immR*/*immA*) system, where the antirepressor acted by proteolytic degradation of the repressor. The system responded to changes in population density, and to DNA-damage-induced SOS response as well. Like in the *Pseudomonas* ICE*clc* example, the two genes were located on the ICE*Bs1*. However, they were rather redundant in other *B. subtilis* mobile genetic elements (MGEs), suggesting that they could act in *trans*. Moreover, a homologue of the system was found in *Staphylococcus haemolyticus*.[29]

In the present case, we did not find any homologues of the *immR*/*immA* system in our strains, either on the SCC*mec* cassette or on the chromosome. Moreover, we also asked whether there might be differences in the MGE contents of different strains, which might identify a specific MGE that would be present in strain N315 (which could activate *ccrAB*), but not in strains COL and MW2 (which lacked *ccrAB* activity), and so drive *ccrAB* expression in *trans* in N315. However, this was not the case. Finally, we also asked whether the *ccrAB* promoter region could carry specific operators for alternative sigma factors, as recently shown in certain *S. aureus* prophages,[30] but we did not find homologous regions either. Thus, the *ccrAB* regulatory elements of SCC*mec* have yet to be identified.

In this regard, and in the context of bistability, one might speculate that the activity of *ccrAB* in only a minority of cells could serendipitously accompany phenotypic variants in heterogeneous bacterial populations. Population heterogeneity appeared critical to ensure survival of a minority of cells (called persisters) under certain stress conditions, such as antibiotic treatment.[31,32] Gene expression is different in antibiotic persisters, which are dormant and tolerant to antibiotics, than in vegetative cells, which are active and killed by the drugs. Analogously, it could be that subpopulations expressing *ccrAB* occur only in specific phenotypic variants along with other housekeeping genes. The question would then arise as to how SCC*mec* cassettes managed to take advantage of this bacterial regulation system, and why was it different in different strains. More insights into the phylogeny of SCC*mec* cassette precursors might help to solve this issue.

While the present experiments were not aimed at identifying the precise molecular mechanisms responsible for *ccrAB* regulation, some clues for them were identified. Indeed, aside from the fact that *trans*-acting determinants were located outside the SCC*mec* cassette, the rate of bistability could vary as a function of growth phase. For instance, it decreased by more than five times (from approx. $\geq$2.5% to $<$0.5%; $P<0.0001$, unpaired *t*-test) between the logarithmic and stationary growth phases in *S. aureus* N315 and coagulase-negative *Staphylococcus* RP62A. Moreover, stress conditions such as high temperature or

treatment with mutagenic mitomycin C also showed some strain-specific trends toward increased rates of bistability, as apparent in Figure 4. Therefore, considering the fact that bistability was affected by various environmental stress conditions it is possible that excision of SCC*mec* is regulated in a way similar to the excision of other MGEs or bacteriophages. Since major differences between the genomes of *S. aureus* strains lay in their MGEs, this could account for differences in *ccrAB* expression in different genetic backgrounds.

The fact that frequency of bistability decreased in late growth was consistent with a recent study showing that spontaneous excision of SCC*mec* in MRSA N315 occurred transiently and early in the logarithmic growth phase, and much less thereafter.[33] These results were obtained by determining the proportion of chromosomes from which the SCC*mec* cassette had been excised compared with total bacterial chromosomes at different timepoints during growth. On the other hand, the present experiments did not reveal an increase in *ccrAB* promoter activity during treatment with β-lactams, as was previously described for transcription of *ccrA* (using β-galactosidase and quantitative RT–PCR) after exposure to β-lactams and vancomycin.[16] However, the two studies differed in design in several ways that may render them complementary rather than contradictory. One of them is that we cloned the promoter region between the start of *ccrAB* and its preceding open reading frame, whereas Higgins *et al.*[16] added the first 21 bp of the *ccrA* gene plus 20 bp of the preceding open reading frame to this region. Therefore, it is conceivable that this larger 'promoter region' contained additional regulatory elements that could alter *ccrAB* promoter activity. Another difference was related to the induction protocols. Indeed, while Higgins *et al.*[16] looked at promoter activity and mRNA expression 15 min after antibiotic addition or temperature shift to 42°C, the present study determined promoter expression in cultures that had experienced much more prolonged exposure to experimental stresses, i.e. throughout growth. Thus, one cannot exclude that *ccrAB* activity was punctually increased at the time of stress exposure, as in the Higgins *et al.*[16] study, but returned to baseline afterwards. Moreover, the present study determined the heterologous 'on' or 'off' activity of the *ccrAB* promoter, whereas Higgins *et al.*[16] measured the sum of all of these individual activities. Although both studies used low copy number plasmids (with a range of approx. 15 to 22 copies per cell), these measurements are not strictly comparable and are amenable to methodology biases. A more thorough comparison would have required integrating each individual data point of light intensity produced by the flow cytometry analysis in the present study. This analysis was attempted, but did not modify the overall results.

Taken together, the most important common message of the two experimental works is that activation of *ccrAB* expression does occur at a substantial rate *in vivo* and is affected by environmental stresses. Moreover, the present results add the notion of heterogeneous gene expression and bistability to the system, and suggest that *ccrAB* activity depends on the microbial background. Therefore, both environmental factors and bacterial background are pertinent with regard to SCC*mec* mobilization, and there might be more or fewer good donors and good recipients present in the environment. In order to achieve a more comprehensive view of SCC*mec* transfer, further work should focus on the molecular mechanisms driven by these two factors as well as on the mechanism (general phage transduction?) underlying the transfer of SCC*mec* from one cell to another.

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Transparency declarations

None to declare.

References

1 Llarrull LI, Fisher JF, Mobashery S. Molecular basis and phenotype of methicillin resistance in *Staphylococcus aureus* and insights into new β-lactams that meet the challenge. *Antimicrob Agents Chemother* 2009; **53**: 4051–63.

2 Kreiswirth B, Kornblum J, Arbeit RD *et al*. Evidence for a clonal origin of methicillin resistance in *Staphylococcus aureus*. *Science* 1993; **259**: 227–30.

3 Fitzgerald JR, Sturdevant DE, Mackie SM *et al*. Evolutionary genomics of *Staphylococcus aureus*: insights into the origin of methicillin-resistant strains and the toxic shock syndrome epidemic. *Proc Natl Acad Sci USA* 2001; **98**: 8821–6.

4 Robinson DA, Enright MC. Evolutionary models of the emergence of methicillin-resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2003; **47**: 3926–34.

5 Enright MC, Robinson DA, Randle G *et al*. The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA). *Proc Natl Acad Sci USA* 2002; **99**: 7687–92.

6 Nubel U, Roumagnac P, Feldkamp M *et al*. Frequent emergence and limited geographic dispersal of methicillin-resistant *Staphylococcus aureus*. *Proc Natl Acad Sci USA* 2008; **105**: 14130–5.

7 Smyth DS, Wong A, Robinson DA. Cross-species spread of SCC*mec* IV subtypes in staphylococci. *Infect Genet Evol* 2011; **11**: 446–53.

8 Katayama Y, Ito T, Hiramatsu K. A new class of genetic element, staphylococcus cassette chromosome *mec*, encodes methicillin resistance in *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2000; **44**: 1549–5.

9 International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC). Classification of staphylococcal cassette chromosome *mec* (SCC*mec*): guidelines for reporting novel SCC*mec* elements. *Antimicrob Agents Chemother* 2009; **53**: 4961–7.

10 Diep BA, Stone GG, Basuino L *et al*. The arginine catabolic mobile element and staphylococcal chromosomal cassette *mec* linkage: convergence of virulence and resistance in the USA300 clone of methicillin-resistant *Staphylococcus aureus*. *J Infect Dis* 2008; **197**: 1523–30.

11 Ito T, Ma XX, Takeuchi F *et al*. Novel type V staphylococcal cassette chromosome *mec* driven by a novel cassette chromosome recombinase, *ccrC*. *Antimicrob Agents Chemother* 2004; **48**: 2637–51.

12 Noto MJ, Archer GL. 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* 2006; **50**: 2782–8.

13 Bloemendaal AL, Brouwer EC, Fluit AC. Methicillin resistance transfer from *Staphylocccus epidermidis* to methicillin-susceptible *Staphylococcus aureus* in a patient during antibiotic therapy. *PLoS ONE* 2010; **5**: e11841.

14 Boundy S, Zhao Q, Fairbanks C *et al*. Spontaneous staphylococcal cassette chromosome *mec* element excision in *Staphylococcus aureus* nasal carriers. *J Clin Microbiol* 2011; **50**: 469–71.

15 Francois P, Uckay I, Iten A *et al*. *In vivo* detection of clonally derived methicillin-resistant/methicillin-susceptible *Staphylococcus aureus* strains is not a rare event. *J Clin Microbiol* 2008; **46**: 1890–1.

16 Higgins PG, Rosato AE, Seifert H *et al*. Differential expression of *ccrA* in methicillin-resistant *Staphylococcus aureus* strains carrying staphylococcal cassette chromosome *mec* type II and IVa elements. *Antimicrob Agents Chemother* 2009; **53**: 4556–8.

17 Berglund C, Soderquist B. The origin of a methicillin-resistant *Staphylococcus aureus* isolate at a neonatal ward in Sweden–possible horizontal transfer of a staphylococcal cassette chromosome *mec* between methicillin-resistant *Staphylococcus haemolyticus* and *Staphylococcus aureus*. *Clin Microbiol Infect* 2008; **14**: 1048–56.

18 Wisplinghoff H, Rosato AE, Enright MC *et al*. Related clones containing SCC*mec* type IV predominate among clinically significant *Staphylococcus epidermidis* isolates. *Antimicrob Agents Chemother* 2003; **47**: 3574–9.

19 Kreiswirth BN, Lofdahl S, Betley MJ *et al*. The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. *Nature* 1983; **305**: 709–12.

20 Kuroda M, Ohta T, Uchiyama I *et al*. Whole genome sequencing of methicillin-resistant *Staphylococcus aureus*. *Lancet* 2001; **357**: 1225–40.

21 Gill SR, Fouts DE, Archer GL *et al*. Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant *Staphylococcus aureus* strain and a biofilm-producing methicillin-resistant *Staphylococcus epidermidis* strain. *J Bacteriol* 2005; **187**: 2426–38.

22 Baba T, Takeuchi F, Kuroda M *et al*. Genome and virulence determinants of high virulence community-acquired MRSA. *Lancet* 2002; **359**: 1819–27.

23 Charpentier E, Anton AI, Barry P *et al*. Novel cassette-based shuttle vector system for gram-positive bacteria. *Appl Env Microbiol* 2004; **70**: 6076–85.

24 Bae T, Glass EM, Schneewind O *et al*. Generating a collection of insertion mutations in the *Staphylococcus aureus* genome using *bursa aurealis*. *Methods Mol Biol* 2008; **416**: 103–16.

25 Stojanov M, Moreillon P, Sakwinska O. Cassette chromosome recombinase expression in MRSA. In: *Abstracts of the Forty-ninth Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, CA, 2009*. Abstract B-032, p. 3. American Society for Microbiology, Washington, DC, USA.

26 Dubnau D, Losick R. Bistability in bacteria. *Mol Microbiol* 2006; **61**: 564–72.

27 Smits WK, Kuipers OP, Veening JW. Phenotypic variation in bacteria: the role of feedback regulation. *Nat Rev Microbiol* 2006; **4**: 259–71.

28 Minoia M, Gaillard M, Reinhard F *et al*. Stochasticity and bistability in horizontal transfer control of a genomic island in *Pseudomonas*. *Proc Natl Acad Sci USA* 2008; **105**: 20792–7.

29 Bose B, Auchtung JM, Lee CA *et al*. A conserved anti-repressor controls horizontal gene transfer by proteolysis. *Mol Microbiol* 2008; **70**: 570–82.

30 Tao L, Wu X, Sun B. Alternative sigma factor σ^H modulates prophage integration and excision in *Staphylococcus aureus*. *PLoS pathog* 2010; **6**: e1000888.

31 Balaban NQ, Merrin J, Chait R *et al*. Bacterial persistence as a phenotypic switch. *Science* 2004; **305**: 1622–5.

32 Gefen O, Balaban NQ. The importance of being persistent: heterogeneity of bacterial populations under antibiotic stress. *FEMS Microbiol Rev* 2009; **33**: 704–17.

33 Stojanov M, Moreillon P, Sakwinska O. Dynamics of excision of the staphylococcal cassette chromosome *mec* (SCC*mec*) in methicillin-resistant *Staphylococcus aureus* (MRSA). In: *Abstracts of the Fifty-second Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, CA, 2012*. Abstract C1–1746, p. 169. American Society for Microbiology, Washington, DC, USA.